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Novel Macrophage Microbicidal Responses Against Gram-Positive Bacteria

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THE UNIVERSITY *of* EDINBURGH

Doctor of Philosophy

2022

Abstract

Antimicrobial resistance is a major global health threat, and there is growing interest in how modulation of the host immune response can enhance pathogen killing and reduce reliance on antimicrobials. One target cell is the macrophage; a key innate immune cell that possesses a range of microbicidal mechanisms and can combine responses for optimal pathogen killing. *Streptococcus pneumoniae* and *Staphylococcus aureus* are important gram-positive pathogens that represent differing intracellular burdens for the macrophage. A key macrophage microbicidal mechanism relevant to the killing of these pathogens is production of reactive oxygen species (ROS). While NADPH oxidase-derived ROS is an early response to infection, mitochondrial ROS (mROS) production is a later response and is enhanced during infection by alterations in mitochondrial dynamics. ROS and mROS can combine with other macrophage responses to facilitate pathogen killing, therefore the significance and potential for such interplay with other host defence mechanisms to enhance macrophage killing of pathogens such as *S. pneumoniae* and *S. aureus* is the focus of this thesis, with specific attention to mitochondrial-associated responses and the microbicidal and immunomodulatory host defence peptide cathelicidin.

The data presented in this thesis show that expression of the *CAMP* gene, encoding cathelicidin, was upregulated by vitamin D in macrophages, was synergistically enhanced by bacterial infection or phenylbutyrate and was impaired by pro-inflammatory cytokines. Cathelicidin directly killed extracellular *S. pneumoniae* and contributed to early macrophage killing of intracellular *S. aureus* when bacterial burden was high. Mitochondrial adaptations to *S. pneumoniae* were

more prevalent in macrophages during later stages of bacterial challenge and included increased mitochondrial fission and increased mROS production. Mitochondrial adaptations to *S. aureus*, which stresses macrophage microbicidal responses to a greater extent than *S. pneumoniae*, were observed during early stages of bacterial challenge. The regulators of canonical fission, dynamin-related protein 1 (Drp1) and mitochondrial fission factor (Mff), failed to influence overall levels of fission in the initial response to *S. aureus*. In contrast, Drp1 regulated localisation of mROS to intracellular *S. aureus* in a subset of macrophages, suggesting roles in mROS delivery to bacterial-containing phagolysosomes. In regard to mechanisms of mROS production, I have provided evidence that reverse electron transport (RET) occurs as an early response to *S. pneumoniae* challenge, but not late *S. pneumoniae*, or *S. aureus* challenge. *S. aureus* enhanced mROS production in macrophages, and while NADPH oxidase-derived ROS was the greater contributor to early killing of *S. aureus*, mROS also contributed to killing. Cathelicidin enhanced microbicidal responses against *S. aureus* particularly when NADPH oxidase-derived ROS generation was impaired, but also appeared to function as a brake on alterations in mitochondrial dynamics and mROS production in the presence of bacteria, therefore potentially regulating mitochondrial homeostasis. Results in this thesis demonstrate that macrophages use ROS, alterations in mitochondrial dynamics and mROS, and cathelicidin to combat *S. pneumoniae* and *S. aureus* infections with pathogen-dependent kinetics. Macrophages adapt responses to different pathogens to ensure a multi-layered immune response to clear pathogens. The work in this thesis provides greater insight into macrophage microbicidal responses to *S. pneumoniae* and *S. aureus* infection and could inform future therapeutic strategies to enhance macrophage microbicidal responses.

Lay Summary

Antibiotics have been used for decades to effectively treat bacterial infections, but in recent years, many bacteria have developed resistance to these treatments. Consequently, infections are becoming more difficult to treat and development of new treatment methods is required. One area of research which has gained much interest is looking at how we can boost our immune systems to fight infection without reliance on antibiotics. When bacterial infection is detected in the body a type of white blood cell that makes up part of our immune system, known as a macrophage, is one of the earliest responders. These cells fight the infection by ingesting and degrading bacteria to kill them, but also use a range of different methods and factors to enhance their killing ability. Many bacteria are able to resist individual methods of killing, therefore macrophages need to apply a combination of methods and factors at different stages in the fight against bacteria in order to kill them effectively.

This thesis focussed on the functions and interactions of three different macrophage mechanisms in fighting bacteria:

- The presence of the host defence peptide cathelicidin, an important protein in cells that can kill bacteria directly and help to enhance other macrophage responses.
- Changes in the structure of the mitochondria. Mitochondria are a type of specialist component in cells, known as organelles, which act as the primary energy generators to power the cell. However, they can also enhance macrophage responses to bacteria by producing chemicals that kill bacteria. The production of these is aided by changes in the mitochondrial structure.

- The production of mitochondrial reactive oxygen species (mROS), which are chemicals produced by mitochondria when they are damaged or placed under stress. These chemicals can help to kill bacteria and activate other macrophage responses.

This thesis investigates how macrophages use combinations of these methods in response to two different bacteria, *Streptococcus pneumoniae (S. pneumoniae)* and *Staphylococcus aureus (S. aureus)*. These are common bacteria which pose a threat to human health as they can cause a wide range of diseases; both can cause pneumonia and sepsis, and *S. aureus* can skin and soft tissue infections. However, they are especially dangerous as they have also developed resistance to several antibiotics. *S. aureus* places more stress on the antimicrobial mechanisms of macrophages than *S. pneumoniae*, therefore comparing and contrasting these bacteria enabled the examination of key components of macrophage responses to different challenges. I have shown that the three mechanisms I have described can contribute to and interact to improve macrophages' ability to kill bacteria. How they do this depends on whether the macrophage is fighting *S. pneumoniae* or *S. aureus*, showing that macrophages can adapt their response to different challenges for the best bacterial killing outcome. Production of the host defence peptide cathelicidin in macrophages can be regulated by multiple factors, such as vitamin D or bacterial infection. Cathelicidin can kill both *S. pneumoniae* and *S. aureus* directly and contributes to the killing of *S. aureus* at an early stage by macrophages. As there is higher stress placed on the macrophage from *S. aureus*, macrophages activate multiple responses at an earlier stage than if they encounter *S. pneumoniae*. Mitochondria usually have a structure resembling long intertwined branches, but

fragmentation of this structure in a process known as fission occurs in response to bacterial infection. My results show and increased production of mROS occurs earlier in response to *S. aureus* than in response to *S. pneumoniae*. The bacterial-killing reactive oxygen species (ROS) chemicals in macrophages are initially produced by a complex of multiple proteins, called nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This happens quickly in macrophages after ingestion of bacteria, whereas mROS is typically produced at a later stage. However, I have shown that mROS produced in the mitochondria also contributes to *S. aureus* killing at an earlier stage, demonstrating that reactive oxygen species from two different sources can contribute *S. aureus* killing at an early stage in the fight against infection. In addition, cathelicidin can contribute more to *S. aureus* killing when the NADPH oxidase protein complex cannot effectively produce reactive oxygen species. However, cathelicidin has no impact on *S. pneumoniae* killing by macrophages at this early stage. This shows that macrophages use additional factors such as cathelicidin to overcome the greater challenge posed by *S. aureus*, especially when important early killing mechanisms are not functional. Cathelicidin also appeared to prevent mitochondrial fragmentation and mROS production triggered by the presence of bacteria, likely as a means of maintaining healthy mitochondria and reducing excessive inflammation. My work in this thesis contributes to the greater understanding of how macrophages use multiple responses in killing *S. pneumoniae* and *S. aureus* bacteria and may also provide useful information for the development of new host-based treatments of infections that boost and enhance our own immune responses, which could reduce the requirement for and reliance on antibiotics.

Acknowledgements

I would like to personally thank the following people for their support during my PhD journey, I would not have made it here without you!

First and foremost, words cannot express my gratitude to my supervisors, Professor David Dockrell and Professor Donald Davidson, for all of their encouragement, advice, and mentorship over the last few years. I have learnt so much, both academically and about myself. Your experience and enthusiasm for the project was inspiring and your unwavering support is one of the main reasons I am able to write this thesis. Thank you.

I am extremely grateful to my thesis committee, Professor Julia Dorin, Professor Debbie Bogaert, and former chair Professor Ian Dransfield. Your insight and advice during our end-of year meetings was invaluable in shaping the direction of my project. Also, my deepest gratitude extends to my funders, the University of Edinburgh and the Medical Research Foundation, without your support this project would not have been possible. Thank you.

A massive thank you to everyone in the Dockrell group and the Davidson/REDJ groups, in particular Jennifer Marshall, Dr Brian McHugh, Dr Clark Russel, and Dr Jamil Jubrail. You were always happy to give advice, provide a helping hand in the lab, and teach me new techniques without which my work would not have been possible. In addition, I could not have asked for a lovelier (or more fun) group of labmates to work alongside and tag-team late night experiments with!

My work would not have been possible without the expertise and support of the Confocal and Light Microscopy (CALM) and Flow Cytometry and Cell Sorting (FACS) facilities in QMRI. Not only was your help and support with program setup and experimental design essential to the success of my experiments, but your passion also taught me to appreciate microscopy and flow cytometry as two of my favourite lab techniques. In addition, my work would not have been possible without the generosity of the blood donors in the QMRI. Your donations allowed me to generate the MDM for all of my work and it has always been highly appreciated. Thank you all.

I must also thank my family and friends for all of their support and patience while I have been a bit off the radar during my PhD, I hope to meet up with you all as soon as I can! In particular, thank you to my best friend Helen and my Hanover Tap pub quiz crew for all the fun chats and gaming/ quiz nights that helped me take a break every so often! Also, I cannot thank my parents enough for their endless love, encouragement, and advice throughout my PhD. You could not have done more to support me through good and difficult times and for that I will be forever grateful. I know you are immensely proud of me. Thank you.

Of course, my thanks would not be complete without acknowledging my wonderful husband Sam. You have quite literally been by my side throughout this PhD, even agreeing to move from Dundee to Edinburgh and find a new job so I could do it! You have always listened to my science ramblings, been understanding of late nights in the lab, made sure I had time to relax when needed and have always genuinely believed in me even when I didn't believe in myself. Also, as a Software Engineer, Sam helped me streamline the code for my image analysis macros, so has

directly contributed to the success of my PhD work! Finally, I have to also thank my beautiful (animal) babies, Harley, Ivy, and Aurora, for the constant joy and companionship you have provided, especially during the writing of this thesis at home. Thank you, I love you!

Declaration

I confirm that this thesis has been composed by myself. All work described herein is my own or, in the cases where projects were undertaken by multiple members of the research group, I made significant contributions to those projects. Where it is indicated throughout this thesis that work was carried out with other members of the group, the results presented reflect my contribution. This work has not been submitted for any other degree or professional qualification.

Katharin Emma Balbirnie-Cumming

22/07/2022

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Abbreviations

=: equals

<: less than

-: minus

x: multiplied by

%: percent

+: plus

°C: degrees Celsius

$\Delta\psi_m$: inner mitochondrial transmembrane potential

25OHD3: 25-hydroxyviatmin D3

acetyl-CoA: acetyl coenzyme A

ADAM10: a disintegrin and metalloproteinase 10

ADP: adenosine diphosphate

Agr: accessory gene regulator

AgrA: accessory gene regulator A

AgrC: accessory gene regulator C

AIP: auto-inducing peptide

AMP: antimicrobial peptide

AMPK: 5' adenosine monophosphate-activated protein kinase

AMR: antimicrobial resistance

ANOVA: analysis of variance

AP-1: activator protein 1

APAF1: apoptotic protease activating factor 1

ASC: apoptosis-associated speck-like protein containing a caspase activation and recruitment domain

ATP: adenosine triphosphate

ATPase: adenosine triphosphatase

Atg5: autophagy related 5

Bak: B-cell lymphoma 2 homologous antagonist/killer

Bax: B-cell lymphoma 2 associated X protein

Bcl-2: B-cell lymphoma 2

Bcl-X: B-cell lymphoma-extra

Bcl-xL: B-cell lymphoma-extra large

BH3: B-cell lymphoma 2 homology domain 3

BHI: brain heart infusion

BioRxiv: BioArchive

BMDM: bone marrow-derived macrophages

C1q: complement component 1q

C3: complement component 3

C3b: complement component 3b

C4: complement component 4

Ca: calcium

CA074-me: CA074-methyl ester

$CaCl_2$: calcium chloride

CaMKIα: calcium/calmodulin-dependent protein kinase I-alpha

CaMKK-β: calcium/calmodulin-dependent protein kinase kinase-beta

CAMP: cathelicidin antimicrobial peptide gene (human)

Camp: cathelicidin antimicrobial peptide gene (mouse)

cAMP: cyclic adenosine monophosphate

CARD: caspase activation and recruitment domain

CbpA: choline binding protein A (see PspC)

CCR2: C-C chemokine receptor type 2

CD11c: cluster of differentiation 11c

CD14: cluster of differentiation 14

CD16: cluster of differentiation 16

CD36: cluster of differentiation 36

Cdc42: cell division control protein 42 homolog

cDNA: complementary deoxyribonucleic acid

C/EBPα: CCAAT enhancer-binding protein α

CFU: colony forming units

CGD: chronic granulomatous disease

CHIPS: Chemotaxis Inhibitory Protein of *Staphylococcus aureus*

CO_2: carbon dioxide

COPD: chronic obstructive pulmonary disease

CR: complement receptor

CREB: cyclic adenosine monophosphate response element-binding protein

CSF-1: colony-stimulating factor-1

CTCF: corrected total cell fluorescence

CXCR1: C-X-C motif chemokine receptor 1

CXCR3: C-X-C motif chemokine receptor 3

CYP: cytochrome P450 mixed-function oxidase

Cyt C: cytochrome c

DAMP: damage-associated molecular pattern

DAPI: 4′,6-diamidino-2-phenylindole

DC: dendritic cell

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

Dnm2: dynamin-2

DPI: diphenyleneiodonium chloride

Drp1: dynamin-related protein 1

E. coli: *Escherichia coli*

ECSIT: evolutionarily conserved signalling intermediate in Toll pathway

EEA1: early endosome antigen 1

Efb: extracellular fibrinogen binding protein

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

ERK1/2: extracellular signal-regulated kinases 1/2

FACS: flow cytometry

FAM: fluorescein amidite

FBS: foetal bovine serum

Fc: fragment crystallisable region

FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

FcεRI: high-affinity immunoglobulin E receptor

FcγR: fragment crystallisable gamma receptor

FcR: fragment crystallisable receptor

FCS: foetal calf serum

Fe: iron

Fis1: mitochondrial fission 1 protein

FPR: formyl peptide receptor

FPR2: formyl peptide receptor 2

FPRL1: formyl peptide receptor-like 1

FPRL2: formyl peptide receptor-like 2

g: grams

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Gata3: GATA binding protein 3

GED: guanosine triphosphatase effector domain

GM-CSF: granulocyte-macrophage colony-stimulating factor

GTPase: guanosine triphosphatase

h: hours

H2O2: hydrogen peroxide

hBD: human β-defensin

HBSS: Hank's Balanced Salt Solution

hCAP18: human cationic antimicrobial protein

HDAC: histone deacetylase

HDP: host defence peptides

HIF: hypoxia inducible factor

HIF-1α: hypoxia inducible factor 1α

HIV: human immunodeficiency virus

Hmp: haem metalloprotease

HNP: human neutrophil peptide

HR1: heptad repeat 1

HR2: heptad repeat 2

HSC: haematopoietic stem cells

IAP-2: inhibitor of apoptosis-2

IDT: Integrated DNA Technologies, Inc.

IFN: interferon

IFN-α: interferon-alpha

IFN-β: interferon-beta

IFN-γ: interferon-gamma

IgA: immunoglobulin A

IgE: immunoglobulin E

IgG: immunoglobulin G

IL-1: interleukin-1

IL-1β: interleukin-1 beta

IL-1RA: interleukin-1 receptor antagonist

IL-4: interleukin-4

IL-5: interleukin-5

IL-6: interleukin-6

IL-9: interleukin-9

IL-10: interleukin-10

IL-12p40: interleukin-12 subunit beta

IL-13: interleukin-13

IL-17: interleukin-17

IL-17A: interleukin-17A

IL-18: interleukin-18

IL-22: interleukin-22

ILC: innate lymphoid cell

iNOS: inducible nitric oxide synthase

IntDen: integrated density

IRF3: Interferon regulatory factor 3

JAK/STAT: Janus kinase/signal transducer and activator of transcription

JC-1: tetraethylbenzimidazolylcarbocyanine iodide

JNK: c-Jun N-terminal kinase

kDa: kilodaltons

LAMP1: lysosomal-associated membrane protein 1

LAMP2: lysosomal-associated membrane protein 2

Ldh1: lactate dehydrogenase 1

LL-37: cathelicidin

LLO: listeriolysin O

L. monocytogenes: *Listeria monocytogenes*

LMP: lysosomal membrane permeabilisation

LPS: lipopolysaccharide

LTA: lipoteichoic acid

LukA: leukocidin A

LukAB: leukocidin AB

LukB: leukocidin B

LukD: leukocidin D

LukE: leukocidin E

LukED: leukocidin ED

LukF: leukocidin F

LukS: leukocidin S

Ly6C: lymphocyte antigen 6C

LytA: autolysin

MALP-2: Mycoplasma-derived lipopeptide 2

MAPK: mitogen-activated protein kinase

MARCO: macrophage receptor with collagenous structure

MAVS: mitochondrial antiviral signalling protein

Mcl-1: myeloid cell leukaemia-1

M-CSF: macrophage colony-stimulating factor

MDM: monocyte-derived macrophages

MDV: mitochondria-derived vesicle

Mff: mitochondrial fission factor

Mfn1: mitofusin 1

Mfn2: mitofusin 2

Mg: magnesium

MHC: major histocompatibility complex

MIC: minimum inhibitory concentration

µg: micrograms

µl: microlitres

MiD49: mitochondrial dynamics protein of 49 kilodaltons

MiD51: mitochondrial dynamics protein of 51 kilodaltons

mg: milligrams

ml: millilitres

mm: millimetres

min: minutes

MIRO1: mitochondrial rho guanosine triphosphatase 1

MitoPQ: mitoparaquat

MitoQ: mitoquinone

Mn: manganese

MOI: multiplicity of infection

MOMP: mitochondrial outer membrane permeabilisation

MPP: mitochondrial processing peptidase

MprF: multiple peptide resistance factor

MrgX2: Mas-related gene X2

mROS: mitochondrial reactive oxygen species

MRSA: methicillin-resistant *Staphylococcus aureus*

Mst1: macrophage stimulating 1

Mst2: macrophage stimulating 2

Mtb: *Mycobacterium tuberculosis*

mTORC1: mammalian target of rapamycin complex 1

Mule: myeloid cell leukaemia-1 ubiquitin ligase E3

NaCl: sodium chloride

NAD+: nicotinamide adenine dinucleotide, oxidised

NADPH: nicotinamide adenine dinucleotide phosphate

ng: nanograms

nm: nanometres

NET: neutrophil extracellular trap

NF-κβ: nuclear factor kappa-light-chain-enhancer of activated B cells

NH_4Cl: ammonium chloride

NK: natural killer

NLR: nucleotide-binding oligomerisation domain-like receptor

NLRP3: nucleotide-binding oligomerisation domain-like receptor family pyrin domain containing 3

NO: nitric oxide

NOD1: nucleotide-binding oligomerisation domain 1

NOD2: nucleotide-binding oligomerisation domain 2

NOS2: nitric oxide synthase 2

NOX1: nicotinamide adenine dinucleotide phosphate oxidase 1

NOX2: nicotinamide adenine dinucleotide phosphate oxidase 2

NOX4: nicotinamide adenine dinucleotide phosphate oxidase 4

NRAMP1: natural resistance-associated macrophage protein 1

1O2: singlet oxygen

O2: oxygen

O2- : superoxide

O3: ozone

OCl-: hypochlorite ion

•OH: hydroxyl radical

OMA1: metalloendopeptidase OMA1, mitochondrial

ONOO-: peroxynitrite

•OOH: peroxyl radical

•OOR: alkoxyl radical

OPA1: optic atrophy 1

P2X7: P2X purinoceptor 7

P. aeruginosa: *Pseudomonas aeruginosa*

PAMP: pathogen-associated molecular pattern

PARL: presenilin-associated rhomboid-like protein

PBA: phenylbutyrate

PBMC: peripheral blood mononuclear cells

PBS: phosphate-buffered saline

PFA: paraformaldehyde

pH: potential hydrogen

PI3K: phosphoinositol-3-kinase

PINK1: phosphatase and tensin homologue-induced kinase 1

PKA: protein kinase A

PLY: pneumolysin

PMN: polymorphonuclear leukocytes

PRR: pattern recognition receptor

PSM: phenol-soluble modulins

PspC: pneumococcal surface protein C

PVL: Panton-Valentine leukocidin

Rab5A: Rat sarcoma-related protein 5A

Rab7: Rat sarcoma-related protein 7

Rac1: Rat sarcoma-related C3 botulinum toxin substrate 1

Rac2: Rat sarcoma-related C3 botulinum toxin substrate 2

Ras: Rat sarcoma

RET: reverse electron transfer

RgrA: retinal G protein-coupled receptor A

RgrB: retinal G protein-coupled receptor B

RgrC: retinal G protein-coupled receptor C

Rhot1: see MIRO1

RIG-I: retinoic acid-inducible gene I

RNA: ribonucleic acid

RNAIII: ribonucleic acid III

RORγt: retinoic acid receptor-related orphan receptor gamma

ROS: reactive oxygen species

RPMI 1640: Roswell Park Memorial Institute 1640 Medium

RT-qPCR: real-time/reverse transcription quantitative polymerase chain reaction

RXR: retinoid X receptor

SaeP: *S. aureus* exoprotein expression P

SaeQ: *S. aureus* exoprotein expression Q

SaeR: *S. aureus* exoprotein expression R

SaeRS: *S. aureus* exoprotein expression RS

SaeS: *S. aureus* exoprotein expression S

S. aureus: *Staphylococcus aureus*

SCFA: short chain fatty acid

SEM: standard error of the mean

siRNA: short interfering ribonucleic acid

SNARE: soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

SOD: superoxide dismutase

SOD1: superoxide dismutase 1

SOD2: superoxide dismutase 2

Spn: *Streptococcus pneumoniae*

S. pneumoniae: *Streptococcus pneumoniae*

SpsA: see PspC

SR-A: scavenger receptor A

TAMRA: Carboxytetramethylrhodamine

TBC1D15: TBC1 domain family member 15

TCA: tricarboxylic acid

γδ T cell: gamma delta T cell

γδ TCR: gamma delta T cell receptor

TCR: T cell receptor

TGF-β: transforming growth factor beta

Th1: T helper 1

Th2: T helper 2

Th17: T helper 17

TLR: Toll-like receptor

TLR1/2: Toll-like receptor 1/2

TLR2: Toll-like receptor 2

TLR4: Toll-like receptor 4

TLR9: Toll-like receptor 9

TMRM: tetramethylrhodamine methyl ester

TNF-α: tumour necrosis factor alpha

TOMM20: translocase of outer mitochondrial membrane 20

TRAF6: tumour necrosis factor receptor-associated factor 6

UK: United Kingdom

U: units

USA: United States of America

VAMP: vesicle-associated membrane protein

VAMP7: vesicle-associated membrane protein 7

VAMP8: vesicle-associated membrane protein 8

v-ATPase: vacuolar adenosine triphosphatase

VDAC: voltage-dependent anion channel

VDR: vitamin D receptor

VDRE: vitamin D response element

x g: relative centrifugal force

Zn: Zinc

Chapter 1

Introduction

1.1 The global burden of antimicrobial resistance

The discovery of the antimicrobial properties of *Penicillium* mould against *Staphylococcus aureus* bacteria by Sir Alexander Fleming in 1928, and the refinement and production of the resultant antimicrobial drug penicillin by Howard Florey and Ernst Chain in the 1940s, marked a pivotal milestone in the advancement of medicine (1,2). Throughout the 20th and 21st centuries, many different antimicrobial drugs have been developed to treat a vast plethora of infections and diseases, which have saved millions of lives globally. In the case of bacteria, antimicrobial drugs most often exert their bactericidal function by targeting and inhibiting essential cellular processes required for survival, such as cell wall synthesis, protein synthesis, nucleic acid synthesis, and various metabolic pathways (3).

However, in parallel to the development of these antimicrobial drugs, the microbes which the drugs are designed to combat have evolved various antimicrobial resistance mechanisms to prevent destruction by these drugs. For example, bacterial cells can confer resistance by genetic means, via the spread of intrinsic resistance possessed by some bacterial strains, by developing resistance in response to selective pressure following exposure to an antimicrobial agent, or by acquiring resistance genes from other bacterial cells via horizontal gene transfer (4,5). Bacteria are also equipped with various mechanisms to resist destruction by

antimicrobials; bacteria have the ability to degrade antimicrobial compounds, prevent entry of the drug into the cell, actively extrude internalised drugs from the cell by the action of membrane-associated efflux pumps, and prevent the drug from interacting with its target by physically protecting the target site or by modifying the target site (6,7).

In more recent times, there has been a significant increase in the development and prevalence of antimicrobial resistant pathogens which are now rendering many of the current antimicrobial drugs ineffective or less effective than in previous years. Although bacteria have various mechanisms which confer resistance to antimicrobials, environmental factors and human behaviour have also played a role in promoting the development of resistance. For example, in healthcare, current antimicrobials can be overprescribed or improperly administered, and patients prescribed antimicrobials for use at home may not complete their entire prescribed course (8). In agriculture, sub-lethal concentrations of antimicrobials are commonly administered to livestock to prophylactically maintain livestock health for optimal food yield, but this results in trace antimicrobial concentrations in the food products that are subsequently consumed (9). Antimicrobials are also commonly used to protect crops, which can lead to the accumulation in antimicrobials in the soil and the subsequent uptake of trace antimicrobials by crops, and the leaching of antimicrobials into water sources via surface water run-off from the field (10). Antimicrobials can also be introduced into crops and soil by the use of manure or sewage-based fertilisers, which can contain trace excreted antimicrobials (10) This results in the presence of trace antimicrobials in crops and water that are eventually consumed, therefore providing another means of intaking trace antimicrobials, and

driving the development of antimicrobial resistance. These practices provide the selective pressure for bacteria to develop resistance mechanisms. In a bacterial population, cells lacking resistance mechanisms may be eliminated by low antimicrobial concentrations but those that have developed resistance mechanisms can persist and subsequently multiply, giving rise to a new population of entirely resistant bacterial cells (11).

The consequences of antimicrobial resistance to human health include the proliferation of microbial infections that are difficult to treat with antimicrobial drugs, the requirement for higher doses of antimicrobial drugs or alternative drugs that may have harsher, more toxic side effects, and ultimately an increase in global mortality from antimicrobial resistant infections, with estimated higher mortality rates than those associated with major diseases such as human immunodeficiency virus (HIV) or malaria (12). As detailed in the UK government report led by Jim O'Neill in 2016, which outlines the recommended actions for tackling antimicrobial resistance, an estimated 700,000 global deaths are reported to be associated with antimicrobial resistant infections. However, by 2050, global deaths associated with antimicrobial resistant infections are projected to reach around 10 million, making such infections one of the leading causes of global deaths (13). Therefore, it is clear that antimicrobial resistance is a serious global health threat that requires a significant degree of attention and research. In 2017, the World Health Organisation published their Priority Pathogen List, highlighting the pathogens that require the greatest research focus to combat antimicrobial resistance (14). The bacteria *Staphylococcus aureus (S. aureus)* and *Streptococcus pneumoniae (S. pneumoniae,* Spn, or the pneumococcus*)* are both highlighted on the list; they are very common pathogens

responsible for a diverse range of conditions and diseases, and they are also resistant to some of the key current front-line antimicrobials. Furthermore, it has been reported that in 2019, both *S. aureus* and *S. pneumoniae* were among the six most prevalent pathogens associated with antimicrobial resistance (AMR) and AMR-associated deaths (12).Therefore, these bacteria are the pathogens of interest and the focus of this project.

1.2 Modulation of host immune response to enhance pathogen killing

It is evident that combatting antimicrobial resistance is a critical global health priority that deserves a great deal of attention in regard to scientific research and development of novel therapeutic methods. There has been much research conducted into developing novel antimicrobial drugs and treatments to circumvent the resistance mechanisms already developed by pathogens; although this field of research is extremely important, it is known that pathogens can eventually adapt and develop resistance to new drugs, and currently there are insufficient numbers of novel drugs in clinical trials (15). However, another highly important field of research aims to understand the mechanisms behind host responses to pathogens and develop host-based therapies and treatments (6,16–18). Understanding host responses to pathogens and targeting therapies to enhance these responses has a number of advantages; pathogens are less likely to develop resistance to the complex and potent antimicrobial responses employed by the host immune system (16). Furthermore, the host immune system possesses a wide range of antimicrobial responses that are activated at different times during infection. This provides the host with a layered immune response which is difficult for pathogens to survive (16). This provides the rationale for focussing studies on the understanding of host

immune responses. A greater understanding of the host response to infection and how these responses can be manipulated to become more efficient, could reduce reliance on current antimicrobial treatments and provide new therapeutic strategies, therefore helping to prevent antimicrobial resistance.

1.3 Overview and clinical significance of *Staphylococcus aureus*

S. aureus is a facultative anaerobic, gram-positive bacterium (19). It asymptomatically colonises the skin and nasal cavities of 20-30% of the general population in many parts of the world, therefore is known a common commensal organism (19,20). However, it can become pathogenic if it breaches the skin or mucosal barrier, for example via wounds. *S. aureus* can cause a variety of diseases in a broad range of people of different ages, sexes, ethnicities, and in patients with other comorbidities, therefore *S. aureus* infections are a risk for a significant proportion of the world's population (20–22).

S. aureus is the causative agent for an array of diseases which can range from mild to severe (20,23). *S. aureus* can cause a range of skin and soft tissue infections; although these are typically less severe examples of *S. aureus* infections, they are the most abundant (23). In addition, they have the potential to develop into more severe infections if *S. aureus* further invades tissue or reaches the bloodstream, therefore skin and soft tissue infections still require care and treatment (24). Common examples of such infections include impetigo, scalded skin syndrome, folliculitis, and cellulitis (25–27). In contrast, *S. aureus* infections can result in more serious and potentially life-threatening conditions, such as pneumonia, bacteraemia, endocarditis and meningitis (20,23). In healthcare settings, *S. aureus* is a primary

causative agent of surgical wound infections and can form chronic biofilm infections associated with invasive medical devices such as intravascular catheters (28–30). The diversity of diseases which *S. aureus* can cause and the widespread potential for the switch from commensal to pathogen illustrates the significance of this bacterium as a global health concern.

1.3.1 Antimicrobial resistance: methicillin-resistant *Staphylococcus aureus* (MRSA)

S. aureus poses a significant clinical challenge due to its ability to rapidly develop resistance to a variety of antimicrobials; for example, the first penicillin-resistant *S. aureus* strains were discovered in 1944, two years after penicillin was discovered as an antimicrobial agent (31). Penicillin is part of the β-lactam antimicrobial group, which structurally contains a β-lactam ring and functions by inhibiting bacterial cell wall synthesis (32). *S. aureus* developed penicillin resistance via enhanced expression of the penicillinase enzyme, which could degrade and therefore inactivate penicillin (7). In the 1950s, a semi-synthetic penicillin derivative termed methicillin was developed that could resist degradation by penicillinase and kill *S. aureus* (33). However, *S. aureus* began to develop resistance to methicillin in the 1960s, and this gave rise to one of the most well-characterised and clinically important antimicrobial-resistant pathogens: methicillin-resistant *S. aureus* (MRSA) (34).

MRSA is well regarded as one of the most prevalent hospital-acquired pathogens which pose a serious threat to hospital staff and patients, in particular elderly or immunocompromised individuals (22). However, in recent years,

community-associated MRSA strains have developed which have had variable impacts on disease burden in different regions of the world (28). For example, the prevalence of community-associated MRSA is relatively low in the UK and other European countries, but it is greater in the USA and Canada. This is due to the clonal expansion of MRSA strains such as USA300, which is the most abundant strain causing community-associated MRSA infections across the USA, but is not the dominant cause of community -associated MRSA infections in other regions of the world (28).Community-associated MRSA strains have been frequently isolated from, and associated with, the development of skin and soft tissue infections in young, healthy individuals (35). Similar to methicillin-sensitive *S. aureus* strains, MRSA primarily colonises the skin and upper respiratory tract, and infection can lead to the manifestation of similar conditions, such as skin and soft tissue infections, endocarditis, bacteraemia, and meningitis (36). However, due to the antimicrobial resistant nature of MRSA, these conditions are significantly harder to treat than their methicillin-sensitive counterparts and can result in significantly increased mortality rates, particularly in elderly individuals (37,38). It has recently been reported that MRSA was responsible for a significant proportion, greater than 100,000, of AMR-associated deaths in 2019, highlighting the significance of MRSA to the global burden of antimicrobial resistance (12). It is evident that MRSA has been a critical global health concern in healthcare settings for a number of years, however the emergence and spread of community-associated strains emphasises the continued importance of this pathogen to global health and the ongoing requirement to develop new antimicrobial strategies to combat antimicrobial-resistant infections.

1.4 Overview of *S. aureus* virulence factors and immune evasion mechanisms

S. aureus possesses a vast armament of factors to promote its survival and persistence within macrophages. These include cytolytic toxins that can kill host cells, and a wide array of virulence factors to destroy or resist many different components of the macrophage host defence response.

1.4.1 Regulation of *S. aureus* toxin and virulence factor expression: accessory gene regulator (*agr*) and *S. aureus* exoprotein expression RS (SaeRS) two-component systems

One of the key regulatory mechanisms of *S. aureus* toxin and virulence factor expression is the activity of two-component systems. There are two key systems associated with toxin and virulence factor regulation in *S. aureus*, termed the *agr* and SaeRS two-component systems (39). Two-component systems are comprised of a sensor histidine kinase located at the cell membrane, and a response regulator located in the cytoplasm. The system is activated via the detection of a stimulus by the sensor histidine kinase, which results in the phosphorylation and activation of the response regulator, which can bind to target genes and act as a transcription factor to induce their expression (39).

The *S. aureus agr* two-component system has been widely characterised as a quorum sensing system but is also involved in the regulation of *S. aureus* virulence (40). Upon detection of high concentrations of auto-inducing peptide (AIP), the sensor histidine kinase AgrC phosphorylates the response regulator AgrA, which binds to deoxyribonucleic acid (DNA) and induces the transcription of the *agr* effector

component, ribonucleic acid III (RNAIII) (39,41,42). RNAIII can subsequently induce and enhance the expression of an array of genes, including those encoding key *S. aureus* toxins and virulence factors.

The SaeRS two-component system has also been reported to be required for *S. aureus* virulence (43). The sensor histidine kinase SaeS is activated upon detection of a number of stimuli, such as host defence peptides, sub-lethal concentrations of antimicrobials, hydrogen peroxide (H_2O_2), and changes in environmental pH and salt concentrations (44–48). The response regulator SaeR is phosphorylated and activates the expression of a range of *S. aureus* virulence factors. Levels of SaeR phosphorylation add an additional layer of regulation to *S. aureus* virulence factor expression, as some genes require low levels of SaeR phosphorylation and other require higher levels (49). Furthermore, it has also been shown that, in addition to kinase activity, SaeS can possess phosphatase activity. Although not required for SaeS kinase activity, activation of phosphatase activity requires SaeS to form a complex with the additional accessory proteins SaeP and SaeQ (50).

1.4.2 *S. aureus* toxins

S. aureus secretes an array of toxins that possess cytolytic activity. These can lead to the destruction of host immune cells such as macrophages, therefore promoting the survival of the pathogen. Examples of the most well-characterised staphylococcal toxins are α-haemolysin, bi-component leukocidins, and phenol soluble modulins (PSMs).

1.4.2.1 α-haemolysin

The 293 amino acid cytolytic toxin α-haemolysin is widely considered to be one of the most important virulence factors expressed by *S. aureus*. Its expression is regulated by the *agr* and SaeRS two-component systems. A-haemolysin is a β-barrel pore-forming toxin that is expressed in a monomeric form and can target the membranes of non-granulocytic eukaryotic cells, including macrophages (51). These monomers can bind to ADAM10, a zinc-dependent disintegrin and metalloprotease on eukaryotic cell surfaces (52). Upon binding, seven of these α-haemolysin monomers can oligomerise to form a heptameric pore to lyse the membrane, allow the efflux of host cell ions, and ultimately kill host cells (53).

1.4.2.2 Bi-component leukocidins

Another group of β-barrel cytolytic toxins expressed by *S. aureus* are the bi-component leukocidins; examples of which include leukocidin AB (LukAB), leukocidin ED (LukED), and Panton-Valentine leukocidin (PVL), the latter of which has been suggested to be a critical virulence determinant in community-acquired MRSA strains, although controversy still remains on this point since some studies fail to demonstrate an important role (19,53,54). The *agr* and SaeRS two-component systems are also important in the regulation of these toxins. Structurally, these toxins are comprised of an S subunit and an F subunit, which heterodimerise to form the complete toxin (55); for the examples highlighted above, LukA, LukE and LukS are the corresponding S subunits, respectively, and LukB, LukD, and LukF are the respective F subunits. The toxin dimers have particular affinity for chemoattractant receptors of phagocytic immune cells which they can bind, then they can interact

with other toxin heterodimers to form an octameric pore in the host cell membrane to facilitate host cell death (56), via highly pro-inflammatory mechanisms such as necroptosis and pyroptosis (57,58). The affinity for receptors on phagocytic immune cells suggests that these toxins are particularly adept at eliminating host cells that can directly kill *S. aureus,* therefore play an important role in mediating *S. aureus* survival (56).

1.4.2.3 Phenol soluble modulins (PSMs)

The PSM group of toxins are also critical for *S. aureus* virulence and are particularly important for the virulence of community-acquired MRSA. These are small, amphipathic, α-helical pore-forming toxins that are regulated by the *agr* two-component system. *S. aureus* expresses seven PSMs that are classified into three categories, α-, β-, and γ-PSMs; *S. aureus* expresses four PSMα (PSMα 1-4), two PSMβ (PSMβ 1 and 2) and one PSMγ (also termed δ-toxin) (59). The PSMs have a broader range of host cell targets than other *S. aureus* toxins as their cytolytic activity does not require interaction with a host cell receptor (19,59,60). Each of the PSM categories possess different degrees of cytolytic ability, based on their charge (19,59,61). The toxins of the PSMα group have the most potent cytolytic activity due to their positive charge; they are more strongly attracted to negatively-charged host cell membranes and can more readily integrate into membranes to form pores. PSMγ possesses a neutral charge, therefore has moderate cytolytic activity. The PSMβ group are negatively charged, therefore are not attracted to host cell membranes, and do not possess cytolytic activity. In addition, PSMs, at non-lytic concentrations, can interact with formyl peptide receptor 2 (FPR2), a G-protein coupled receptor located on the surface of innate immune cells such as neutrophils

and macrophages, and induce a variety of pro-inflammatory host defence responses, including activation and chemotaxis of immune cells and production of pro-inflammatory cytokines (59,62).

1.4.3 *S. aureus* immune evasion mechanisms

In addition to producing a range of toxins with cytolytic activity that directly target host immune cells, *S. aureus* also possesses a wide array of virulence factors that have evolved to counteract different host immune mechanisms in various immune cell types such as macrophages and neutrophils, therefore allowing *S. aureus* to evade host immune detection, neutralise bactericidal responses and tolerate harsh conditions generated by the host immune system. As this project focuses on macrophages, examples of *S. aureus* immune evasion mechanisms against macrophage responses are described below. The fact that *S. aureus* possesses different mechanisms to counteract multiple components of the macrophage host defence response further illustrates the requirement of a multi-step, layered host immune response with spatially and temporally separated steps to efficiently enhance the clearance of bacterial infections.

1.4.3.1 Inhibition of chemotaxis

The process of chemotaxis is important for the recruitment of immune cells such as neutrophils and monocytes to infection sites; pathogens, such as bacteria, and also immune cells in the infection microenvironment, generate chemoattractant compounds, which can be detected by circulating immune cells to attract them to the site (63). *S. aureus* can inhibit the chemotaxis of immune cells via the expression of the chemotaxis inhibitory protein of *S. aureus* (CHIPS). This 14.1 kilodalton (kDa)

protein is expressed in more than 60% of clinical isolates (63), and inhibits chemotaxis by binding to chemoattractant receptors on the surface of immune cells, such as the C5a receptor which detects the complement-associated chemoattractant C5a, and the formyl peptide receptor (FPR), which interacts with formylated peptides such as those secreted by bacteria (64,65). Inhibition of chemotaxis can promote *S. aureus* survival, as it limits the number of host immune cells recruited to the infection site, but it may also play a host anti-inflammatory role by reducing the recruitment of neutrophils which can promote excessive inflammatory responses and host tissue damage in some inflammatory diseases (64).

1.4.3.2 Evasion of bacterial internalisation

S. aureus can be opsonised by a variety of components and proteins, including immune system components such as antibodies and the complement component 3b (C3b) protein. Opsonins can recognise and bind to bacterial surface components, then interact with phagocyte surface receptors to enhance pathogen recognition and internalisation. *S. aureus* can prevent its internalisation by macrophages by preventing the recognition of these bacterial surface-associated opsonins by their macrophage surface receptors and disrupting the process of internalisation. Protein A prevents detection of immunoglobulin G (IgG)-opsonised bacteria by binding to the fragment crystallisable (Fc) region of the IgG antibody (66,67). The Fc region of IgG is then masked and inaccessible to macrophage fragment crystallisable gamma receptor (FcγRs), therefore FcγR-mediated internalisation is prevented. Extracellular fibrinogen binding protein (Efb) prevents complement-mediated internalisation by interacting with and masking C3b bound to the bacterial surface, therefore the C3b cannot be detected by complement

receptors (CRs) (68). Furthermore, *S. aureus* can utilise Efb to recruit the host blood plasma glycoprotein, fibrinogen, which *S. aureus* can use to cover bacterial surface-bound C3b opsonins in a fibrinogen coat to prevent CR detection (69).

1.4.3.3 Evasion of phagolysosomal killing

Following bacterial internalisation, macrophage bacteria-containing phagosomes undergo a series of maturation steps to generate a hostile environment for the bacterium and ultimately mediate pathogen killing. *S. aureus* possesses mechanisms to counteract these stages of phagosomal maturation, which include recruitment of phagosomal maturation factors, phagosomal acidification, lysosomal recruitment, and pathogen killing by lysosomal hydrolases. *S. aureus* can generate ammonia to neutralise the acidity of the phagosomal microenvironment; ammonia can be generated by the hydrolysis of arginine to citrulline by arginine deiminase, or via the hydrolysis of urea by urease (70,71). Furthermore, *S. aureus* can actively impair the maturation of the phagolysosome; it has been shown that *S. aureus*-containing phagosomes may be unable to fuse with lysosomes, therefore cannot acquire and activate the highly antimicrobial lysosomal factors such as cathepsin D and β-glucuronidase (72). It is also possible that in some contexts, *S. aureus*-containing phagosome and lysosome fusion still occurs, but *S. aureus* can impair the accumulation of lysosomal factors within the phagosomal compartment, as shown in a model of THP-1 macrophage-like cell infection with *S. aureus* USA300 (73). This study suggested possible mechanisms for this impairment; *S. aureus* may be able to redirect the transport of lysosomal components away from the phagosomal compartment, so although phagosome and lysosome fusion occurs, the lysosomal

factors are not retained, and it is also possible that *S. aureus* may degrade the lysosomal hydrolases to promote its survival in the phagosome (73).

1.4.3.4 Evasion of reactive oxygen and nitrogen species

The generation of reactive oxygen and nitrogen species are key components of the macrophage host defence response, and these reactive species possess both bactericidal and immunomodulatory functions. *S. aureus* is equipped with several similar enzymes to those that host cells also possess to regulate reactive species generation and prevent reactive species toxicity. *S. aureus* expresses superoxide dismutases (SODs) and catalase to eliminate superoxide ($O2^-$) and H_2O_2, respectively (74,75). Peroxiredoxin neutralises H_2O_2 via reversible oxidation of peroxiredoxin by H_2O_2 (76). Haem metalloprotease (Hmp) is a flavohaemoglobin enzyme that scavenges nitric oxide (NO) and detoxifies NO by oxidising it to nitrate (77). *S. aureus* can also adapt to nitrosative stress via switching from respiration to glycolysis for adenosine triphosphate (ATP) production (78,79). This is mediated by the action of lactic acid dehydrogenase 1 (Ldh1), the expression of which can be induced by high NO concentrations. Ldh1 catalyses the reduction of pyruvate to L-lactate during fermentation, and the ATP generated promotes the maintenance of redox homeostasis and growth of *S. aureus* (78).

1.4.3.5 Evasion of host defence peptides

Macrophages generate a range of antimicrobial host defence peptides possessing both bactericidal and immunomodulatory functionality. Many of these host defence peptides are cationic and amphipathic and exert their bactericidal action by forming pores in bacterial membranes. This is mediated by the attraction

and binding of peptides to the membrane surface via the difference in the positive charges of the peptides and the negative charges of the bacterial membranes, followed by peptide insertion into the membrane and conformational changes to form the pore. (80). *S. aureus* expresses a series of enzymes that can alter the bacterial cell membrane and peptidoglycan to generate a more positive charge, which acts to repel cationic host defence peptides and prevent pore formation. Multiple peptide resistance factor (MprF) is a bacterial integral membrane protein that increases the positive charge of peptidoglycan via the addition of a positively charged lysine residue to peptidoglycan structures (81). A group of four enzymes encoded by the *dlt* operon, DltA, DltB, DltC, and DltD, modify teichoic aids by adding positively charged D-alanine residues to neutralise the bacterial surface negative charge (82). Furthermore, *S. aureus* also expresses proteases that can degrade antimicrobial host defence peptides completely. This has been demonstrated for human cathelicidin, an antimicrobial host defence peptide, where the *S. aureus*-associated metalloprotease aureolysin and the glutamylendopeptidase V8 protease were able to efficiently degrade cathelicidin *in vitro*, thereby promoting *S. aureus* resistance to cathelicidin and enhancing bacterial survival (83).

1.5 Overview and clinical significance of *Streptococcus pneumoniae*

S. pneumoniae is a gram-positive bacterium that asymptomatically colonises the upper respiratory tract in healthy individuals. However, *S. pneumoniae* can become pathogenic if migration of the bacterium to areas such as the lungs, bloodstream, or the meninges occurs (84). *S. pneumoniae* can cause a variety of diseases in a wide range of individuals of different ages, sexes, and ethnicities. However certain demographics are particularly at risk of pneumococcal disease and

are more susceptible to the more life-threatening pneumococcal conditions; these include young children, elderly adults, immunocompromised individuals, and patients with pre-existing co-morbidities (85–87).

Pneumococcal diseases can range from less severe local infections to invasive life-threatening conditions. Examples of less severe manifestations of pneumococcal infection include sinusitis and acute otitis media (88), however, the more severe conditions comprise the majority of the pneumococcal disease burden requiring hospitalisation. *S. pneumoniae* is the leading cause of community-acquired pneumonia, which has been associated with significant hospitalisation and mortality rates particularly in older adults and patients with co-morbidities (85,86). *S. pneumoniae* is also the leading causative agent of meningitis in older adults and is a significant cause of other invasive diseases such as bacteraemia and sepsis (89–92). Pneumococcal infections present a significant global health challenge as they cause a range of life-threatening conditions that most commonly affect more vulnerable populations.

1.5.1 Antimicrobial resistance in *S. pneumoniae*

Antimicrobial resistance has become increasingly prevalent *in S. pneumoniae* over recent decades, thereby posing significant challenges to the treatment of pneumococcal diseases. Early evidence of penicillin resistance *in S. pneumoniae* was identified in Boston in 1965, with subsequent resistant strains rapidly discovered across the world (93). It has been reported that *S. pneumoniae* possess six penicillin-binding proteins. These cytoplasmic membrane proteins are involved in bacterial peptidoglycan synthesis and are the targets of penicillin, however bacteria

such as *S. pneumoniae* have developed penicillin-binding proteins with low affinity for penicillin, which contributes to the development of penicillin resistance (94). These penicillin-binding proteins are not only the targets of penicillin, but for many other β-lactam antimicrobials (94), therefore these low affinity penicillin binding proteins can confer resistance to a broader range of β-lactam antimicrobials than penicillin alone.

Widespread resistance of *S. pneumoniae* to antimicrobials of the macrolide group has also been reported. Macrolides are classified by their macrocyclic lactone ring structure and function as bacteriostatic agents that inhibit bacterial growth and impair protein elongation and synthesis, by binding to the 50S bacterial ribosomal RNA subunit and preventing its interactions with transfer RNA molecules (95). The most common macrolide antibiotic used globally has been erythromycin, which was discovered and isolated from *Streptomyces erythraeus* bacteria in 1952, although newer macrolides such as clarithromycin or azithromycin are now more often used in developed countries (95). However, in more recent years, macrolide-resistant *S. pneumoniae* has been identified as a contributor to pneumococcal disease cases globally, with particular evidence reported from across Europe (96). Macrolide resistance has been shown to be associated with the expression and activity of *S. pneumoniae* antimicrobial efflux pumps encoded by the *mef(A)* and *mef(E)* genes, which confer resistance via the removal of antimicrobial molecules from bacterial cells, and also via the expression of a ribosomal RNA methylase encoded by the *erm(B)* gene, which modifies the 23S ribosomal RNA in bacteria, therefore altering the bacterial ribosome and rendering the bacterium resistant to the antimicrobial effects of the macrolides (88,97,98).

To illustrate the crucial importance of correct antimicrobial use to preventing the development of antimicrobial resistance, a 2007 study investigated the relationship between antimicrobial use and *S. pneumoniae* antimicrobial resistance in 15 European countries (99). It was reported that antimicrobial-resistant *S. pneumoniae* was more prevalent in countries with higher rates of antimicrobial drug prescription and use, therefore demonstrating a strong correlation between antimicrobial use and the development of resistant bacteria. This illustrates that antimicrobial use has a direct impact of the development of antimicrobial resistance and emphasises the importance of careful use of antimicrobials to protect their efficacy and longevity.

1.6 *S. pneumoniae* virulence factors and immune evasion mechanisms

S. pneumoniae possesses an array of virulence factors and immune evasion mechanisms to prevent its clearance by innate immune cells such as macrophages. These factors can enhance bacterial colonisation in the host, promote bacterial virulence, kill host cells, and counteract host defence responses.

1.6.1 Pneumolysin

The pneumolysin toxin is widely regarded as one of the most important virulence factors expressed by *S. pneumoniae* (100). It is a 53 kDa cholesterol-dependent cytolysin; this toxin binds to cholesterol associated with host cell membranes and oligomerises with other pneumolysin monomers to form large pores in host cell membranes (101,102). This therefore results in loss of host cell integrity and can lead to cell death. The importance of pneumolysin to *S. pneumoniae* virulence has been demonstrated in studies utilising pneumolysin-deficient bacterial

strains in various models. Reduced bacterial numbers have been found in the lungs and respiratory tract of mice inoculated with *S. pneumoniae* lacking pneumolysin compared to wild-type *S. pneumoniae* (103). Pneumolysin-deficient *S. pneumoniae* have also been shown to induce weaker inflammatory responses and result in delayed immune cell recruitment to lungs in murine models of respiratory tract infection (103–105).

In addition to cytolytic activity, pneumolysin can also induce host cell immunomodulatory functions at sub-lytic concentrations. Pneumolysin has the ability to activate the complement pathway, which can enhance *S. pneumoniae* pathogenesis in the lung by inducing greater levels of inflammation in lung tissue (106,107). Low pneumolysin concentrations have been suggested to interact with macrophages via activation of Toll-like receptor 4 (TLR4), in a similar manner to lipopolysaccharide (LPS). The subsequent signalling cascade can therefore induce an array of pro-inflammatory macrophage responses, such as the production of pro-inflammatory cytokines and the generation of reactive species (108); this can ultimately result in reduced susceptibility to pneumococcal infection. More recent studies have highlighted the nucleotide-binding oligomerisation domain (NOD)-like receptor (NLR) family pyrin domain-containing 3 (NLRP3) inflammasome as the key receptor for pneumolysin and pneumolysin-mediated inflammasome activation and interleukin 1 beta (IL-1β) production can modulate and enhance host defence responses to pneumococcal infection (109). However, pneumococcal serotypes that express toxins with reduced haemolytic functionality have limited effects on inflammasome activation and IL-1β production, and these serotypes have been associated with more invasive pneumococcal disease, although because of the

reduced inflammatory responses, the invasive disease may be better tolerated (109).Therefore, although pneumolysin is a key *S. pneumoniae* virulence determinant that can enhance bacterial pathogenicity and survival, at low concentrations it can activate host immune cell responses to enhance clearance of the pathogen; however these responses can be limited during infection with pneumococcal strains expressing less haemolytic toxins. Furthermore, pneumolysin has been shown to enhance macrophage clearance of intracellular *S. pneumoniae* by inducing lysosomal membrane permeabilisation (LMP), which is independent of its cytolytic activity (110). This induces macrophage apoptosis, an important host defence strategy to clear persistent intracellular bacteria and limit excessive inflammation. This further illustrates the importance of pneumolysin as a mediator of host immune cell activation and enhanced clearance of *S. pneumoniae.*

1.6.2 Autolysins: LytA

Autolysins are a diverse group of lytic enzymes present in a wide range of bacteria, and they function as bacterial cell wall degrading enzymes by breaking down peptidoglycan structures (111). One of the most well-characterised autolysins is LytA, a key virulence factor in *S. pneumoniae*, as shown by reduced virulence of LytA-deficient bacteria *in vitro* (112,113). LytA can degrade components of the *S. pneumoniae* cell wall, which can result in bacterial cell death and release of bacterial pathogen-associated molecular patterns (PAMPs) (111,114). These can subsequently activate pro-inflammatory host immune responses and induce excessive inflammation that can damage host cells and tissues (111). In addition, the degradative action of LytA can also promote pneumococcal virulence further by

mediating the release of other *S. pneumoniae* virulence factors, such as pneumolysin, from the bacterial cell (115).

1.6.3 Polysaccharide capsule

The polysaccharide capsule is regarded as the primary virulence factor in *S. pneumoniae*; it allows the bacterium to evade internalisation by host immune cells by preventing the binding of opsonins such as C3b and antibodies (116,117). The opsonins can recognise and bind pneumococcal cell wall components, but the polysaccharide capsule inhibits binding of the opsonins to the bacterial surface. The result is reduced clearance of bacteria by phagocytes and enhanced extracellular persistence (108). In addition, the polysaccharide capsule can promote pneumococcal colonisation and persistence via by promoting pneumococcal escape from mucus which prevents the aggregation and mucociliary clearance of bacteria, and allows bacteria to reach the surface of epithelial cells (118). The capsule can also enhance resistance to antimicrobial compounds and peptides by preventing contact with or ingress into the bacterial cell (119).

1.6.4 Pilus

The pilus is an important part of bacterial cell anatomy and is commonly found in many different bacterial species. In *S. pneumoniae*, the pilus is encoded on the *rlr* gene locus of the pneumococcal pathogenicity island I, and the pilus consists of three structural components, retinal G protein-coupled A, C, and C (RgrA, RgrB, and RgrC), that are assembled by sortase enzymes (120). It has been reported that the pneumococcal pilus, in particular the RgrA component, acts as an adhesin, and is required for optimal adherence of *S. pneumoniae* to host cells, and therefore is

important for enhanced pneumococcal colonisation and virulence (121). Furthermore, the pneumococcal pilus can induce pro-inflammatory host defence responses, such as the production of pro-inflammatory cytokines, upon detection by immune cells such as macrophages (122).

1.6.5 Choline binding proteins- choline binding protein A and pneumococcal surface protein A

Choline binding proteins are a group of proteins that consist of a choline binding segment at the C terminus, a flexible protein linker, and the peptide functional domain at the N terminus. The choline binding segment can interact with choline residues of lipoteichoic acid (LTA) in *S. pneumoniae* and other gram-positive bacteria, therefore allowing these proteins to be anchored to the bacterial surface (111). The pneumococcal autolysin LytA is one example of a choline binding protein (123), but choline binding protein A (CbpA), also known as pneumococcal surface protein C (PspC) or SpsA, has been described as one of the most abundant choline binding proteins in *S. pneumoniae*. CbpA has been shown play a role in the virulence of *S. pneumoniae* by enhancing adhesion to and colonisation of host tissues (124). In the context of host cells that have been activated by cytokine signalling, adhesion is facilitated by the binding of the C-terminal choline region to *S. pneumoniae* LTA, and the N-terminal functional region to host cells, which can promote pneumococcal colonisation and development of pneumococcal disease (111). CbpA can alter host immune responses by binding to and sequestering secretory immunoglobulin A (IgA); this is the most abundant immunoglobulin on mammalian mucosal surfaces and primarily mediates mucosal surface defence by

preventing interactions of pathogens and toxins with epithelial cells (125,126). CbpA can also inhibit pneumococcal opsonisation by complement components by recruiting and binding to complement factor H, an inhibitor of complement alternative activation that prevents complement factor binding to the opsonin C3b (127).

Pneumococcal surface protein A (PspA) is another choline binding protein that has been shown to be important in *S. pneumoniae* virulence. PspA prevents activation of the complement system by *S. pneumoniae*. PspA possess a highly charged N-terminal that extends from the bacterial cell surface, which can inhibit complement activation by *S. pneumoniae* (128). PspA has been reported to inhibit complement-mediated opsonophagocytosis of *S. pneumoniae* by impairing C3b deposition and preventing the generation of complement component 3 (C3) convertases, which are required for complement alternative activation (129). PspA-mediated inhibition of complement has been shown to reduce complement-mediated phagocytosis of *S. pneumoniae,* and therefore reduced pathogen clearance (130). Furthermore, PspA can also bind to lactoferrin, an important glycoprotein in host phagocytic cells and mucosal secretions that transports and sequesters iron (Fe), using this as a source of Fe, which is an essential factor for pneumococcal growth (131). In order to overcome the limitation of Fe availability, PspA on *S. pneumoniae* can act as a lactoferrin receptor, and the PspA/lactoferrin interactions can allow *S. pneumoniae* to obtain and utilise lactoferrin-associated Fe for bacterial growth and colonisation (131).

1.6.6 Hyaluronidase and neuraminidase

Hyaluronidases and neuraminidases are groups of proteases that are expressed on the surface of *S. pneumoniae* bacteria. These are well-characterised examples of LPTXG-anchored surface protein virulence factors in *S. pneumoniae*; the amino acid sequence of proteins in this group contains a LPXTG motif primarily at the C terminus, which is required for covalent linkage to peptidoglycan on the bacterial cell surface (132). Hyaluronidases can mediate host tissue permeability and enhanced pneumococcal invasion and colonisation via their ability to degrade hyaluronic acid, a component of the extracellular matrix and host connective tissue (101,133). Furthermore, hyaluronidases can interact with pro-inflammatory cytokines and chemokines, such as tumour necrosis factor-alpha (TNF-α) and IL-1β, which can further enhance cytokine secretion from host cells and facilitate enhanced tissue inflammation during pneumococcal pneumonia (108).

Neuraminidases exert their virulence effects by cleaving N-acetylneuraminic acids from components of the host cell membrane such as glycolipids, lipoproteins and oligosaccharides (134). This can result in damage to host cells and subsequent host cell dysfunction, but neuraminidase activity can also lead to increased exposure of host cell membrane components such as galactose on epithelial cell membranes, which can enhance pneumococcal colonisation and biofilm formation (134–136). *S. pneumoniae* interactions with such components and the enhanced pneumococcal adhesion and colonisation can lead to the induction of host pro-inflammatory responses.

1.7 Overview of the human immune system

The human immune system comprises several different layers of protection against pathogens. Broadly, the immune system is organised into two categories of responses. The innate immune system, which consists of physical barriers such as the skin and mucosal surfaces, and fast-responding innate immune cells, such as macrophages and neutrophils, are the first lines of defence against invading pathogens (137). The innate response provides more rapid generalised responses, while the adaptive immune system, which consists of components such as T and B cells, provides longer-term and more specialised immunity. Cells such as cytotoxic T cells are involved directly in pathogen killing, and antibody production by B cells facilitates opsonophagocytosis and other mechanisms of pathogen killing (16). The adaptive immune system also facilitates the development of immunological memory, which help to mount an effective immune response against previously-encountered pathogens (137). Recent research also emphasises that the innate system also shows immunological memory, through epigenetic imprinting and development of trained immunity and tolerance (138–140). The innate immune system, in particular the macrophage, is the focus of this project, therefore the key components of the innate immune system, their functions in host defence, and the particular roles of the macrophage will be discussed.

1.7.1 Physical barriers

Physical barriers are one of the most essential components in host defence, as they are constantly in contact with the external environment and are the components that pathogens first encounter. A key immunological function is to

prevent invasion by pathogens. The function of the skin as a protective barrier is well known, but other bodily systems such as the respiratory tract, digestive system, and the reproductive and urinary tracts are also in constant contact with the environment, therefore are lined with epithelial tissue which can prevent pathogen infiltration. These barriers are also equipped with a range of antimicrobial factors, including host defence peptides, hydrolytic enzymes such as lysozyme, and low pH secretions (141,142). Epithelial surfaces contain pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and signal inflammatory responses via cytokine and chemokine expression (143,144). Furthermore, systems such as the respiratory and gastrointestinal tracts are lined with a specialised mucosal barrier which can facilitate host defence by capturing and trapping pathogens that have been inhaled or ingested, respectively, in the mucus, as well as assisting in pathogen clearance due the presence of antimicrobial factors (141). Another aspect of the physical barriers is that, in most cases, they are colonised with commensal flora that also exerts a barrier to pathobionts, by allowing colonisation resistance (145). Although these barriers are very effective host defence components, they can still be breached by pathogens if barrier integrity is compromised, for example by wounding (146) or by pathogens that directly invade, as in respiratory tract pathogens (147). Therefore, the cells of the innate immune system have specialised antimicrobial functions to eliminate pathogens and protect the host, complementing the epithelial barriers.

1.7.2 Neutrophils

Neutrophils develop from myeloid progenitor cells in the bone marrow and are released in abundance, where they can circulate in the blood and rapidly migrate to sites of infection (148,149). Neutrophils are the most abundant innate immune cell

type, but they are short lived, therefore the bone marrow continuously produces neutrophils to maintain the population (150). Neutrophil antimicrobial responses are rapidly activated upon detection of a pathogen. Neutrophils express an array of receptors to detect various microbial components, such as TLRs, fragment crystallisable receptors (FcRs) and CRs, the latter two of which detect opsonins bound to bacteria, which lead to the induction of neutrophil antimicrobial responses upon receptor activation (151,152). Similar to macrophages, neutrophils are adept at phagocytosis and killing of pathogens (153), and they also generate potent and effective reactive oxygen species (ROS) to mediate pathogen clearance (154). However, neutrophils also possess two antimicrobial mechanisms that are distinct from tissue macrophage responses; these are the release of antimicrobial effector molecules from intracellular granules, and the use of neutrophil extracellular traps (NETs). Mature neutrophils possess granules containing pre-synthesised antimicrobial effectors molecules; these include host defence peptides, proteases, and myeloperoxidase, which functions to generate highly potent chlorinated reactive oxygen species such as hypochlorous acid (155). Upon activation, neutrophils can be induced to degranulate and release some of their effector molecules to eliminate pathogens quickly and efficiently (155). NETs are comprised of neutrophil DNA from the de-condensation of chromatin; these DNA structures are coated with antimicrobial effector molecules including serine proteases such as cathepsin G and neutrophil elastase, and antimicrobial host defence peptides such as cathelicidin found within the granules, which are expelled from the cell into the extracellular environment (156). NETs function to physically catch and trap extracellular pathogens within the DNA structure, and the antimicrobial effectors coating the DNA can mediate pathogen clearance (157).

1.7.3 Eosinophils

Eosinophils are particularly important in the clearance of parasitic infections, but they also possess multiple effector functions that promote an inflammatory response as a result of detection of bacterial pathogens, upon activation of eosinophils by factors such as cytokines and antibodies (149). Eosinophils are derived in the bone marrow from a cellular precursor for both eosinophils and basophils, which can then differentiate into eosinophils (158). Reports have shown that IL-5 is the key cytokine for the development of eosinophils and for the promotion of eosinophil release into the bloodstream following development (159,160). Upon activation, circulating eosinophils can be recruited to sites of infection and contribute to the inflammatory response via the production of pro-inflammatory cytokines and chemokines (149). Eosinophils also produce peptides such as eosinophil cationic protein and eosinophil peroxidase that are involved in mediating other immune cell functions and host defence responses, including inhibition of T cell proliferation, antibody synthesis by B cells and subsequent activation of mast cell degranulation, and the generation of reactive oxygen and nitrogen species (149,161,162).

1.7.4 Mast cells and basophils

Mast cells are tissue-resident granulocytes that initially develop as immature circulating mast cells, then fully differentiate into mature mast cells following migration into tissues (149). Mast cells are considered to be one of the primary "guardian" cells of the innate immune system, as they are one of the first immune cell types to come into contact with a pathogen or foreign agent (163). Basophils possess very similar functions to mast cells, although basophils are primarily located

in the circulatory system, where they comprise less than 1% of the circulating leukocyte population (164).The primary mechanism of mast cell and basophil activation is the interaction between B cell-derived immunoglobulin E (IgE) antibodies and high-affinity immunoglobulin E receptor (FcεRI); mast cell and basophil activation in this manner is most often observed in response to allergic reactions (164,165). Upon recognition and binding of IgE, FcεRIs can aggregate on mast cell and basophil surfaces, and the binding of allergens to FcεRI/IgE complexes activates mast cell and basophil immune effector functions (166). In addition to FcεRI, mast cells also express other receptors, such as TLRs, that can recognise and interact with other factors, such as PAMPs, to activate mast cell immune responses (165). The primary function of activated mast cells and basophils is rapid degranulation and release of immune effector molecules (164,167). These include chemokines to enhance the recruitment of other immune cells such as neutrophils and macrophages to the infection site (168), and histamine, heparin, vascular endothelial growth factor (VEGF) and pro-inflammatory cytokines, which can all promote enhanced permeability of vascular structures at the infection site to allow for more efficient migration of recruited immune cells (164,165,168). In addition to releasing granule contents, activated mast cells can also synthesise pro-inflammatory cytokines and lipid mediators to promote host inflammatory responses (169). Mast cells can contribute directly to pathogen killing via mechanisms such as phagocytosis, ROS production, expression of antimicrobial peptides and proteases, and formation of extracellular traps, and can modulate other host defence responses such as enhancing mucus production at mucosal surfaces and promoting immune cell recruitment via chemokine production and enhancement of vascular permeability (170). Furthermore, mast cells can promote adaptive immune responses by

presenting antigens to T cells themselves or by promoting the maturation and migration of dendritic cells (DCs) and T cells to facilitate DC-mediated T cell activation (170).

1.7.5 Natural killer (NK) cells

NK cells are distinct from other innate immune cells described so far as they do not originate from a myeloid progenitor. NK cells develop from the same lymphoid progenitor as T and B cells, however, NK cells do not possess the same adaptive immune functions of T and B cells due to the absence of receptors for antigen recognition (171). NK cells have potent and rapid killing functionality against damaged or dysfunctional host cells, such as those infected by intracellular pathogens or tumour cells. Clearance of these damaged cells is critical in the maintenance of healthy host tissues, but NK cells require careful regulation due to their potent cytotoxic functions. NK cells can detect and probe signals derived from both healthy and damaged host cells with receptors on the NK cell surface (172). Healthy cells primarily produce signals to inhibit NK cell cytotoxic functions, while damaged cells produce signals that activate NK cell cytotoxic functions, therefore NK cells can utilise the proportions of activation and inhibitory signals they receive to determine if cells are healthy or dysfunctional, and if clearance of dysfunctional cells is required (149). Furthermore, NK cells can modulate the immune responses of other innate immune cells such as macrophages; for example, NK cells in adipose tissue can promote pro-inflammatory macrophage polarisation via the production of pro-inflammatory cytokines such as TNF-α (173), while interferon-gamma (IFN-γ) produced by NK cells resident in the bone marrow can prime monocytes prior to their egress and migration to the gastrointestinal tract, to exert more anti-inflammatory

responses during gastrointestinal infection, such as production of anti-inflammatory interleukin 10 (IL-10) (174).Conversely, other immune cells can also assist in the regulation of NK cell activity; for example, NK cell interactions with DCs promote NK cell cytotoxicity, pro-inflammatory cytokine production, and activation of T cell adaptive immune responses (175).

1.7.6 Innate lymphoid cells (ILCs) and gamma delta (γδ) T cells

NK cells represent the most well-known example of lymphoid-derived innate immune cells, however the discovery and characterisation of other innate lymphoid cells (ILCs) in recent years has significantly broadened our understanding of this group. Similar to NK cells. ILCs are lymphoid-derived cells that lack the antigen recognition receptors typically found on adaptive immune cells (176). ILCs are primarily tissue-resident cells, and are particularly abundant in lymphoid, epithelial, and mucosal tissues in order to rapidly react to pathogen invasion (177,178). Three groups of ILCs have been described to date; each of these groups can be correlated to different subsets of helper T cells as they share similar functions, while NK cells correlate with cytotoxic T cells. However, the difference between ILCs and T cells lies in the speed of immune responses; T cell adaptive immune responses can take days to manifest while ILCs can respond rapidly upon detection of cytokines and other signals from other tissue—resident innate immune cells (176). Group 1 ILCs (ILC1) correspond to T helper 1 (Th1) cells, and elicit a highly pro-inflammatory response against intracellular pathogens via the production of high levels of IFN-γ and TNF-α (177,179). Group 2 ILCs (ILC2) correspond to T helper 2 (Th2) cells and are adept at mounting an immune response against parasites and helminths via the expression of the transcription factor GATA-binding protein 3 (Gata3) and a number

of interleukins (IL-4, IL-5, IL-9, and IL-13) as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) (177,179). By expressing IL-13 and amphiregulin, ILC2 also play roles in host tissue repair following infection with helminths or viruses (178). Group 3 ILCs (ILC3) correspond to T helper 17 (Th17) cells; they express retinoic acid receptor-related orphan receptor-gamma (RORγt) and primarily respond to extracellular microbes via expression of interleukins (IL-17 and IL-22), and GM-CSF (177,179). The cytokines produced by ILCs can influence the immune phenotypes and functions of other innate immune cells in the local environment. For example, in the context of macrophages, adipose tissue-resident ILC1s have been shown to promote classical pro-inflammatory macrophage activation via ILC1 production of IFN-γ, which can drive insulin resistance in obesity (180). In the intestinal environment, GM-CSF produced by ILC3s can also drive the polarisation of macrophages to the classical pro-inflammatory phenotype, while macrophage wound healing mechanisms are inhibited (181). In addition, GM-CSF-activated pro-inflammatory macrophages can in turn promote further activation of ILC3s in a positive feedback loop (181), which illustrates the importance of ILC-derived cytokine production in the modulation of other innate immune responses.

In addition to ILCs, which are innate immune cells that share developmental origins and similar functions to different T cell types, a small subset of tissue-resident T cells, termed gamma delta (γδ) T cells due to their T cells receptors consisting of γ and δ chain heterodimers rather than the conventional α and β chain heterodimers, exhibit features and functions that are characteristic of both innate and adaptive immune responses (182,183). γδ T cells are particularly concentrated in the epithelial and mucosal tissue of barrier sites, and can be activated in response to a wide range

of stimuli; γδ T cells are capable of recognising major histocompatibility complex (MHC)-associated antigens via their T cell receptors (TCRs) like other T cells, but the gamma delta T cell receptor (γδ TCR) can also respond to antigens independent of MHCs (182,184). In addition, γδ T cells are also capable of responding to the detection of PAMPs and damage-associated molecular patterns (DAMPs) via expression of PRRs (184). The effector functions of activated γδ T cells associated with innate immunity include the direct killing of infected or dysfunctional cells via expression of molecules such as granzymes or perforin, the production of pro-inflammatory cytokines to modulate the inflammatory responses of other immune cells, and the production of anti-inflammatory cytokines and epithelial cell growth factors to dampen pro-inflammatory responses from immune cells and promote tissue regeneration, respectively (184). γδ T cells also have the capacity to develop into antigen presenting cells that can present antigen to and activate other γδ T cells and also classic αβ T cells (183). Furthermore, some γδ T cells activated upon pathogen recognition can mature into cells more closely resembling classic adaptive T cells; they function to expand and develop into a long-lasting memory T cell population in the tissue, which provides enhanced immunity and more efficient responses if the pathogen is encountered again (182).

1.7.7 Monocytes

Monocytes are mononuclear phagocytic cells that develop from myeloid progenitors in the bone marrow, then they are released into the bloodstream (185). These circulatory monocytes can be recruited to various tissues or sites of infection where they can further develop and mature into macrophages or DCs (149). Human monocytes can be categorised into three subsets based on their expression of the

surface receptors cluster of differentiation 14 (CD14) and 16 (CD16). Monocytes expressing high levels of CD14 but lacking CD16 expression represent the majority (80-90%) of circulating monocytes and are known as the classical inflammatory monocytes (186–188). Monocytes expressing low levels of CD14 but expressing higher levels of CD16 exhibit a more anti-inflammatory phenotype and are known as non-classical monocytes; these have been shown to act as precursors in the development of alternatively-activated macrophages (187), as well as patrolling blood vessels to cellular debris present in the vascular system (149,189). Monocytes expressing high levels of CD14 while also expressing CD16 surface markers are known as intermediate monocytes and express the highest level of antigen presenting molecules while also secreting pro-inflammatory cytokines such as TNF-α , IL-1β and interleukin 6 (IL-6) in response to TLR signalling (188,190,191). In mice, the markers distinguishing between murine monocyte subsets differ from those in humans, however studies have identified correlations between these murine subsets and some human subsets. Examples of the most well described murine monocyte markers are C-C chemokine receptor 2 (CCR2), L-selectin, C-X-C chemokine receptor 1 (CXCR1), and lymphocyte antigen 6C (Ly6C) which is a component of the GR1 epitope (192). Murine monocytes expressing higher levels of CCR2, L-selectin, and Ly6C but lower levels of C-X-C chemokine receptor 3 (CXCR3) were shown to correlate with human classical inflammatory monocytes, while those expressing lower levels of CCR2, L-selectin, and Ly6C and higher levels of CXCR3 correlated with the human intermediate monocyte subset (192). Further information on the role of monocytes in the context of macrophage origins and development is presented in the Macrophage section of this thesis.

1.7.8 Dendritic cells (DCs)

DCs are widely regarded as the professional antigen presenting cells of the innate immune system and represent an important link between innate and adaptive immune responses (193,194). Morphologically, DCs possess many cellular protrusions, termed dendrites, that provide the cell with a large surface area to facilitate contact points with other immune cells in the extracellular environment, in particular T cells (195,196). DCs are present in abundance in physical barrier tissues such as the skin and internal epithelial surfaces, which allows them to react quickly to pathogens that breach physical barriers (194). DCs can internalise pathogen-derived antigenic components (197), which can subsequently be degraded into small peptide fragments and loaded onto MHC molecules within the DC (198). These MHC/antigen complexes are transported to DC surfaces which allows for the antigen to be presented to other immune cells. Immature DCs can migrate into host tissue to capture antigens, then they can migrate to the lymph nodes and spleen, regions with a high abundance of adaptive immune cells, in order to present the antigen to T cells (194,199). Once a T cell has recognised the presented antigen, T cell responses can be activated to mediate an adaptive immune responses against the newly identified pathogenic agent (137,200); such responses include the killing of infected cells containing intracellular pathogens, the production of various cytokines to modulate other immune cell responses and the development of memory cells to mediate efficient immune responses upon subsequent encounters with the pathogen (137,201,202).

1.7.9 Macrophages

Macrophages are large, mononuclear professional phagocytes that are key players in the first line of cellular defence in tissues. Macrophages are capable of engulfing large particles (greater than 0.5 micrometres) (203), such as bacteria or apoptotic cells to facilitate pathogen clearance or resolution of inflammation. Macrophages utilise several rapid and delayed antimicrobial mechanisms to effectively clear bacteria (204), which demonstrates the importance of a layered innate immune response to efficiently eliminate pathogens.

1.7.9.1 Origins and differentiation of tissue-resident macrophage populations

Macrophages can be found in almost all tissue types where they reside as tissue-specific resident macrophage populations (205). Tissue-resident macrophages share core functions in the detection, engulfment and clearance of pathogens (206). However, each tissue-resident population possesses adaptations to fulfil specific functions in their particular tissue niche (185,205,206). Examples include alveolar macrophages, which are adapted for efficient clearance of pathogens in the lung environment (207). Microglia, specialist macrophages of the central nervous system, are adapted to regulate neuron synaptic activity (208). Cardiac macrophages maintain heart muscle integrity and mediate adaptation to myocardial injury (209). Kupffer cells contribute to the detoxification functions of the liver and are critical for the maintenance of hepatocyte health (210). Osteoclasts, macrophages specialising in generation and maintenance of bone tissue, can remodel bone tissue and promote bone tissue reabsorption via the activity of the cysteine protease cathepsin K (211).

Many tissue-resident macrophage populations originate from yolk sac-derived progenitors arising during early embryonic development. At this stage, macrophages are the sole immune cells generated, as the yolk sac-derived progenitors are limited to the generation of macrophages and erythrocytes (212). However, as embryonic development progresses, hematopoietic stem cells (HSC) are generated which migrate to the foetal liver and serve as the progenitor for all immune cell types (212). Following the completion of embryonic development, bone marrow-derived HSC become the primary progenitors of immune cells (213).

In adults, macrophages can also arise from the differentiation of peripheral blood mononuclear cell (PBMC)-derived monocytes, which themselves are derived from the HSC in the bone marrow and are subsequently released into the bloodstream (185,212). Circulatory monocytes can mature into a blood-resident population of macrophages that play important roles in vascular homeostasis, by patrolling the vascular endothelium and clearing dysfunctional endothelial cells (189). Circulating monocytes can also be recruited to and migrate into tissues both in steady state and in response to infection (192). Therefore, monocytes can maintain tissue macrophage populations in steady state conditions at certain sites such as the intestine and act as an important source of highly pro-inflammatory macrophages during infection (186). At other sites such as the lung, they may only be required when local resident alveolar macrophages or intermediate interstitial macrophages are depleted (214).

Tissue-resident macrophage populations can become depleted during infection; a key role for circulating monocytes is the replenishment of the depleted tissue-resident macrophage population (192). This has been demonstrated for a

number of tissue resident populations, including cardiac and alveolar macrophages (212). The steady state cardiac macrophage population is predominantly of embryonic origin, but following depletion, circulating monocytes can infiltrate the cardiac tissue, proliferate and replenish this tissue-resident population, therefore the new cardiac macrophage population becomes predominantly adult monocyte-derived (212). Alveolar macrophages have also been shown to have an embryonic yolk sac origin but from a later foetal liver-derived stage (214). In a murine model of alveolar macrophage depletion and reconstitution utilising diphtheria receptor expression driven by cluster of differentiation 11c (CD11c) to induce alveolar macrophage depletion, bone marrow-derived monocytes were shown to replenish depleted alveolar macrophage populations, but this process required an intermediate step of monocyte differentiation; monocytes differentiated into interstitial parenchymal lung macrophages first, then were recruited to alveolae to complete differentiation into alveolar macrophages (215). In contrast, more recent studies have demonstrated that the predominant re-population mechanism for macrophages in the lung is the proliferation of the remaining resident macrophage population pre-existing in the tissue, without the requirement for monocyte infiltration (216). Although these reports describe different mechanisms for resident macrophage re-population in the lung, as well as varying extents of depletion of the resident lung population, it remains evident that both the presence of specialised tissue-resident macrophage populations, and recruited monocytes contribute to the maintenance of tissue resident macrophage populations. Both play roles, depending on the conditions, in the repopulation of the alveolar niche, and both are essential for optimal host tissue homeostasis and innate immunity. Moreover, it has been shown that under conditions of alternative activation, tissue-resident macrophage

proliferation is favoured over the recruitment of monocytes to generate macrophages, which is favoured during classical activation (217).

1.7.9.2 Macrophage activation

Macrophages can be activated, or polarised, by a variety of stimuli during infection, for example by cytokines, microbial components or live bacteria (by so-called vita-PAMPS) to display particular phenotypic traits and exert particular functions (218). Traditionally, polarised macrophage states have been described as either classically-activated or alternatively-activated, typically exhibiting pro-inflammatory and anti-inflammatory functions respectively (219). However, macrophage polarisation is not a fixed binary process; macrophages exhibit a high degree of plasticity and a range of stimulus specific activation states (185). Their phenotypes and functions can change over time depending on the requirements of the host and the environmental signals they are exposed to (185). Furthermore, macrophages may be activated to exhibit traits that are characteristic of different macrophage activation types simultaneously. Therefore, macrophage activation can be more accurately described as a spectrum to generate macrophages with a wide variety of specialised phenotypes and functions (185). This allows the macrophage to tailor responses to diverse stimuli and pathogens, which illustrates that macrophages can play roles in mediating all aspects of host immunity.

1.7.9.2.1 Classically-activated macrophages

The term “classically-activated” is widely used to refer to macrophages that are activated to exhibit a highly pro-inflammatory phenotype (185). These macrophages are characterised by their highly efficient microbicidal functionality and

enhanced antimicrobial effector mechanisms, such as production of pro-inflammatory cytokines, nutrient limitation, and reactive species production (185,219). Macrophage antimicrobial mechanisms are described in more detail below. In addition, these macrophages undergo a shift in their metabolism; although the primary mechanism for energy generation in many eukaryotic cells is ATP generation mediated by oxidative phosphorylation in the mitochondria, classically-activated macrophages switch to a glycolytic pathway of ATP generation, thus allowing the mitochondria to function in a more antimicrobial capacity via enhanced mitochondrial reactive oxygen species (mROS) production (220); this is discussed in more detail in the mitochondrial section of this thesis.

The polarisation of macrophages to the classically-activated phenotype is induced by a range of stimuli; these include the pro-inflammatory cytokines IFN-γ and TNF-α (219). It has been reported that in the early stages of the inflammatory response, cells such as ILC1s and NK cells secrete IFN-γ in response to stimuli such as cellular stress or infection, and this IFN-γ can prime macrophages and promote macrophage pro-inflammatory responses (179,185,221). In addition, γδ T cells are also an important source of pro-inflammatory cytokines such as IFN-γ, particularly at the barrier sites in which they are most abundant such as the skin or mucosal surfaces (182). IFN-γ is important for early macrophage activation but is short-lived, so in order to sustain a population of pro-inflammatory macrophages, a later IFN-γ signal is also required (185). It has been shown that this signal is typically derived from T helper 1 (Th-1) cells of the adaptive immune system, which illustrates a link between innate and adaptive immunity in promoting macrophage host defence responses. Initial reports suggested that although IFN-γ can prime macrophages, it

was insufficient to fully activate them alone (219). Therefore, the second signal of TNF-α is required to promote macrophage activation. Exogenous TNF-α in the inflammatory environment can provide this signal, but it has been reported that the TNF-α signal typically derives from macrophages themselves, via the activation of TLR signalling by, for example, PAMPs such as bacterial LPS or LTA (219). This demonstrates that multiple pro-inflammatory signals are required to function cooperatively to enhance macrophage host defence responses. This model, however, was likely influenced by the markers used to define activation state and, with a broader range of markers, IFN-γ stimulation may result in a more polarised phenotype than when combined with LPS (222).This has led to recognition that analysis needs to reflect the cell origin, the specific stimulus and the markers used and these need to be standardised between studies. It also leads to the conclusion that different stimuli will produce variations in the classical activation state (222).

However, classically-activated macrophage inflammatory responses require careful regulation because if uncontrolled, the strong pro-inflammatory responses can result in excessive host cell and tissue damage. It has been shown that excessive pro-inflammatory cytokine production by these macrophages can promote the development of T helper 17 (Th-17) cells (223). Th-17 cells generate high levels of the cytokine IL-17, which can promote the recruitment of neutrophils to infection sites and while designed to aid pathogen clearance can result in excessive inflammatory responses that can damage host tissues. The production of anti-inflammatory cytokines, such as transforming growth factor-beta (TGF-β) and IL-10, can be produced by a variety of cells including macrophages and can prevent excessive inflammatory responses by dampening macrophage activation (224,225).

For example, TGF-β has been shown to be produced by macrophages following the phagocytosis of apoptotic neutrophils, and this TGF-β production can lead to the inhibition of pro-inflammatory cytokine production, therefore dampen the macrophage inflammatory response (226).

1.7.9.2.2 Alternatively-activated macrophages

In contrast to classical activation, macrophages can also be polarised to exhibit a more anti-inflammatory phenotype and these are known as “alternatively-activated” macrophages (227). These macrophages are much less efficient at pathogen killing than their classically-activated counterparts and produce fewer antimicrobial factors such as pro-inflammatory cytokines and reactive species, but they are adept at producing anti-inflammatory cytokines such as IL-10 and interleukin 1 receptor antagonist (IL-1RA), dampening pro-inflammatory responses, and also promoting wound healing and tissue repair (228).

The primary signal promoting alternative macrophage activation is IL-4 and while granulocytes such as neutrophils and eosinophils are important generators in innate immunity (229), ILC2s and T helper 2 (Th-2) cells serve as the primary producers of IL-4 (179,185). This further demonstrates the importance of adaptive immune responses, in particular those from T helper cells, in the regulation of innate immune responses, as both Th-1 and Th-2 cells have been shown to influence pro- and anti-inflammatory macrophage activation, respectively. Stimuli that induce alternative activation such as IL-4, alter macrophage phenotype through reprogramming of metabolism (220). In contrast to classical activation, this is by enhancing mitochondrial oxidative phosphorylation and downregulating glycolysis

(220). A key mechanism by which IL-4 induced-alternatively activated macrophages exert their effects is via the induction of arginase activity (230). Arginase transcription can be induced by enhanced expression of anti-inflammatory cytokines such as IL-4 and TGF-β (230,231), and arginase catalyses the conversion of arginine to ornithine (228). Ornithine can act as a precursor for the production of polyamines and collagen, which are important factors for the production of the extracellular matrix, wound healing, and tissue repair (232). Arginase also plays a role in immunomodulation alongside its roles in wound healing; arginase-mediated arginine depletion can impair arginine-dependent immune responses (233). For example, arginine has been shown to be required for the proliferation of cytotoxic T cells which are important mediators of pro-inflammatory adaptive immune responses, therefore catabolism of arginine can inhibit cytotoxic T cell proliferation and dampen pro-inflammatory responses (234). In macrophages, NO production via inducible nitric oxide synthase (iNOS) requires arginine as an iNOS substrate, therefore arginine depletion inhibits NOS function and NO production (235). In addition, alternatively-activated macrophages produce anti-inflammatory cytokines such as IL-10 and IL-1RA, which, as mentioned previously, can act as negative regulatory signals to inhibit pro-inflammatory responses, which can therefore prevent inflammation-mediated host tissue damage (219).

1.8 Macrophage antimicrobial responses

Macrophages possess a vast array of mechanisms to ensure efficient clearance of pathogens. Some of these mechanisms are microbicidal and facilitate direct pathogen killing, and others function to weaken or counteract the defence mechanisms of the pathogen or enhance other host defence responses.

1.8.1 Phagocytic receptors and induction of phagocytosis

The phagocytosis and killing of pathogens by macrophages is a well-characterised early host defence strategy and is widely considered to be one of the most important mechanisms for pathogen clearance in innate immunity. Broadly, the term phagocytosis has been used to describe the recognition and engulfment of pathogens which leads to subsequent killing of the pathogen in the internal phagolysosomal compartment (203). More specifically, phagocytosis refers to the first stages of the process, pathogen recognition and engulfment, which subsequently leads to macrophage activation and induction of antimicrobial responses that lead to pathogen clearance (236). This is a complex process that involves a series of tightly regulated steps to ensure effective pathogen internalisation and killing.

Macrophages possess various cell surface and intracellular receptors that are designed to recognise specific microbial PAMPs. In bacteria, common examples of PAMPs include peptidoglycan, flagellin, and LPS present in gram-negative bacterial cell walls, and LTA present in gram-positive cell walls (203). These PAMPs can be recognised by receptors on the surface of or inside phagocytic cells, such as TLRs, and NLRs (137). Ten TLRs have been described in human cells (151). Some TLRs reside in the cell membrane and recognise pathogens in the extracellular environment, such as TLRs 1, 2, 4, 5 and 6, and others are located in endosomal membranes in the intracellular environment, such as TLRs 3, 7, 8, 9, and 10 (137). In macrophages, activation of TLR signalling can lead to a number of downstream effector responses such as classical macrophage activation, and production of pro-inflammatory cytokines and chemokines that play roles in modulating macrophage

pro-inflammatory responses (237). NLRs reside in the cytosol and following priming and activation form a macromolecular complex including caspases that can induce effects such as processing of interleukin 1 (IL-1) family cytokines or induction of cell death by pyroptosis (238). In the case of NLRP3, which detects a range of bacterial pathogens and microbial products including pore-forming toxins such as pneumolysin, priming follows TLR4 stimulation which induces the activation of NF-κβ and production of the pro-inflammatory cytokines IL-1β and interleukin 18 (IL-18) as well as upregulating NLRP3 expression. Priming also ensures NLRP3 deubiquitination (239). Following this, formation of the NLRP3–macromolecular inflammasome complex, containing a NLRP3 sensor protein subunit, caspase-1 and apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC), occurs after activation by appropriate triggers such as mitochondrial recruitment, mROS, mitochondrial cardiolipin, lysosomal cathepsins or potassium ion efflux (137,239).

Furthermore, in addition to direct recognition of bacterial pathogens by macrophage receptors, bacterial cells can be recognised following opsonisation; host defence components such as antibodies and proteins of the complement cascade can bind to structures on the bacterial cell surface, and these decorated bacteria can be recognised by macrophage opsonic receptors via recognition of the host defence components (240,241). The most well-characterised receptors facilitating the recognition of opsonised bacterial pathogens by macrophages are FcγRs and CRs (203). FcγRs recognises the Fc region of antibodies, therefore can respond to the binding of antibody-opsonised bacteria to the receptor (203). Bacteria that have been opsonised by complement proteins such as complement component 1q (C1q), C3,

C3b and complement component 4 (C4) are detected via binding to CRs on macrophages. In particular, the integrin family members CR3 and CR4 can mediate internalisation of pathogens opsonised with the C3bi complement protein (203). In many cases, as for example with opsonised *S. pneumoniae,* it is a range of opsonic receptors working in combination that maximise phagocytosis (242). In addition, macrophages also possess a number of non-opsonic receptors that can detect pathogens without the requirement for opsonisation (243). The mannose receptor, a member of the C-type lectin receptor superfamily, is considered to be an important example of a non-opsonic PRR (244). The extracellular portion of the receptor can detect and bind to mannose commonly found on the surfaces of bacterial and fungal microorganisms, while the cytoplasmic portion facilitates receptor internalisation upon pathogen binding. And receptor recycling between the macrophage plasma membrane and the early endosome membrane (244). Macrophages also express scavenger receptors, which describes a large group that consists of a variety of transmembrane glycoproteins with diverse pathogen recognition ability. Some of the most well-characterised examples of scavenger receptors in macrophages include the class A receptors scavenger receptor A (SR-A) and macrophage receptor with collagenous structure (MARCO), and the class B receptor cluster of differentiation 36 (CD36) (245). SR-A binds to LPS and LTA on bacterial surfaces to mediate non-opsonic phagocytosis of the pathogens (245). MARCO is constitutively expressed on macrophage subsets, but its expression can be greatly enhanced in macrophages via TLR activation by microbial stimuli (245,246). MARCO can bind LPS and also whole gram-positive and gram-negative bacterial cells (245,247). CD36 binds to LTA from gram-positive bacteria and macrophage-activating lipopeptide 2 (MALP-2), a lipopeptide from *Mycoplasma fermentans* (248). This receptor has been shown to act

as a TLR2 co-receptor, as CD36 was required for LTA- and MALP-2-mediated activation of TLR2 signalling in response to microbial diacylglycerides (245).

1.8.2 Phagosome formation

Following the recognition of bacterial pathogens by macrophages, signalling pathways are induced to facilitate the formation of the phagosome that ultimately is required to enable subsequent processing of the ingested bacteria. This involves a series of sequential steps of phagosomal maturation (249,250) This has been described in the most detail following FcγR-mediated receptor binding to IgG-opsonised bacteria (251,252). FcγR engagement activates a kinase cascade involving activation of Src family kinases and phosphoionisitide 3-kinase (PI3K) (253), resulting in the activation of the Rat sarcoma (Ras)-homologous (Rho)-family guanosine triphosphatases (GTPases), Ras-related C3 botulinum toxin substrate 1 (Rac1) and 2 (Rac2), and cell division control protein 42 homolog (Cdc42). These GTPases enable the remodelling of the actin cytoskeleton, a key component in the phagosome formation process (254). Actin polymerisation is induced at the site of pathogen recognition and binding, and this leads to the extension of pseudopod structures from the macrophage that envelop the surface-bound pathogen (254). Finally, phagosome sealing occurs to complete the internalisation process; another cytoskeletal component, myosin, is required to mediate the final scission of the newly-formed phagosome from the cell membrane (204).

1.8.3 Phagosome maturation and pathogen killing

The newly-formed phagosome undergoes a series of maturation steps to develop the acidic and degradative environment required to clear the internalised

pathogen (249). This involves the fusion of the phagosome in a sequential manner with different endosomal compartments within the cell, and this is represented schematically in Figure 1.1.

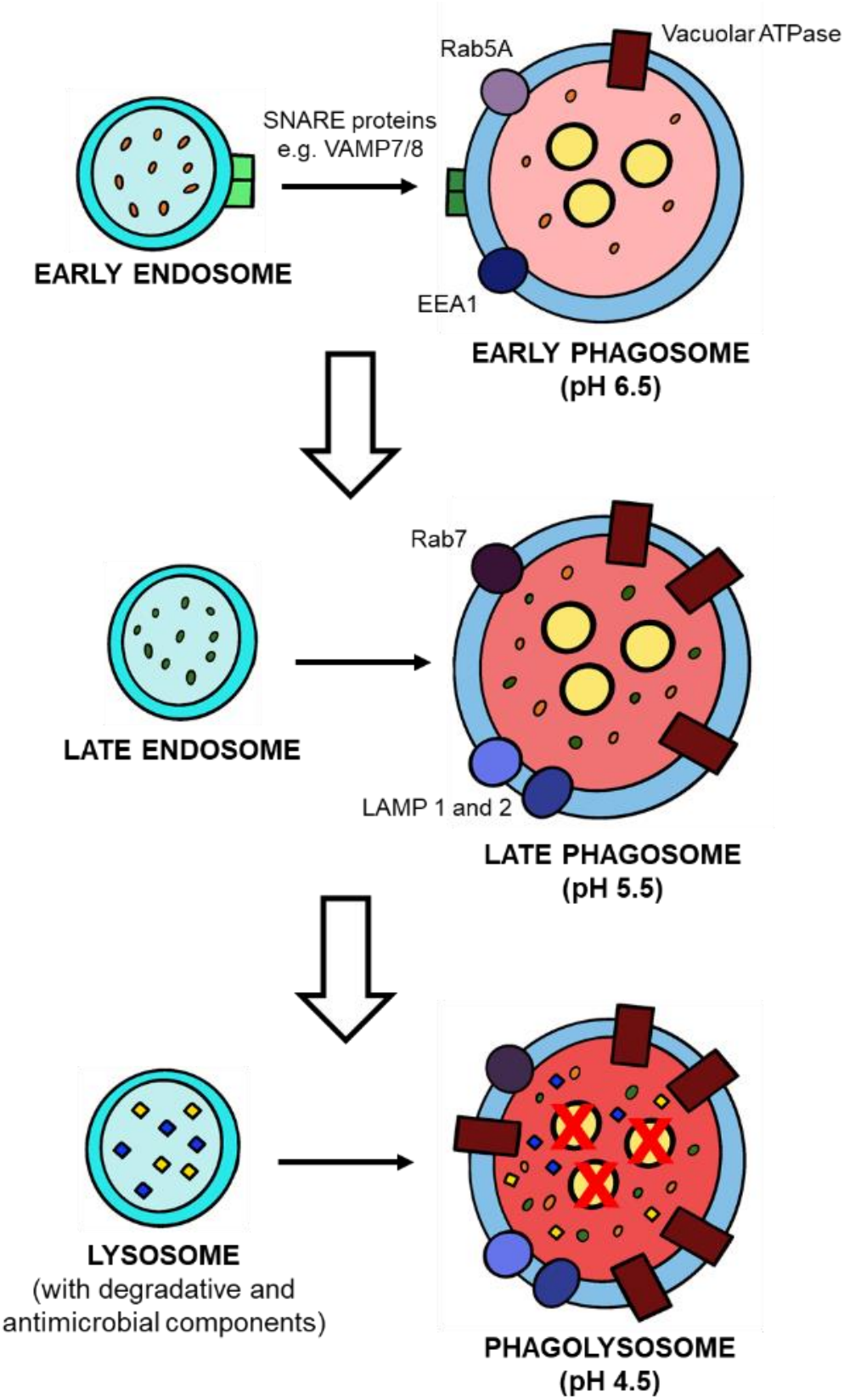


Figure 1.1: Schematic representation of the stages of phagosomal maturation following bacterial internalisation by macrophages.

The early phagosome is characterised by a mildly acidic phagosomal lumen, low levels of hydrolytic enzymes and the presence of the small GTPase Rat sarcoma-related protein 5A (Rab5A), a characteristic marker of early endosomes (249,255). Rab5A facilitates the first stages of phagosomal maturation by recruiting early endosome antigen 1 (EEA1) to the phagosome (204,256). The subsequent binding of various effectors leads to the recruitment and fusion of early endosomes that are required for phagosome maturation (204). The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) group of proteins play a critical role in vesicle fusion. SNARE proteins present on the incoming vesicle membrane and on the target vesicle membrane interact and form a protein complex which facilitates vesicular fusion (204). Vesicle associated membrane proteins (VAMPs) 7 and 8 (VAMP7 and VAMP8) have been highlighted as important SNAREs in the fusion of early endosomes to phagosomes (204). Rab5A is eventually lost and another small GTPase, Rat sarcoma-related protein 7 (Rab7), is acquired by the early phagosome which marks the progression to the late phagosome stage (257). Rab7 is required for maturation via the promotion of lysosomal fusion, as inhibition of Rab7 results in failed phagosomal maturation (258). Late phagosomes are also characterised by the acquisition of lysosome-associated membrane proteins 1 and 2 (LAMP1 and LAMP2), which have also been shown to be required for Rab7 recruitment to early phagosomes and successful phagosome maturation (259). The late phagosome also acquires an increasing density of vacuolar adenosine triphosphatases (v-ATPases) on its surface, which enhances acidification of the phagosomal lumen (260). Finally, phagosomal maturation is completed by the formation of the phagolysosome, a highly microbicidal organelle equipped with factors to facilitate pathogen degradation and clearance (249). Lysosomes

containing degradative enzymes and antimicrobial proteins, as well as hydrogen ions, are recruited to late phagosomes and fuse in a Rab7-dependent manner (249), which results in the release of these microbicidal factors into the phagosomal compartment; some of these will be detailed later in this section. Furthermore, additional v-ATPases are recruited to the phagolysosome which further acidifies the phagosomal lumen (249,260) to an extent that produces a hostile antimicrobial microenvironment, but also provides favourable conditions for the activity of some degradative enzymes that function optimally at an acidic pH (204). The killing of pathogens also facilitates additional host defence responses via the release of microbial components that can activate intracellular PRRs such as TLRs 3,7, and 9 and the NLRs nucleotide-binding oligomerisation domain 1 and 2 (NOD1 and NOD2) (261). To illustrate this, it has been shown in a murine macrophage model of *S. aureus* challenge that initial macrophage immune responses were induced by *S. aureus* interaction with TLR2 on the macrophage surface, and a second later inflammatory response was triggered upon interaction of degraded *S. aureus* components with TLR2 and TLR9, which resulted in enhanced macrophage inflammatory responses (261). Pathogen degradation was required for this second wave of signalling, as the TLR2 and TLR9 ligands were only released from a degradation-sensitive mutant, nut not, wild-type *S. aureus* (261). This further demonstrates that macrophages can utilise different inflammatory responses at different times during bacterial infection to mediate pathogen clearance.

It is evident that phagocytosis and phagolysosomal clearance of pathogens are essential macrophage microbicidal responses in innate immunity, however they do have some limitations. Although macrophages are adept at bacterial clearance,

phagolysosomal killing is a finite process (20), and pathogens have means to subvert individual components of the macrophage response (20,21). This has been demonstrated in a variety of models. In a macrophage model of *S. aureus* infection, although macrophages could sustain bacterial phagocytic uptake when challenged with a high bacterial burden, they could not sustain phagolysosomal killing, which therefore resulted in a persistent intracellular population of *S. aureus* (72). In addition, reduced phagolysosomal killing was associated with impairment of phagosomal maturation and acidification, and reduced activity of the lysosomal cysteine protease cathepsin D (72). *Mycobacterium tuberculosis* (Mtb) is widely regarded as a persistent intracellular pathogen that can survive and replicate within macrophages, and studies have shown that Mtb can prevent phagosome maturation from the early phagosome stage (262). These phagosomes possessed Rab5A but were unable to recruit the Rab5A-associated factors required for maturation progression, such as EEA1 (262). Furthermore, Mtb has the ability to escape the phagosome and alter other macrophage host defence responses to benefit its persistence (263). *Listeria monocytogenes (L. monocytogenes)* also prevents phagosomal maturation and promotes phagosomal escape by degrading the phagosomal membrane via the action of its pore-forming cytolytic toxin listeriolysin O (LLO), and via the action of phospholipases (264). Therefore, in these contexts, it is likely that a combination of antimicrobial responses in the macrophage, or a combination of responses from macrophages and from other immune cells are required to combat these subversions and enhance bacterial clearance.

1.8.4 Antimicrobial proteins

Successful phagosomal maturation and bacterial killing also involves the acquisition and activity of various antimicrobial proteins with different antimicrobial functions. The lysosome delivers a number of these antimicrobial proteins to bacteria-containing phagosomes (265), and the cathepsin family of proteases are some of the most well characterised and abundant lysosomal proteins. Cathepsins consist of cysteine, serine, and aspartate proteases and exhibit both antimicrobial and immunomodulatory effects to promote clearance of internalised bacteria (204). In a model of *S. aureus*-infected macrophages, the cysteine protease cathepsin L was shown to contribute to the killing of *S. aureus* by macrophages (266). Cathepsin K was required to promote the induction of pro-inflammatory cytokine production to achieve pro-inflammatory host defence responses in the presence of *S. aureus* (266). Cathepsin B plays an important role in the enhancement of IL-1β expression via activation of the NLRP3 inflammasome in macrophages and epithelial cells (267,268), but it has now been reported that multiple different cathepsins can mediate IL-1β production via NLRP3 inflammasome activation in macrophages (267). The NLRP3 inflammasome is a multi-protein complex (as described above) that is assembled in response to various stimuli, such as PAMPs and DAMPs. The NLRP3 inflammasome promotes macrophage antimicrobial responses by activating the proteolytic enzyme caspase-1, which leads to the processing of the leaderless IL-1 superfamily cytokines, particularly the pro-inflammatory cytokines IL-1β and IL-18 from their inactive pro-forms (269,270). The aspartate protease cathepsin D has been shown to promote the clearance of *L. monocytogenes* via cathepsin D-mediated degradation of LLO (271). Cathepsin D is also required for optimal

clearance of pneumococci in macrophages as it contributes the induction of apoptosis-associated killing (272).

The lysosome also possesses hydrolase enzymes that are designed to target and degrade different bacterial cell wall and membrane components, such as carbohydrates and lipids (249). A well-described carbohydrate-associated hydrolase is lysozyme, which functions by hydrolysing glycosidic linkages between peptidoglycan cell wall molecules. This weakens and destabilises the peptidoglycan wall of bacterial cells which can result in cell lysis (273). Phospholipases catalyse the hydrolysis of fatty acids in phospholipids, and a common example is phospholipase A2. This enzyme can directly target bacterial phospholipid membranes or can be activated in response to pro-inflammatory stimuli to generate pro-inflammatory lipid mediators, as shown in a macrophage model following LPS induction (274).

The cationic antimicrobial host defence peptides defensins and cathelicidin are also key players in macrophage host defence responses. Humans express two subclasses of defensins, α- and β-defensins (275). The α-defensins, human neutrophil peptide 1-4 (HNP 1-4) are primarily expressed in neutrophils and stored in granules, and are released upon pathogen-stimulated degranulation (275). Human β-defensins (hBDs) vary in expression; hBD1 is constitutively expressed, while hBDs 2,3, and 4 are expressed at low levels in steady state conditions. However, the expression of all four hBDs can be enhanced in response to inflammatory stimuli such as pro-inflammatory cytokines, PAMPs and DAMPs (275). Defensins exhibit direct bactericidal effects via the ability to form pores in bacterial cell membranes, and also possess immunomodulatory properties to enhance other host defence

responses (275). Cathelicidin is discussed in depth in the host defence peptides section of this thesis.

1.8.5 Nutrient limitation and metal sequestration

Many bacterial pathogens require access to nutrients and metal ions for survival. For example, some bacterial enzymes that contribute to bacterial cell survival and resistance to host defence responses require the integration of metal ions for their activity (249). Therefore, the macrophage has mechanisms to limit the availability of such nutrients in the macrophage to prevent nutrient acquisition by pathogens. A key mechanism for metal ion sequestration in macrophages is the expulsion of metal ions from the phagosome, in particular zinc (Zn^{2+}), iron (Fe^{2+}), and manganese (Mn^{2+}) cations (249). This is facilitated by the integral membrane protein natural resistance-associated macrophage protein 1 (NRAMP1) which is located in the phagosomal membrane. NRAMP1 functions to actively transport metal cations out of the phagosome, therefore depleting the metal ion concentration in the phagosomal lumen (276). In the context of Fe, following transport into the macrophage cytosol, Fe can be sequestered and stored in the macrophage by binding to ferritin proteins, which renders Fe unusable to pathogens (277). However, Fe can be released by lysosomal degradation of ferritin, demonstrating that macrophages have additional mechanisms to modulate Fe concentrations and availability (278). In addition to NRAMP1-mediated expulsion of Fe from phagosomes, the integral membrane protein ferroportin located on the plasma membrane can expel Fe from the cell completely, therefore diminishing the intracellular Fe pool (279). Ferroportin expression is regulated in response to pro-inflammatory stimuli; PAMP-mediated TLR signalling was shown to induce the

production of hepcidin in macrophages, a protein which can degrade ferroportin to prevent Fe expulsion from the cell and inhibit Fe availability for extracellular pathogens (279). However, to prevent Fe acquisition by intracellular pathogens, the production of iNOS-derived NO can re-establish ferroportin expression (280), therefore different macrophage host defence responses, NO production and Fe limitation, can interact to enhance macrophage antimicrobial responses.

1.8.6 Reactive species in macrophages- NADPH oxidase-derived ROS and iNOS-derived NO

The initial source of ROS produced in phagocytic cells is derived from the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system (204,281), while mitochondrial ROS represents a critical later source of ROS in macrophages and is discussed in detail in the mitochondria section. NADPH oxidase is a membrane-bound multi-protein complex which can be located on the macrophage cell membrane as well as the phagosomal membrane, and it results in the formation of superoxide (281). Structurally, this complex consists of a series of protein subunits: $p22^{phox}$ and $gp91^{phox}$, which are membrane-bound components involved in the transfer of NADP—associated electrons to molecular oxygen to generate superoxide radicals, and the cytosolic regulatory subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Ras-related C3 botulinum toxin substrates 1 and 2 (Rac1 and Rac2) (282). The NADPH oxidase complex is rapidly assembled on cell and phagosomal membranes following the detection of pathogens by phagocytic receptors and subsequent induction of downstream signalling (117). This NADPH oxidase-derived ROS possesses microbicidal activity against internalised pathogens in macrophages; NADPH oxidase rapidly releases an oxidative burst into the phagosome following

recruitment to the phagosome membrane, and the high superoxide concentration within the phagosome can help clear internalised pathogens (283). In addition to direct microbicidal functions, NADPH oxidase-derived ROS can also mediate other macrophage inflammatory responses. ROS from the oxidative burst can lead to the production of pro-inflammatory cytokines via modulation of a number of signalling pathways implicated in inflammatory responses, such as the hypoxia inducible factor (HIF) pathway, the NF-κβ pathway, the mitogen activated protein kinase (MAPK) pathway, and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (284,285). It can also provide a source of ROS to activate the inflammasome (239). Upregulation of pro-inflammatory cytokine production can enhance immune cell activation, induction of pro-inflammatory responses, and promote innate immune cell recruitment to infection sites. Classically-activated macrophages are adept at generating robust, sustained oxidative bursts compared to alternatively-activated macrophages (286), which indicates the importance of this process in facilitating an effective pro-inflammatory host defence response against intracellular pathogens.

However, there are limitations to the microbicidal effects of ROS in macrophages. NADPH-oxidase derived ROS may have limited functionality against bacteria that are resistant to reactive species due to well-developed antioxidant mechanisms to prevent ROS toxicity, for example *Salmonella enterica* and *S. pneumoniae* (287,288), and therefore may be insufficient to kill such bacteria on their own. Furthermore, the ROS produced by macrophages is less potent than the ROS produced by other immune cells such as monocytes or neutrophils (289,290), as most macrophages do not express the myeloperoxidase enzyme and therefore

cannot make the more potent halogenated species, such as hypochlorous acid (249). To combat this, the macrophage may combine ROS with other microbicidal strategies (291,292).

In addition to ROS, macrophages also generate reactive nitrogen species, particularly NO, as a microbicidal mechanism to clear intracellular bacteria. As mentioned above, NO is synthesised in macrophages by the enzyme iNOS, also termed nitric oxide synthase 2 (NOS2) (293). The catalytic activity of iNOS is induced by pro-inflammatory stimuli, such as detection of PAMPs, bacterial internalisation or signalling via pro-inflammatory cytokines (294). iNOS functions as a homodimeric structure, and catalyses the conversion of arginine into citrulline and NO (295). iNOS is localised to phagosomal membranes, facing the cytosolic side of the membrane; therefore, NO must diffuse through the phagosomal membrane to elicit its microbicidal effect on intracellular bacteria (249). Furthermore, as pathogens can circumvent NO and superoxide responses individually, NO can interact with superoxide within the phagosome to generate the more highly reactive species peroxynitrite (ONOO-), which possesses more potent microbicidal activity than superoxide and NO individually (281). However, NO production is usually delayed, much later than NADPH-oxidase derived ROS (292), and human macrophages generate less NO than some other animal species such as rodents (293). Therefore, although the generation of ROS and its combination with NO is important for pathogen clearance in macrophages, the limitations on the timing, localisation and potency of reactive species production give rise to the possibility that reactive species may need to interact with additional antimicrobial mechanisms in the macrophage to fully enhance pathogen killing. This provides the rationale for

studying the interactions of ROS and different macrophage antimicrobial responses, and their effects on modulating and enhancing host defence responses against bacterial pathogens.

1.8.7 Apoptosis-associated killing

The mitochondrial, or intrinsic, pathway of apoptosis is a critical process for clearance of pathogens in macrophages, in particular the clearance of persistent intracellular bacteria (292,296). The mitochondrial pathway of apoptosis is mediated by a series of processes including mitochondrial outer membrane permeabilisation (MOMP), cytochrome c release from the mitochondrial intermembrane space and activation of caspases. Induction of this apoptotic pathway requires the activation of B-cell lymphoma 2 (Bcl-2) family proteins B-cell lymphoma 2 associated X protein (Bax) and B-cell lymphoma 2 homologous antagonist (Bak), which are pro-apoptotic factors that associate with the mitochondrial outer membrane (297,298). Activated Bax and Bak are recruited to the mitochondrial outer membrane and induce MOMP (299,300). This facilitates the release of cytochrome c from the mitochondrial intermembrane space into the cytosol, where it binds to apoptotic protease activating factor 1 (APAF1) to form an apoptosome complex (301). This complex can cleave pro-caspase 9 into the active caspase 9 form, which subsequently activates other caspases such as caspase 3, 6, and 7 (302). These activated caspases primarily promote apoptosis by activating factors to facilitate nuclear fragmentation, chromatin condensation, and membrane blebbing (303–305). In addition, these caspases can also degrade mitochondrial-associated proteins including complexes of the electron transport chain (302), therefore resulting in extensive mitochondrial dysfunction, enhanced mROS production, and ultimately cell death.

The Dockrell group has extensively studied the role of apoptosis-associated killing in the context of late-stage *S. pneumoniae* infection of macrophages. *S. pneumoniae* can persist intracellularly after phagolysosomal killing mechanisms have been exhausted, so the apoptosis-associated killing pathway is a critical macrophage response for optimal clearance of S*. pneumoniae*. The *S. pneumoniae* pore-forming toxin pneumolysin (PLY) has been shown to contribute to LMP, an upstream event that can result in the induction of apoptosis, in response to phagocytosis of *S. pneumoniae* and in response to TLR signalling (110). PLY-mediated LMP occurred independently of PLY's pore-forming activity, and LMP promoted cell death by apoptosis rather than necrosis; the induction of apoptosis limited further activation of inflammatory responses, which can be enhanced by the induction of necrosis (110). In addition, cathepsin D was shown to be activated and released from the phagolysosome by LMP. Cathepsin D mediated the induction of macrophage apoptosis-associated killing mechanisms by promoting the degradation of the anti-apoptotic protein myeloid cell leukaemia-1 (Mcl-1) by its ubiquitin ligase myeloid cell leukaemia-1 ubiquitin ligase E3 (Mule) (272). Mcl-1 is a member of the Bcl-2 protein family, and it can heterodimerise with Bax and Bak to prevent induction of MOMP (306). Degradation of Mcl-1 can therefore promote Bax/Bak interactions with mitochondria and induction of MOMP (306). The importance of this pathway in the clearance of *S. pneumoniae* was emphasised by the fact that inhibition or knockout of cathepsin D inhibited the induction of macrophage apoptosis and bacterial killing, and inhibition of apoptosis by overexpression of Mcl-1 enhanced macrophage susceptibility to infection (292). However, apoptosis could be re-induced and bacterial killing increased via the addition of clodronate or B-cell lymphoma 2 homology domain 3 (BH3) mimetics, therefore highlighting a

pharmacological means of modulating apoptosis to enhance bacterial killing (292). Interestingly, apoptosis-associated killing mechanisms were induced in response to *S. pneumoniae* and *Haemophilus influenzae*, but not in response to *S. aureus* (292), suggesting that other macrophage host defence mechanisms are required to combat *S. aureus* infection.

1.9 Overview of mitochondria

Mitochondria are specialist organelles with essential roles in cellular energy generation and metabolic regulation in eukaryotic cells (307). These are double membrane-bound organelles that consist of an outer and inner mitochondrial membrane, separated by an intermembrane space. The inner mitochondrial membrane is organised into folded structures termed cristae and encloses the mitochondrial matrix. It has been reported that mitochondria evolved from an endosymbiotic relationship between eukaryotic cells and alphaproteobacteria; therefore, mitochondria still contain a portion of their own DNA that is distinct from nuclear DNA (307).

In steady state conditions, the mitochondria are primarily responsible for the generation of cellular energy in the form of ATP by aerobic respiration and oxidative phosphorylation (308). The latter refers to the generation of ATP via the transfer of electrons through the mitochondrial cristae-bound electron transport chain, a series of protein complexes that facilitate these redox reactions, and the subsequent proton flow to drive ATP synthase activity. This process is represented schematically in Figure 1.2. Mitochondria also play roles in a number of other cellular process (309,310), such as the synthesis of fatty acids and acetylation of proteins, which

requires the generation of acetyl coenzyme A (acetyl-CoA) from the tricarboxylic acid (TCA) cycle-derived citrate (311,312); regulation of the cell cycle, where mitochondrial biogenesis increases to provide the high energy demand required for cell division (313); and regulation of calcium (Ca) homeostasis via uptake and release of Ca ions from mitochondria depending on cellular Ca demand (314).

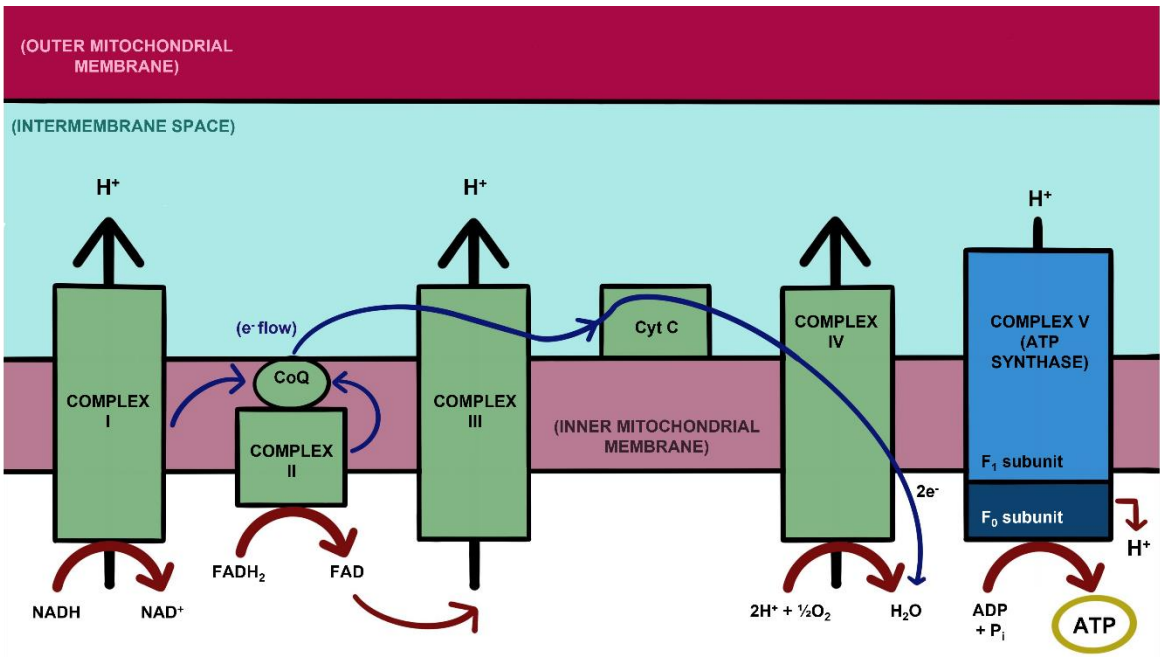


Figure 1.2: Schematic representation of the mitochondrial electron transport chain and the process of oxidative phosphorylation for ATP generation (315,316)**.** The electron transport chain consists of Complexes I to IV, Co-enzyme Q, and cytochrome C. The reduction of NADH and $FADH_2$ by Complexes I and II, respectively, provides the electrons which are transferred through the chain. Upon oxidation of oxygen as the terminal electron acceptor, an electrochemical gradient is generated that can be utilised by Complex V, also termed ATP synthase. The electrochemical gradient is used to generate a proton gradient across the inner mitochondrial membrane, and the resulting proton motive force drives ATP synthesis by the phosphorylation of adenosine diphosphate (ADP).

1.9.1 Mitochondrial metabolism and function in host defence responses in macrophages

Although mitochondria are specialist organelles whose key function is in metabolic regulation, they are also important effectors of host responses to pathogens in macrophages. As mentioned previously, the mitochondrial pathway of apoptosis is a critical macrophage antimicrobial response against persistent intracellular pathogens. However, during infection, mitochondria mediate other responses facilitating host defence.

Mitochondria play a role in pathogen sensing, in particular the detection of viral pathogens and subsequent activation of antiviral immune responses. Viral RNA is detected by the cytosolic receptor retinoic acid-inducible gene I (RIG-1); upon viral RNA binding, RIG-I activates mitochondrial antiviral signalling protein (MAVS) located on the mitochondrial outer membrane (317). The N-terminal domains of both RIG-I and MAVS contain CARDs, and activation of MAVS is facilitated by the interactions between RIG-I and MAVS CARDs (309,318). Activation of MAVS results in the phosphorylation and activation of transcription factors such as NF-κB and interferon regulatory factor 3 (IRF3), which upregulate the expression of type-I interferons (IFNs) such as interferon-alpha (IFN-α) and interferon-beta (IFN-β) (318). These cytokines are important in the antiviral immune response as they inhibit viral replication (318). Therefore, mitochondria are equipped to play a highly important role in antiviral signalling and activation of antiviral immune responses.

Low levels of mROS are produced as a by-product of oxidative phosphorylation, and this mROS can contribute to maintaining cellular homeostasis

(319,320). However, excessive mROS production can occur as a result of, for example, bacterial infection, cellular damage, and mitochondrial dysfunction (321). Excessive mROS accumulation can result in oxidative stress, which can lead to damage of essential cellular components such as DNA, and if cellular damage is extensive, cell death (284). Conversely, in the context of bacterial infection, an increase in mROS production plays essential roles in mediating host defence responses in immune cells such as macrophages to enhance pathogen clearance, both via direct bacterial killing and by modulation of other inflammatory responses through signalling roles (309,322); this will be discussed in more detail below.

Macrophage function is influenced by changes in metabolism. An important metabolic alteration in classically-activated macrophages is a shift from oxidative phosphorylation to glycolytic metabolism for ATP generation (198,323). Glycolytic metabolism is less efficient than oxidative phosphorylation (324), but it has been shown to promote rapid activation of pro-inflammatory macrophages at sites of infection (198). Glycolysis also promotes rapid ATP generation and a switch in mitochondrial function from ATP generation to increased mROS production in response to infection (325–327). In addition, there are breaks in the Krebs cycle in mitochondria of classically-activated macrophages which result in the accumulation of the Krebs cycle intermediates citrate and succinate (328), which both play roles in enhancing macrophage pro-inflammatory responses. Citrate accumulation enhances the generation of NADPH, which can subsequently be used to generate NO via iNOS and ROS via NADPH oxidase (198). Succinate accumulation enhances the expression of hypoxia inducible factor 1α (HIF-1α), which can act as a transcription factor and induce the expression of IL-1β via binding to the IL-1β gene promoter

(329). In addition to direct transcriptional activation of IL-1β, it has been shown that activation of glycolysis in macrophages, mediated by activation of the serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1) and the glycolytic enzyme hexokinase, is critical for activation of the NLRP3 inflammasome and subsequent activation of IL-1β (330). These processes demonstrate that there are close links between mitochondrial function, mitochondrial metabolism, and macrophage activation; the coordination and interactions of these are critical for the enhancement of host defence responses.

1.10 Mitochondrial dynamics

Mitochondria are dynamic organelles that undergo constant fission and fusion in response to changing cellular needs; this helps to regulate cellular functions including survival and metabolism (331). Fission is the process of splitting mitochondria and is required to increase mitochondrial numbers within a cell, duplicate mitochondria during cellular division, and segregate damaged mitochondria during mitophagy (332,333). Fusion is important for maintaining mitochondrial integrity, enhancing ATP generation if cellular energy demands are high, and replenishing mitochondrial DNA and other mitochondrial components following events that disrupt mitochondrial homeostasis, for example, cell division or mitophagy (334,335). The fusion process requires strict coordination of tethering of both mitochondrial outer and inner membranes in a sequential manner (336,337). These fission and fusion processes are tightly regulated and are mediated by different molecules; fission involves the splitting of mitochondria by the recruitment, oligomerisation and constriction of mitochondria by dynamin related protein 1 (Drp1) via interactions with membrane proteins mitochondrial fission 1 protein (Fis1) and

mitochondrial fission factor (Mff) (338,339), and fusion involves the tethering of mitochondria together by the dimerisation of mitofusins 1 and 2 (Mfn 1 and 2) on outer mitochondrial membranes and optic atrophy 1 (OPA1) on inner mitochondrial membranes (337). The fission and fusion processes are represented schematically in Figure 1.3.

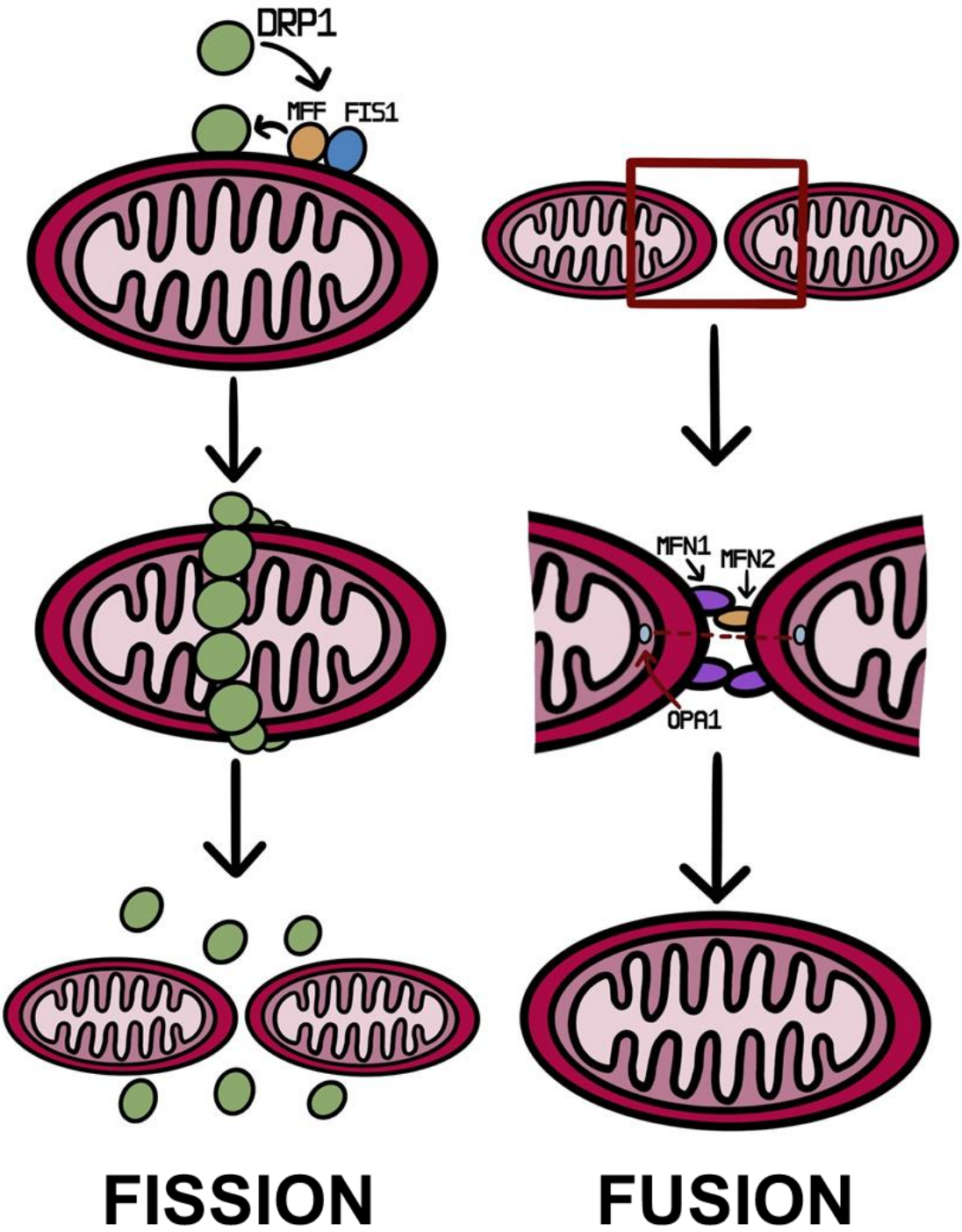


Figure 1.3: Schematic representation of the processes of fission and fusion, highlighting the key factors involved in fission (Drp1, Fis1, Mff) and fusion (Mfn1, Mfn2, OPA1).

1.10.1 Drp1 and its receptors Fis1 and Mff

The most well-characterised mitochondrial fission pathway involves the recruitment and activity of Drp1 (331,340–342). This 80 kDa protein consists of 699 amino acids and contains three domains (332,343); an N-terminal GTPase domain, which is required for Drp1-mediated mitochondrial constriction and scission; a dynamin-like middle domain, which is involved in the oligomerisation of Drp1 units, and a C-terminal GTPase effector (GED)/assembly domain, which is important for Drp1 targeting and recruitment to mitochondria and also the regulation of Drp1 GTPase activity (343). Drp1 knockouts in mouse embryos have been shown to be lethal (344), suggesting that mitochondrial fission, and the involvement of Drp1, are essential for survival.

Drp1 is primarily located in the cytoplasm in the form of dimers or tetramers (345,346), but is recruited to outer mitochondrial membranes where multiple Drp1 units can self-assemble into oligomeric helical rings that surround mitochondria at mitochondrial division sites (347). Structurally, the middle and C-terminal GED domains fold and interact to create a stalk-like structure, which results in the orientation of the GTPase region away from the mitochondrial membrane (348), then Drp1 units oligomerise into helical rings around mitochondria via binding of the stalk-like structures (349). GTP hydrolysis occurs via the interactions between the GTPase regions of these different Drp1 subunits (347). Through Drp1's GTPase activity, the structure changes conformation; the helix compresses and constricts to facilitate mitochondrial fission (350). Although Drp1 activity is important for the constriction of mitochondria during fission, more recently it has been shown that Drp1 alone may be unable to complete the scission process following membrane

constriction (351), and another member of the dynamin protein family, dynamin-2 (Dnm2), is required to facilitate mitochondrial scission (352).

Mitochondrial fission occurs at particular sites on the mitochondria, which are commonly the contact sites between the mitochondria and the endoplasmic reticulum (ER) (353). Mitochondrial-ER interactions mediate lipid synthesis, and the uptake of ER-derived Ca into mitochondria, where Ca can act as an important signalling molecule in the regulation of mitochondrial metabolism (354). The constriction of mitochondria can be induced by this ER contact; the ER tubules can encircle mitochondria and induce the initial constriction event prior to the oligomerisation of Drp1 units at these sites (353). Furthermore, ER tubules constrict mitochondria to the diameter required for the assembly of Drp1 oligomeric structures; Drp1's helical conformation around mitochondria is larger in diameter than those created by other dynamin protein such as dynamin-1, but it is the correct size to form around mitochondrial fission sites following initial constriction (355). It has also been shown that the cytoskeletal components actin and myosin II are recruited to mitochondria-ER contact sites and provide the forces required for initial constriction via actin polymerisation and myosin motor functionality (356,357). It is evident that mitochondria-ER contact sites are important for the initiation of the fission process, then the dynamin family proteins Drp1 and Dnm2 function co-ordinately to complete fission in a sequential manner; Drp1 mediates the further constriction of mitochondria at fission sites following the action of initial ER tubule constriction, then Dnm2 is transiently localised to these constricted sites to mediate final scission (352). This illustrates that although the role of Drp1 has been widely described, multiple

components and processes such as ER tubule constriction and other dynamin family proteins are required to facilitate mitochondrial fission.

In order to facilitate fission, Drp1 requires specific targeting to mitochondrial outer membranes from the cytosol. Mitochondrial outer membranes have receptors that recruit and interact with Drp1; the most well-characterised are the integral adapter proteins Fis1 and Mff (356,358–360). Fis1 and Mff are anchored into the outer mitochondrial membrane by their C-terminal transmembrane domains while the N-terminal domains are oriented into the cytosol in order to interact with Drp1. Fis1 is diffusely localised across the entire outer membrane, while Mff localisation is more punctate and concentrated at sites of Drp1 accumulation (358). These proteins act as platforms to facilitate Drp1 recruitment and oligomerisation during fission. However, reports investigating Fis1 and Mff in mammalian cell line models have suggested that Mff is the dominant receptor for Drp1; knockdown of Fis1 expression did not affect Drp1 recruitment (361), while knockdown of Mff expression resulted in a loss of Drp1-enriched foci (358,362). Furthermore, it has been shown that Mff functions as the primary Drp1 receptor in tandem with adaptor proteins termed mitochondrial dynamics 49 (MiD49) and 51 (MiD51), which contribute to the recruitment of Drp1 to mitochondria and mediate the assembly and stabilisation of Drp1 oligomers (363).

Fission can also be regulated by the post-translational modification of Drp1 by phosphorylation of specific serine residues, which can alter Drp1 activity. The phosphorylation of two different serine residues has contrasting effects on Drp1; phosphorylation of serine 616 by cyclin B1-cyclin-dependent kinase enhances Drp1 activity (364), while phosphorylation of serine 637 by protein kinase A inhibits Drp1

activity (365). The phosphorylation of serine 616 and dephosphorylation of serine 637 have been shown to facilitate Drp1 recruitment to mitochondria in addition to influencing Drp1 activity (366). Furthermore, a link between Ca metabolism, Drp1 phosphorylation and mitochondrial fission has been described; calcineurin is a Ca-sensitive protein phosphatase and can activate Drp1 via dephosphorylation of serine 637 (364), while Ca/calmodulin-dependent protein kinase Iα (CaMKIα) phosphorylates serine 637 upon voltage-dependent anion channel (VDAC)-mediated Ca influx into mitochondria (367). Therefore, alterations in Ca homeostasis can contribute to the regulation of mitochondrial dynamics by modulating Drp1 phosphorylation.

1.10.1.1 Drp1-independent fission mechanisms

Some studies have now begun to investigate Drp1-independent fission. One study identified a protein termed TBC1 domain family member 15 (TBC1D15) as a Fis1-binding protein (368). This protein was found to co-localise to mitochondria when stabilised by the interaction with Fis1 in HeLa cells. Knockdown of TBC1D15 had similar effects to Fis1 knockdown; mitochondrial networks developed a highly branched structure independently of Drp1. Therefore, fission could be activated using many of the canonical components but without the activity of Drp1. In a model of HeLa cells challenged with the *L. monocytogenes* pore-forming toxin LLO, mitochondrial fission was observed in the absence of Drp1 oligomers, when Mff was degraded, and in cells with impaired Drp1 function (369). Interestingly, mitochondrial-ER contact sites marked LLO-induced fragmentation sites despite the absence of Drp1, suggesting that the ER can also regulate the fission process independently of Drp1. Another study also identified a mitochondrial inner membrane protein

homologue in *Drosophila melanogaster*, PMI, to be involved in regulation of mitochondrial morphology independently of the canonical Drp1 and Mfn pathways (370). PMI was shown to promote fission, as inactivation of PMI resulted in highly elongated mitochondrial networks, and in a PMI-null fly model, mitochondrial networks could not be restored in the absence of OPA1 or altered by Drp1 overexpression. These studies illustrate that in a variety of models, mitochondrial fission can occur by other mechanisms independently of Drp1. However, Drp1 still remains the key regulator of fission (332), so Drp1-independent fission mechanisms still require ongoing investigation and whether there is a non-canonical Drp-1 independent form of fission is still debated.

1.10.2 Mitofusins

The dynamin-like GTPases mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) are integral mitochondrial outer membrane proteins required for the first process of outer membrane fusion (371,372). Structurally, Mfns contain a GTPase domain at the N-terminal end, and two transmembrane domains at the C-terminal end, which are important for localisation of the protein to the outer mitochondrial membrane (373,374). The N-terminal and C-terminal domains are flanked by two coiled coil motif regions with two heptad repeats; these domains are termed heptad repeat 1 (HR1) and 2 (HR2) (371). The transmembrane domains traverse the outer membrane twice which positions the protein's functional domains into the cytosol, therefore providing access for Mfns on adjacent mitochondrial membranes to interact and facilitate tethering (374). Mfns can form homodimers or heterodimers with Mfns on adjacent mitochondria (372), and this interaction occurs via anti-parallel linkages between the HR2 regions (371). Both of the N-terminal and C-terminal domains of

Mfn2, but not Mfn1, are oriented into the cytosol, which allows for interactions between HR1 and HR2 within the same Mfn2 molecule (342). HR1-HR2 interactions within the same Mfn2 molecule renders the HR2 region inaccessible for interactions with other Mfn2 molecules on adjacent mitochondria, therefore acts as a fusion regulatory mechanism (342). Drp1 has been suggested to play an unexpected role in the fusion process by interacting with the HR1 region of Mfn2, which mediates the dissociation of HR1 from HR2 and renders HR2 accessible to facilitate fusion (342).

In addition to mitochondrial tethering, Mfn2 can also be found in abundance at mitochondria-ER contact sites. In a similar manner to mitochondrial membrane tethering, mitochondrial-ER tethering is mediated by the homodimeric or heterodimeric interactions of Mfn2 localised on ER membranes with Mfn1 or Mfn2 on mitochondria (375). Therefore, alongside its role in the regulation of mitochondrial dynamics, Mfn2 also plays an important role in Ca-mediated regulation of mitochondrial metabolism by mediating mitochondria-ER tethering and mitochondrial uptake of ER-associated Ca (375).

1.10.3 OPA1

Inner membrane fusion is mediated by the inner mitochondrial membrane dynamin-like GTPase OPA1 (336,337). This protein consists of an N-terminal transmembrane domain which anchors it into the inner membrane and allows the rest of the protein to reside in the mitochondrial intermembrane space, and a GTPase domain located in the C-terminal region (376). This N-terminal domain of OPA1 possesses an alternative splicing domain which allows for the generation of eight alternative splicing mRNA variants, and each variant can be proteolytically

processed into two isoforms which are both required for fusion (377,378); the long isoform remains tethered to the inner membrane, and the soluble short isoform is released into the intermembrane space. Proteolysis of OPA1 occurs in the mitochondrial intermembrane space by the metalloproteases, metalloendopeptidase OMA1 (OMA1) and YME1 (379). OPA1 also contains two coiled coil regions, one at the N-terminal end following the transmembrane domain and one at the C-terminal end following the GTPase domain (366). OPA1 facilitates the tethering of two inner mitochondrial membranes to complete the fusion process; Mfn-mediated fusion of outer mitochondrial membranes brings the OPA1 molecules on inner mitochondrial membranes into close proximity to facilitate inner membrane fusion (337). Both coiled coil domains, but in particular the C-terminal domain, are involved in the homodimeric interactions between the adjacent OPA1 molecules (380).

OPA1 also has additional roles in the regulation of mitochondrial structure, metabolism and function that are independent of its fusion activity; OPA1 can regulate and remodel mitochondrial cristae structures via the regulation of cristae tight junction diameter (381). By maintaining tight junction integrity, OPA1 can protect cells from apoptosis by preventing cytochrome C release (381). OPA1 can also regulate Ca metabolism by reducing the uptake of Ca into mitochondria via tight junction modulation (382). OPA1 can modulate the assembly of electron transport chain complexes in the cristae, particularly ATP synthase, in response to changing metabolic conditions and cellular energy demand (383). Furthermore, regulation of ATP synthase assembly via OPA1 cristae modulation can prevent excessive mROS accumulation (384), thereby OPA1 can influence mROS production via alterations to the mitochondrial cristae structure.

1.11 Mitochondrial dynamics in the modulation of macrophage function, mitochondrial metabolism, and host defence responses

Mitochondrial dynamics play a variety of roles in the modulation of macrophage function and host defence responses. Changes in mitochondrial morphology can influence macrophage activation status and mitochondrial metabolism. Classically-activated macrophages have been shown to exhibit more fragmented mitochondria while alternatively-activated macrophage mitochondrial networks are more branched; one study confirmed these phenotypes in vivo, in a mouse model of LPS challenge (385). Prior to LPS challenge, resident macrophages exhibited an alternatively-activated phenotype and elongated mitochondria, and oxidative phosphorylation activity for ATP generation was observed. Following LPS challenge, macrophages exhibited more fragmented mitochondrial networks and higher rates of glycolysis, which are characteristic of classically-activated macrophages. Greater mitochondrial fragmentation is also associated with enhancement of macrophage pro-inflammatory responses, including increased production of cellular and mitochondrial ROS (386). Knockdown of fusion components such as Mfn2 and OPA1 promote mitochondrial metabolic changes associated with classical activation, including reduced oxygen consumption, Krebs cycle activity and oxidative phosphorylation capacity, loss of mitochondrial inner transmembrane potential ($\Delta\psi_m$), and higher rates of glycolysis (366). Therefore, disruption of mitochondrial dynamics and homeostasis can modulate mitochondrial energy generation and pro-inflammatory responses, which illustrates the importance of regulating fission and fusion to regulate cellular function.

Changes in mitochondrial dynamics also influence apoptosis, which as mentioned previously, is an important host defence response in macrophages to clear intracellular bacteria. It has been shown that fission occurs upstream of characteristic apoptotic processes such as caspase activation, suggesting that fission may be involved in the initial early stages of apoptosis (387). As described above, fission can promote mitochondrial outer membrane permeabilisation and loss of $\Delta\psi_m$, and it has been shown that as a result, fission can induce cytochrome c release from mitochondria which subsequently activates caspase 9 and downstream apoptotic processes (366). Therefore, activation of fission can lead to enhanced apoptosis. In addition, the inhibition of fusion components can also enhance apoptotic processes. Mfn2 can interact with the pro-apoptotic Bax protein during apoptosis, where Bax inhibits Mfn2 fusion activity (388). Depletion of OPA1 results in enhanced apoptosis, while OPA1-mediated cristae re-modelling can also inhibit apoptosis and release of cytochrome c by promoting tight junctions in cristae (381). Promoting fusion protects against apoptosis; the anti-apoptotic factor B-cell lymphoma-extra (Bcl-X) has been shown to induce greater levels of fusion to prevent apoptosis (389). It is evident that modulation of mitochondrial dynamics has important consequences for the activation of apoptosis, but it has been shown that this is not sufficient to solely induce apoptosis. However, this demonstrates that fission and fusion components can participate in the apoptotic process independently of their canonical roles in mitochondrial dynamics (366).

Alongside its roles in initiating apoptosis, fission can also promote the clearance of other apoptotic cells by macrophages in a process known as efferocytosis; this process is important in the resolution of inflammation, as removal

of apoptotic cells prevents host cell and tissue damage via secondary necrosis and further inflammation (390). Macrophage uptake of apoptotic cells was shown to induce fission, and fission was shown to be a critical process allowing macrophages to efficiently clear high levels of apoptotic cells (390). Deficiencies in the fission pathway resulting in macrophages unable to internalise multiple apoptotic cells. Fission-deficient macrophages also exhibited impaired phagosome sealing following apoptotic cell internalisation, and impaired degradation of apoptotic cells; this was associated with defective intraphagosomal ROS production. Therefore, as well as promoting apoptosis, enhancement of fission can also promote the resolution of inflammation, which further illustrates the importance of mitochondrial dynamics to modulation of macrophage host defence responses.

Cell death can occur in response to excessive cellular damage, and mitochondrial dysfunction can be one trigger contributing high levels of ROS generation and altered protein homeostasis ('proteostasis'); however, mitochondria possess quality control mechanisms to protect themselves from damage and dysfunction, thereby protecting the whole cell (391). If damage to mitochondria from, for example, misfolded proteins or ROS accumulation, is not excessive, mitochondria can selectively segregate and remove damaged portions for degradation in a specialised form of autophagy, termed mitophagy (392). The most well-characterised mitophagy pathway involves the mitochondrial serine/threonine kinase phosphatase and tensin homologue-induced kinase 1 (PINK1) and the cytosolic E3 ubiquitin ligase Parkin (391,393). In steady state conditions, PINK1 is continuously degraded via the proteolytic action of mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL) proteins in the mitochondria, in a

mechanism that involves import of PINK1 from the outer mitochondrial membrane. PINK1 subsequently translocates to the cytosol and is degraded by the proteasome (391). However, following mitochondrial damage or dysfunction, a loss of $\Delta\psi_m$ leads to reduced mitochondrial import and cleavage of PINK1 and accumulation of PINK1 on the outer mitochondrial membrane. PINK1 becomes activated via autophosphorylation; activated PINK1 phosphorylates and activates outer mitochondrial membrane ubiquitin at serine 65 (394), which subsequently recruits Parkin to the mitochondria (391). Parkin is activated by PINK1 via phosphorylation and by its E3 ubiquitin ligase activity, it polyubiquitinates a variety of mitochondrial protein substrates, including mitochondrial fusion components, mitochondrial rho guanosine triphosphatase 1 (MIRO1/Rhot1) which leads to inhibition of mitochondrial motility, and VDAC 1, therefore targeting them for proteasomal degradation (393). Ubiquitination and degradation of Mfn 1 and 2 enhances fission, which can facilitate the process of segregation of mitochondrial portions during mitophagy. It also prevents the segregated, damaged mitochondrial portions from re-fusing to the healthy mitochondrial network, and the enhanced fragmentation process more easily facilitates the clearance of these portions by autophagosomes (393). Historically, it was thought that Drp1 was required to facilitate mitophagy, but recent studies have described Drp1-independent mitophagy mechanisms. One study in a HeLa cell model has now suggested that Drp1 participates in mitophagy, but rather than facilitating the scission of mitochondria, it preserves the healthy portions of the mitochondrial network by limiting PINK1 and Parkin activity to the specific regions of the mitochondrial network that are damaged or contain misfolded or mutated proteins, and therefore require segregation (333). This was validated in Drp1-knockout cells; loss of Drp1 resulted in excessive PINK1 and Parkin activity across

the whole mitochondrial network and enhanced rates of mitophagy (333). Therefore, mitochondrial dynamics also play a central role in maintaining mitochondrial homeostasis and facilitating mitochondrial quality control.

1.12 Mitochondrial reactive oxygen species (mROS)

The generation of ROS is important for efficient bacterial clearance in macrophages (322), and mitochondria are a primary source of ROS production in immune cells such as macrophages (309) The term 'reactive oxygen species' is widely used to describe a collection of highly reactive molecules that contain one or more oxygen atoms. One of the most prevalent reactive oxygen species is superoxide, which is produced by the one-electron reduction of molecular oxygen (320). Superoxide is typically converted to H_2O_2 by reaction with water molecules or by the action of superoxide dismutase enzymes (SODs) but can act as a precursor to additional ROS subspecies (322). ROS molecules can be classified into two different subsets: free radicals, such as superoxide, hydroxyl radical (OH•), alkoxyl (•OOR) and peroxyl radicals (•OOH); and non-radicals such as H_2O_2, singlet oxygen ($^1O_2$), ozone (O_3) and hypochlorite anion (OCl^-) (322).

1.12.1 Mechanisms of mROS production and regulation

The mitochondria serve as a highly important site of ROS production, and the primary ROS from mitochondria is H_2O_2 which is generated by the detoxification of superoxide.

Regular electron transport involves the flow of electrons from their entry point at Complex I to Complex IV of the electron transport chain with oxygen as a final

electron acceptor. However, disruption of the electron transport chain in response to, for example, mitochondrial dysfunction, alterations in mitochondrial metabolism, or induction of apoptosis, can interrupt electron flow and result in the premature release of electrons from the chain. Superoxide is generated by the single electron reduction of molecular oxygen and is primarily produced by the premature release of electrons from Complexes I and III, and their interactions with oxygen (395,396). Complex I-derived superoxide is confined to the mitochondrial matrix, as superoxide is unable to cross the mitochondrial inner membrane; however, Complex III-derived superoxide can be liberated from both the mitochondrial matrix and the intermembrane space (397). Superoxide released into the intermembrane space has the ability to exit the mitochondria into the cytosol via VDACs in the mitochondrial outer membrane (398,399). In addition, it is possible for electrons to flow back from Complex II to Complex I, thereby reducing oxidised nicotinamide adenine dinucleotide (NAD+) to NADH and generating high levels of ROS via Complex I; this process is known as reverse electron transfer (RET), as occurs during ischemia reperfusion injury (400).

Superoxide is cytotoxic at high concentrations; therefore, cells possess antioxidant mechanisms to protect themselves from oxidative stress. Superoxide dismutases (SODs) are key enzymes in the regulation of superoxide production. Three SODs with different mitochondrial and cellular localisations act to neutralise superoxide; SOD1 can be found in both the mitochondrial intermembrane space and the cytosol, SOD2 resides in the mitochondrial matrix, and SOD3 is anchored to the extracellular matrix (398). SODs neutralise superoxide by catalysing the reaction

between two superoxide anions in the presence of hydrogen, thereby resulting in the generation of H_2O_2 and oxygen (322).

H_2O_2 is an important component of the host defence response to infection and participates in the oxidative burst to mediate pathogen clearance. As mentioned previously, superoxide cannot cross membranes except via channels such as VDACs, and they are also highly reactive and very short lived molecules (322,399). H_2O_2 is more stable and can diffuse across membranes, therefore is more suited to a variety of signalling roles within a cell (322,398). However, H_2O_2 can still be cytotoxic if not regulated, therefore additional enzymatic antioxidant mechanisms targeting the neutralisation of H_2O_2 are also prevalent within cells. Peroxiredoxins can be oxidised by H_2O_2 which generates H_2O, and oxidised peroxiredoxins can be reversibly reduced by thioredoxin to restore enzymatic activity (76). Glutathione peroxidases function in a similar manner to peroxiredoxins- oxidation by H_2O_2 releases H_2O, and glutathione peroxidases are reduced by glutathione (398). Catalase, which is localised to peroxisomes, catalyses the degradation of H_2O_2 into H_2O and O_2 (398).

1.12.2 Antimicrobial effects of mROS

mROS possess both microbicidal and signalling properties, which illustrates the importance of ROS in facilitating an efficient and effective host immune response to pathogens. mROS has direct bacterial killing functionality to directly clear pathogens, including pathogens that have been internalised by phagocytic immune cells such as macrophages. A key mechanism for facilitating mROS production and its bactericidal effects against intracellular bacteria in macrophages has been

characterised, and this process involves the activation of the TRAF6-ECSIT pathway via TLR signalling (401). PAMPs such as LPS and LTA derived from bacteria internalised by macrophages activate signalling via TLRs 1/2, 2 and 4 present in the phagosomal membrane. This leads to recruitment of mitochondria to the phagosome, and the recruitment of the TLR signalling intermediate component TNF receptor-associated factor 6 (TRAF6) to the mitochondria. Here, TRAF6 interacts with evolutionarily conserved signalling intermediate in Toll pathway (ECSIT) present on mitochondrial outer membranes; ECSIT has been shown to interact with mitochondrial proteins and assist in the assembly of electron transport chain Complex I. TRAF6 possesses E3 ubiquitin ligase activity, therefore can modify ECSIT by polyubiquitination. This modification to ESCIT and resulting loss in activity alters the activity of the oxidative phosphorylation machinery, resulting in an increase in Complex I-derived superoxide generation. Superoxide cannot cross membranes, but once superoxide is converted to H_2O_2, it can cross the mitochondrial and phagosomal membranes to participate in bacterial killing directly (401).

The importance of mROS antimicrobial functions in bacterial clearance have been demonstrated in a number of intracellular bacterial models. In *Escherichia coli*- or *L. monocytogenes* -infected murine bone marrow-derived macrophages (BMDM), mROS was shown to participate in the clearance of intracellular bacteria following mitochondrial recruitment to phagosomes, mediated by the phagosome-localised kinases macrophage stimulating 1 and 2 (Mst1 and Mst2) (402). BMDM lacking these kinases exhibited higher bacterial burdens and reduced mROS production than the wild-type counterparts (402). Enhancement of mROS production has been shown to be required for the delayed phase microbicidal response to intracellular *S.*

pneumoniae in alveolar macrophages following the exhaustion of early stage phagolysosomal killing mechanisms; mROS production was shown to be regulated by the expression of the anti-apoptotic protein Mcl-1 (403). In alveolar macrophages from chronic obstructive pulmonary disease (COPD) patients, Mcl-1 expression levels were enhanced, and this was associated with reduced mROS production and impaired bacterial clearance (403). Mtb is known to be a persistent intracellular pathogen of macrophages; it has the ability to replicate within macrophage phagosomes, and escape from phagosomes into the cytosol (204). Studies in Mtb-infected zebrafish macrophages have shown that TNF-induced production of mROS and opening of the mitochondrial permeability transition pore (mPTP) to allow mROS to translocate to the cytosol, where it could participate in the killing of cytosolic Mtb (404,405). Conversely, in a Mtb-infected BMDM model, it was shown that mROS did not have a direct antibacterial role in Mtb clearance (406). However, it has been suggested that since Mtb can persist intracellularly and circumvent macrophage defence responses such as NADPH oxidase-derived ROS and phagolysosomal degradation, the macrophage can induce excessive levels of ROS production and cell death in order to clear the pathogen and prevent further infection (322).

A more recent mechanism for activation of mROS production and killing of bacteria internalised by macrophages has been described in a model of MRSA infection (407). MRSA internalisation induced an enhancement in ROS production. In order to kill internalised MRSA, mROS were transported to bacteria-containing phagosomes by the formation of mitochondria-derived vesicles (MDVs) (407). MDVs are small, double-membrane cargo transport vesicles that form and detach from mitochondria independently of the Drp1-mediated mitochondrial fission mechanism

(408,409). These vesicles specialise in the transport of mitochondrial proteins and lipids to other organelles, including transport of cargo to lysosomes for degradation (409). The formation of MDVs for transport to lysosomes requires the activity of Pink1 and Parkin (410), and the production of MDVs in this manner has been shown to mediate mitochondrial quality control; the transport of only the dysfunctional proteins for degradation may be sufficient to preserve the whole organelle. In the context of MDV-mediated delivery of mROS to MRSA-containing macrophage phagosomes, activation of TLR signalling was shown to be necessary to induce vesicle formation (402), then Pink1 and Parkin activity was required to complete the process (410). PINK1/Parkin-mediated vesicle formation was shown to occur independently of their mitophagy functionality (410), demonstrating that these components are multifunctional; in addition to mitochondrial quality control, they can also participate in mROS-associated bacterial killing.

These studies all illustrate the importance of mROS for bacterial killing of intracellular bacteria in macrophages, and also demonstrate critical links between innate immune signalling, modulation of mitochondrial structure and function, mROS production and enhanced macrophage host defence responses.

1.12.3 Signalling functions of mitochondrial ROS

mROS have an abundance of signalling functions in addition to roles in direct bacterial killing. The release of mitochondrial components such as mROS, mitochondrial DNA, or cardiolipin during infection can act as DAMPs (411). TLRs are important transmembrane receptors that not only detect and bind PAMPs, but also DAMPs, which can modulate the host innate immune response via TLR signalling

action. mROS act as important innate immune signalling molecules and have been shown to enhance the production of pro-inflammatory cytokines and chemokines via activation of the MAPK cascade by inhibiting MAPK phosphatases (269,412). Sustained MAPK activation leads to activation of the downstream signalling intermediates extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). These kinases can subsequently promote the activation of a wide range of transcription factors, which ultimately results in the production of pro-inflammatory cytokines and chemokines that are important for eliciting an effective pro-inflammatory innate immune response (269,412).

Alongside activation of cytokine production through modifying MAPK signalling, mROS innate immune signalling can also enhance inflammasome signalling and leaderless IL-1 superfamily cytokine production in macrophages. One well characterised example is the activation of the NLRP3 inflammasome. It has been suggested that mitochondrial ROS is the primary ROS driving NLRP3 inflammasome activation, as studies have shown that the subunits nicotinamide adenine dinucleotide phosphate oxidase 1,2, and 4 (NOX1, NOX2 and NOX4) did not contribute to ROS-mediated inflammasome activation (413), and enhancement of mROS via inhibition of oxidative phosphorylation led to increased caspase-1 activation and subsequent processing of IL-1β and IL-18 (270). Furthermore, it has been reported that the process of mitophagy plays a role in regulating mROS-mediated inflammasome activation; damaged or dysfunctional mitochondria, which serve as the mROS generators promoting inflammasome activation, can be targeted for proteasomal degradation by the mitophagy machinery, thereby reducing mROS

production and subsequent inflammasome activation (270,414). In contrast, the NLRP3 inflammasome can inhibit mitophagy, therefore dysfunctional mitochondria are not cleared, and this promotes enhanced mROS production and inflammasome activation (415). These studies emphasise the importance of mROS in the modulation of host defence mechanisms and demonstrate the requirement of interactions of different responses to facilitate a highly effective macrophage innate immune response.

1.13 Summary of mitochondria

It is very clear that mitochondria are essential in all aspects of macrophage function. The roles of mitochondria in cellular metabolism and energy generation have been well established, but mitochondria are also central to regulating macrophage immune responses to pathogens. Changes in mitochondrial metabolism and mitochondrial fission and fusion dynamics regulate the activation state of macrophages and therefore regulate macrophage functions. Mitochondrial ROS production is important for direct killing of intracellular pathogens and can act in combination with other macrophage responses to enhance pathogen killing but due to its prominent signalling functions, it can also induce other macrophage immune responses. In addition, apoptosis-associated killing mediated by mitochondrial outer membrane permeabilisation, and cytochrome c release is a key delayed macrophage response that is required for the clearance of persistent intracellular pathogens. The diversity of mitochondrial functions in both cellular homeostasis and in response to pathogens demonstrates the crucial importance of the mitochondria in mediating innate immune responses in macrophages.

1.14 Summary of interactions between macrophage activation status, mROS, mitochondrial dynamics and macrophage antimicrobial responses

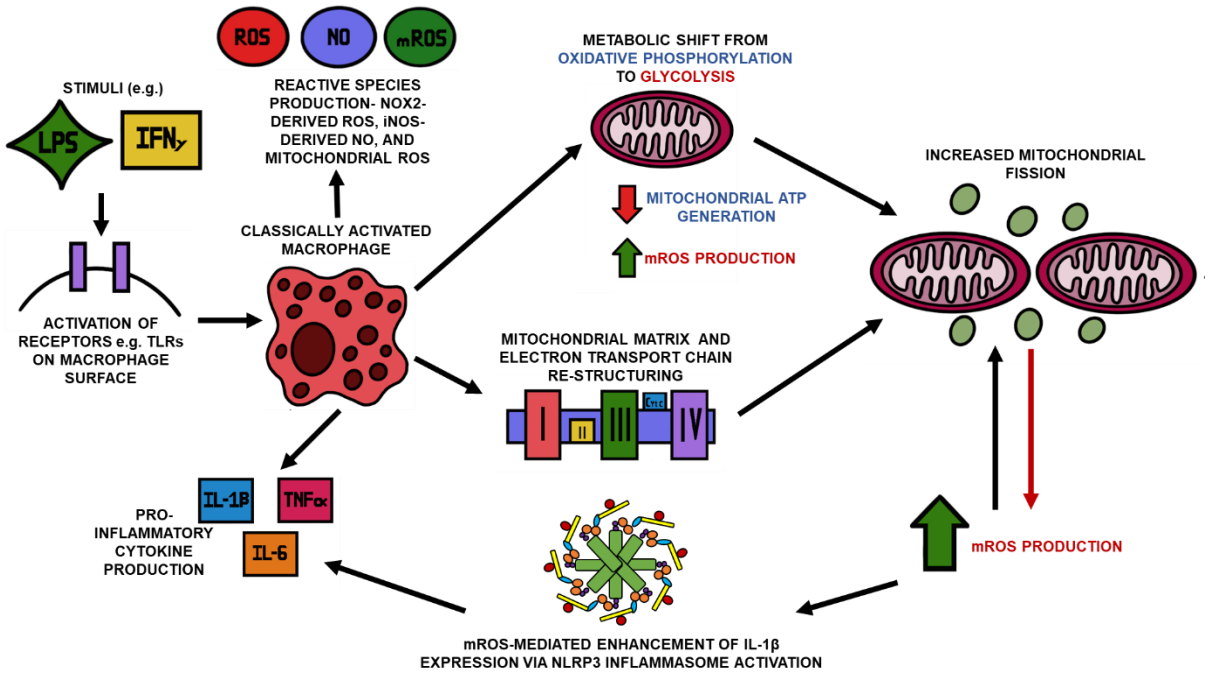


Figure 1.4: Schematic summary of interactions between macrophage activation status, macrophage antimicrobial responses, metabolic changes, mROS production and mitochondrial fission.

As described above, the balance of fission and fusion is linked to changes in mitochondrial metabolism and macrophage antimicrobial responses. This is represented schematically in Figure 1.4. Classically-activated macrophages are activated in response to pro-inflammatory stimuli such as LPS or IFN-γ, which promotes a more pro-inflammatory phenotype characterised by the production of pro-inflammatory cytokines, the result of signalling via surface TLR and other PRRs, to induce macrophage-pro-inflammatory responses, generation of reactive oxygen and nitrogen species and a metabolic shift to glycolytic energy generation to promote

enhanced ROS production from mitochondria (198,204,219). Enhanced mROS production can directly kill intracellular pathogens, but is also important in the signalling of macrophage pro-inflammatory responses, such as the enhanced expression of the pro-inflammatory cytokine IL-1β via mROS-mediated activation of the NLRP3 inflammasome (270,413). The mitochondrial matrix structure is altered in classically-activated macrophage mitochondria, which disrupts the electron transport chain and increases mROS production (325–327). This structural re-organisation of the electron transport chain, and the increase in mROS production via the metabolic shift to glycolysis, which are associated with classically-activated macrophages, can lead to increased fission (385). In addition to increased fission mediated by increased mROS production, enhancement of mitochondrial fission can also feedback to induce mROS production (386), demonstrating that mitochondrial fission and mROS production can enhance each other. This therefore demonstrates that the complex interweaving of macrophage activation status, metabolic status, and mitochondrial dynamics are vital for the promotion and enhancement of macrophage antimicrobial responses to clear infections.

1.15 Overview of host defence peptides (HDP)

In addition to mitochondria, another key component of the human innate immune system is the diverse array of host defence peptides (HDP), also termed antimicrobial peptides (AMP) that play a variety of roles in the host response to pathogens. The term antimicrobial peptide was widely accepted in the field in the late 20th century, especially after the characterisation of three peptides from the giant silk moth in 1980 that possessed direct bactericidal activity (416). However, as AMP research has developed over the last three decades, the understanding of AMP

antimicrobial functions has expanded and it is evident that in addition to their direct bacterial killing functionality, AMP also possess a wide variety of immunomodulatory functions that contribute to enhanced bacterial killing in host defence (417,418). Therefore, the term host defence peptide has now gained more popularity to reflect the diverse functions of these peptides, and this is the term that will be used throughout.

HDP are short, amphipathic peptides typically between 12 and 80 amino acids in length (419). They exist in a range of structural forms and are expressed in almost all cell and tissue types (420). The most studied HDP in humans are defensins and cathelicidin. Both of these groups are cationic and amphipathic, and possess a positive ionic charge which plays an important role in their antimicrobial functionality (417); this will be discussed in more detail later in this section. The work in this thesis focuses on human cathelicidin. Humans express only one cathelicidin, therefore it is very well characterised and there have been several studies carried out demonstrating that cathelicidin has the ability to interact with other host defence mechanisms to enhance bacterial killing; these will also be discussed in more detail.

1.15.1 Cathelicidin structure, expression, and localisation

Human cathelicidin, also termed LL-37, is a 4.5 kDa peptide consisting of 37 amino acids. Structurally, it forms amphipathic α-helices upon interaction with lipid membranes and it has been reported that this α-helical structure is associated with cathelicidin antimicrobial activity (421). Cathelicidin is encoded by the cathelicidin antimicrobial peptide (*CAMP)* gene on human chromosome 3p21.3 and begins as a peptide precursor, an 18 kDa peptide termed human cationic antimicrobial peptide

18 (hCAP18) (422). hCAP18 consists of a N-terminal signal sequence of 30 amino acids and a cathelin domain of 101 amino acids, which collectively comprise the pro-region, and a C-terminal cathelicidin antimicrobial domain of 37 amino acids, which once cleaved becomes the active peptide (423). The *CAMP* gene is comprised of four exons, the first three of which encode the hCAP18 pro-region (the N-terminal signal and cathelin domains), while the fourth encodes the antimicrobial region (424).

Cathelicidin is widely expressed in a diverse range of tissues, bodily fluids, and cell types including epithelial cells and immune cells (425). Of note, the locations and cell types where cathelicidin is known to be expressed in abundance are well regarded as first-line physical barriers to infection, including the skin, airway surface epithelia and lining fluid, and rapid-acting innate immune cells such as neutrophils and macrophages; this emphasises the importance of this peptide in the innate host immune response to infection (425).

Inflammatory stimuli such as whole bacteria, bacterial components such as LPS, pro-inflammatory cytokines and wounding can all induce hCAP18/cathelicidin expression in a variety of immune and epithelial cells (426,427). A primary site of cathelicidin expression is in neutrophils where it is constitutively expressed and stored in the hCAP18 pro-peptide form within specific granules (428)**.** This provide a supply of inactive peptide which is rapidly released and cleaved to form active peptide when required, as a stimulus such as bacterial infection stimulates neutrophil degranulation, therefore hCAP18 is released and can be processed to active cathelicidin to aid in bacterial clearance. The processing of hCAP18 into active cathelicidin occurs by cleavage of the cathelicidin domain from the pro-peptide by proteinase 3 (429). This serine protease is located primarily within neutrophil

azurophil granules and has been shown to exhibit this cathelicidin cleavage ability exclusively from other molecules within the granule (429). In addition to storage in cellular granules, hCAP18 can be produced in a secretory form; it can be induced in other immune cell types when appropriate stimuli are detected, for example in monocytes, macrophages, B cells and some subsets of T cells (430,431).

The regulation of *CAMP* gene expression is complex. The *CAMP* gene promoter region contains binding sites for a wide range of transcription factors. In human cells and tissues, particularly macrophages, airway epithelial cells and keratinocytes, one of the most well described regulatory pathways for *CAMP* gene expression is vitamin D metabolism and subsequent activation of the *CAMP* gene promoter (432). In addition, the short chain fatty acid butyrate has also been reported to modulate *CAMP* gene expression, both via modulation of *CAMP* gene promoter activation and by epigenetic modifications to histone acetylation (433,434). Furthermore, other factors, such as cyclic adenosine monophosphate (cAMP) signalling, ER stress, and the presence of pro-inflammatory cytokines can modulate *CAMP* gene expression independently of the above mechanisms or by combining with these mechanisms (435–437). Each of these methods of *CAMP* gene regulation will be discussed in more detail subsequently. The fact that there are multiple signalling pathways, transcription factors and mechanisms to regulate *CAMP* gene expression in most human cell and tissue types emphasises the importance of cathelicidin in host cell immunity.

1.15.1.1 Expression via vitamin D metabolism

Vitamin D metabolism has been well described as an important pathway for the induction of cathelicidin from the human *CAMP* gene, but not the murine *Camp* gene, as the human *CAMP* gene possesses a vitamin D response element (VDRE) in its promoter (438). This mechanism plays an important role in the experiments detailed in this thesis; therefore, this section will provide an overview of vitamin D metabolism and induction of *CAMP* gene expression.

Vitamin D metabolism begins in the skin, where vitamin D3, also termed cholecalciferol, is produced from its precursor 7-dehydrocholesterol via the action of UVB radiation from the sun. 7-dehydroxycholesterol absorbs UVB light which results in cleavage of one of the bonds in the compound's B ring, and this alteration produces the vitamin D3 that can be further processed into its active form (439,440). Vitamin D3 can also be obtained directly from the diet.

The UVB-mediated production of vitamin D3 from 7-dehydrocholesterol is not an enzymatic process, but the three main steps that occur subsequently depend on the activity of three cytochrome P450 mixed-function oxidases (CYPs): the 25-hydroxylase CYP27A1, the 1α-hydroxylase CYP27B1 and the 24-hydroxylase CYP24A1, all of which are located in the mitochondria (440). The specific 25-hydroxylation of vitamin D3 by CYP27A1 occurs in the liver, and this hydroxylation step converts vitamin D3 into 25-hydroxyvitamin D3 (25OHD3). The CYP27A1 enzyme is the only known mitochondrial-associated 25-hydroxylase, but it is not confined to the liver; it is found widely throughout the body (440).

25OHD3 does not possess the ability to induce gene expression, so it is further hydroxylated into the biologically active 1,25-dihydroxyvitamin D3, also termed calcitriol (440). The only known enzyme with 25OHD3 1α-hydroxylase activity is CYP27B1 (441)**.** As for CYP27A1 in the liver, CYP27B1-mediated calcitriol production is primarily carried out in the kidney, but the enzyme is also expressed in a wide variety of cell and tissue types such as epithelial cells of the skin, immune cells, vascular tissue, and gastrointestinal tissue (442).

Calcitriol has a strong affinity for the vitamin D receptor (VDR), which leads to the expression of vitamin D3-inducible genes such as *CAMP.* VDR is a transcription factor of the steroid hormone nuclear receptor family (438). This protein consists of three domains; a N-terminal DNA binding domain with two zinc fingers, which binds to the grooves of DNA at distinct locations known as VDREs, thereby facilitating the activation of vitamin D-susceptible genes; a 12-helical ligand binding domain at the C-terminal; and a hinge region which links the two domains. The final C-terminal helix provides gating functionality to enclose a bound ligand and provides the opportunity for co-activators to bind, but also allows for the calcitriol/VDR complex to interact with its heterodimeric partner, retinoid X receptor (RXR) (440,443). This heterodimeric complex then induces the expression of genes such as *CAMP* by binding to the VDRE in the gene promoter region. This illustrates a clear link between metabolism and enhancement of innate immune responses and can be utilised easily *in vitro* to induce *CAMP* gene expression which was very useful for carrying out some of the experiments detailed in this thesis. To provide further evidence of a link between innate immune cell activation and *CAMP* gene expression, it has been reported that the activation of TLR 1/2 in human monocytes

and macrophages leads to enhanced expression of the 1α-hydroxylase enzyme CYP27B1 and VDR which, as described above, can lead to potentially enhanced vitamin D metabolism and therefore subsequently enhanced *CAMP* gene expression (444).

Calcitriol can be toxic to cells if concentrations are excessive, therefore calcitriol production requires careful regulation. Calcitriol plays a role in its own regulation by acting as a strong inducer for the only known 24-hydroxylase in the vitamin D3 metabolic pathway, CYP24A1 (440). The CYP24A1 gene promoter contains two VDREs, therefore can be activated by the binding of VDR/RXR heterodimers (445). Calcitriol is degraded by the 24-hydroxylation activity of CYP24A1, therefore this 24-hydroxylase enzyme is important for the regulation of calcitriol levels and prevention of toxic calcitriol accumulation (440).

1.15.1.2 Expression via the action of butyrate

Another well-known inducer of *CAMP* gene expression is the short chain fatty acid butyrate. Short chain fatty acids (SCFAs) are small carboxylic acid molecules primarily produced by anaerobic bacteria in the gastrointestinal tract following the metabolism of indigestible fibre and polysaccharides from the host diet (446). Butyrate is one of the most abundantly-produced and potent SCFAs; it is a four-carbon aliphatic SCFA that has been reported to epigenetically regulate the expression of genes via its action as a histone deacetylase (HDAC) inhibitor (447).

The term “epigenetics” refers to mechanisms regulating gene expression that are reversible and do not require alterations to a genetic sequence. Epigenetic regulation includes DNA methylation, post-translational modification of histone tails

and production of microRNAs (139). One such example of an epigenetic mechanism of gene regulation is the modification of histones by acetylation. Histones play an essential role in chromatin structure by interacting with nuclear DNA, which wraps around histones to form nucleosome structures. These nucleosomes can then pack into chromatin fibres, which allows for the protection, organisation, and storage of DNA within eukaryotic cells (448). Chromatin structure is heterogeneous and changes in the structure can modulate the accessibility of genes for expression (448). The two primary conformations can be termed euchromatin and heterochromatin. Euchromatin refers to a loose, uncondensed chromatin structure which allows for transcription of genes by providing access for components such as transcription factors to bind to DNA, while heterochromatin describes the more densely-packed chromatin structures that do not provide access to DNA for gene transcription but facilitate the organisation and storage of DNA (448). However, histones also play an important role in gene regulation by influencing the accessibility of genes to transcription factors and this can be altered by post-translational modifications of histones (449). Lysine residues in histones can be acetylated by histone acetyltransferases (HATs), using acetyl-CoA as an acetyl group donor (449). This weakens the histone-DNA interaction which relaxes the tightly wound DNA, therefore providing access for transcription factors to interact with gene promoter regions and activate gene expression (450). Conversely, this process can be reversed by deacetylation of histone lysine residues by histone deacetylases, therefore histone-DNA interactions are restored, and DNA is re-wound thus gene promoter regions are no longer accessible for activation. However, this deacetylation process can be impaired by inhibition of histone deacetylases by

HDAC inhibitors such as butyrate, which sustains the open chromatin conformation and prolongs the accessibility of genes for activation (450,451).

Butyrate's epigenetic modulation of histone acetylation, through inhibition of HDAC enzymatic activity, has been shown to play a particularly important role in *CAMP* gene expression in, for example, epithelial and endothelial cells, but it has been shown in a variety of studies that butyrate-mediated *CAMP* gene expression can occur by other mechanisms, including in macrophages. A hydroxylated derivative of butyrate, 4-hydroxybutyrate, can enhance BMDM expression of the murine analogue to human *CAMP*, termed *Camp* (434). This enhancement occurred via *Camp* transcriptional activation by 4-hydroxybutyrate, rather than via its HDAC inhibitory activity. In a THP1 macrophage-like cell line and *Mycobacterium bovis* infection model, another butyrate derivative, sodium butyrate, mediated its effect via HDAC inhibition but rather than inhibiting HDAC enzymatic function, it inhibited HDAC expression, thereby indirectly preventing the deacetylation of histones (452). In addition, the clinically available derivative PBA was shown to influence *CAMP* gene expression via MAPK signalling. Three MAPK signalling cascades, extracellular signal-regulated kinases 1/2 (ERK1/2), JNK and p38 kinase pathways, can mediate the activation of activator protein-1 (AP-1), which can lead to induction of the *CAMP* gene promoter (450). It has been reported that *CAMP* gene activation by sodium butyrate can occur via these pathways (450), and a later study showed that PBA-mediated *CAMP* gene activation can occur in a similar manner (451). Interestingly, studies have shown that PBA and 1,25-dihydroxyvitamin D3 can act in a synergistic manner to enhance *CAMP* gene expression in a number of cell types such as keratinocytes, primary immune cells, and epithelial cells (451,453,454).

Mechanistically, this synergy is possibly due to the fact that VDR is required for PBA-mediated upregulation of *CAMP* gene expression as PBA enhances VDR transcription factor activity to enhance the expression of *CAMP* and *CYP24A1* genes, and the synergy of PBA and calcitriol results in further enhanced gene expression and subsequent secretion of hCAP18 protein (451,455).These studies illustrate that butyrate is multi-functional with regard to the mechanisms it employs to enhance *CAMP* gene expression, and it also has the ability to act in synergy with other factors and compounds to potentially enhance *CAMP* gene expression further.

1.15.1.3 Other factors which influence *CAMP* gene expression mechanisms

Although vitamin D3 metabolism and butyrate activity are the primary reported mechanisms for the induction of *CAMP* gene expression, there are other factors that can induce *CAMP* gene expression independent of these pathways, or that can further influence *CAMP* gene expression by these pathways.

The cyclic AMP (cAMP) secondary messenger protein has been reported to play a role in *CAMP* gene expression in epithelial cells via activation of protein kinase A (PKA), ERK and MAPK signalling pathways, subsequent induction of transcription factors cAMP response element binding protein (CREB) and AP-1, and their activation by phosphorylation (435). *CAMP* can be transcriptionally regulated by binding of CREB and AP-1 to its promoter and subsequent transcriptional activation. Furthermore, cAMP early repressor has been suggested to play a role in the inhibition of sodium butyrate-mediated *CAMP* gene expression by bacterial toxins in epithelial cells, and cAMP early repressor was shown to bind to the *CAMP* gene promoter and inhibit transcription in a competitive manner with CREB and AP-1

(435). This describes an alternative, vitamin D3-independent signalling pathway that can upregulate and modulate *CAMP* gene expression.

ER stress has been reported to regulate *CAMP* gene expression by a signalling pathway independent of vitamin D3 in epithelial cells (437). The ER has essential roles in protein and lipid synthesis and protein folding within eukaryotic cells, as well as roles in metabolic regulation via intracellular Ca release (456,457). Fluctuations in Ca levels can result in ER stress via an inappropriate accumulation of protein, and this stress can lead to dysfunctional ER and apoptosis if ER homeostasis is not restored (457). ER stress can upregulate *CAMP* gene expression by activating NF-κB signalling, MAPK activation to phosphorylate and subsequently activate CCAAT enhancer-binding protein α (C/EBPα) (437). Interestingly, ER stress was shown to inhibit activation of VDR by 1,25-dihydroxyvitamin D3 (437). Therefore, this study showed that ER stress-mediated *CAMP* gene upregulation is a distinct signalling mechanism that does not require calcitriol or VDR activation.

It is evident that pro-inflammatory cytokines are key components of the inflammatory microenvironment, and cytokines have been reported to influence vitamin D3-mediated *CAMP* gene expression in epithelial cells. In one study, pro-inflammatory interleukin 17A (IL-17A) derived from Th17 cells enhanced *CAMP* gene expression in keratinocytes, in a mechanism mediated by 1,25-dihydroxyvitamin D3, via IL-17A receptor signalling (458). Both IL-17A and vitamin D3 enhanced *CAMP* gene expression by signalling through the MEK-ERK pathway. In contrast, in airway epithelial cells, the presence of TNF-α and IL-1β attenuated vitamin D3-mediated *CAMP* gene expression; the mechanism of this occurrence was shown to be TNF-α- and IL-1β-mediated upregulation of vitamin D3 degradation enzyme CYP274A1 and

downregulation of CYP27B1, which converts inactive 25-hydroxyvitamin D3 to the active 1,25-dihydroxyvitamin D3 (436). Therefore, it is evident that in different contexts, different pro-inflammatory cytokines can influence vitamin D3-mediated *CAMP* gene expression in different, and sometimes contrasting ways.

1.15.2 Bactericidal mechanisms of cathelicidin

Cathelicidin possesses microbicidal properties against many types of microorganisms such as bacteria, viruses, and fungi. It is known to have the ability to kill a wide variety of both gram-positive and gram-negative bacteria, a process which typically occurs very rapidly in response to pathogen detection (459). Cathelicidin can exert its microbicidal effects on bacteria by forming pores in the bacterial cell membrane, which allows for leakage of essential cell contents and subsequent cell death (460). The mechanism by which cathelicidin forms pores has been well characterised and is known as a toroidal pore mechanism (461).

Cathelicidin pore formation and subsequent bacterial killing consists of three main steps: attraction of cathelicidin to the bacterial cell, attachment of cathelicidin to the bacterial cell surface, and insertion of the peptide into the membrane to form a pore (461). Since cathelicidin is a cationic HDP, it possesses an overall positive charge. The surfaces of both gram-positive and gram-negative bacteria possess an overall negative charge due to the presence of anionic phospholipids such as cardiolipin and phosphatidylglycerols, and phosphate groups on LTA and LPS, respectively (462). It is likely that the initial attraction of cathelicidin to bacterial surfaces is facilitated by electrostatic interactions due to the differences in charges. Cathelicidin can also interact with eukaryotic cells, although cathelicidin is less toxic

to eukaryotic cells than prokaryotic cells (461). Eukaryotic cell membranes contain a plethora of zwitterionic lipids such as cholesterol and phosphocholines, therefore the overall charge is more neutral. As the charge differences are important for cathelicidin interactions with bacteria, and eukaryotic cells exhibit a comparative lack of charge difference, cathelicidin appears to be more selective for, and exert a greater effect on, bacterial cells (461).

Cathelicidin pore-forming activity requires access to gram-negative bacterial inner membranes and gram-positive cytoplasmic membranes. Gram-negative bacteria possess an additional outer membrane that cathelicidin must traverse initially, and both gram-negative and gram-positive bacteria possess peptidoglycan cell walls that cathelicidin must be able to move through in order to make contact with the inner and cytoplasmic membranes respectively (462). There are studies that suggest that cathelicidin is more potent against gram-negative bacteria than gram-positive bacteria and a possible explanation for this is due to the presence of a thicker cell wall layer on gram-positive bacteria; although the gram-positive cell wall possesses a negative charge and can electrostatically attract cathelicidin, the density and composition of the cell wall may provide additional challenges for cathelicidin during transition to the bacterial membrane (80).

Once cathelicidin reaches the bacterial inner membrane of gram-negative bacteria or cytoplasmic membrane of gram-positive bacteria, it can then bind to the membrane lipid bilayer. Cathelicidin forms pores in bacterial membranes by a toroidal pore mechanism. Cathelicidin accumulates on membranes and binds parallel to the membrane surface, in contact with the phospholipid head groups (461). At high membrane-associated peptide concentrations, cathelicidin induces

positive curvature strain in the membrane (463); this induces the lipids to bend inwards along with allowing cathelicidin to insert into the membrane, which forms a transmembrane pore that is lined with both cathelicidin peptides and lipid head groups that resembles a toroid shape, hence it is termed the toroidal pore mechanism (462).

The presence of the toroidal pores in the bacterial membrane subsequently facilitates several processes that are detrimental to the bacterial cell. Evidently, pore formation in the membrane results in prominent membrane perturbation and leakage, which can result in loss of cellular mass and essential components for survival (464). Cathelicidin-induced toroidal pores are sizeable and have been reported to allow the passage of particles up to 9 nm (425). Furthermore, cathelicidin can utilise electrostatic interactions to interfere with protein complexes of the electron transport chain within the membrane and subsequent ATP generation by breaking down the proton motive force that mediates ATP synthesis, thereby interfering with bacterial cell metabolism and energy generation which can dramatically impair bacterial survival (464). Breakdown of the proton motive force by cathelicidin also results in increased superoxide generation from the bacterium (464). This latter mechanism links cathelicidin activity and alterations in cellular metabolism, electron transport chain function, superoxide production and ATP generation. As it is possible that the evolutionary origins of mitochondria are linked to bacterial cell structure and function (307), this provides some rationale to explore links between cathelicidin activity and mitochondrial function.

In addition to these more direct effects on bacterial cells, cathelicidin has been shown to promote bacterial killing by synergising with current antimicrobial

treatments. In one study, treatment of *S. pneumoniae*-mediated and *S. aureus*-mediated meningitis patients with the cephalosporin-group drug ceftaroline not only resulted in successful treatment, but ceftaroline also enhanced the susceptibility of *S. pneumoniae* to cathelicidin's bactericidal effects by altering the bacterial surface charge (465). Other studies have shown that β-lactamase inhibitor compounds enhance cathelicidin bactericidal activity against MRSA (466), while β-lactam antibiotics such as nafcillin could aid in the treatment of MSSA-mediated bacteraemia via their antibiotic capacity as well as sensitise bacteria to cathelicidin (467). These studies provide further evidence in support of studying cathelicidin's potential synergy with other antimicrobial mechanisms, particularly against *S. pneumoniae* and *S. aureus.*

1.15.3 Immunomodulatory functions of cathelicidin

Cathelicidin has an extensive range of immunomodulatory properties in addition to its direct antimicrobial properties (468); the multifunctionality of this peptide further emphasises its role as a key component of host defence.

An early study into the immunomodulatory roles of cathelicidin showed that cathelicidin could inhibit bacteria LPS- and LTA-mediated TNF-α production in macrophages, and modulate expression of several genes, with a focus on upregulating genes encoding chemokines and associated receptors (469). This demonstrates that cathelicidin can regulate macrophage pro-inflammatory cytokine production and enhance recruitment of other immune cells to the infection site via chemotaxis, and therefore play a role in the regulation of inflammatory responses (469). In regard to cathelicidin's regulation of cytokine production, it is known that

cathelicidin can modulate signalling from receptors such as TLRs. LPS and LTA are both well-known endotoxins that act as PAMPs which can be detected by and bind to TLRs 4 and 2 respectively, which leads to the activation of downstream signalling pathways and expression of pro-inflammatory cytokines (470,471). As shown in the study above, cathelicidin is a strong inhibitor of LPS and LTA (469); it is now known that cathelicidin has the ability to bind to these endotoxins and neutralise them, therefore they cannot interact with their TLRs and activate pro-inflammatory cytokine signalling (418,472–474). In addition, cathelicidin was also shown to prevent *Escherichia coli* (*E. coli)*-mediated sepsis in a rat model by neutralising *E. coli* LPS, and its therapeutic effect were comparable or better compared to current antimicrobials used in the treatment of sepsis (475). This illustrates the importance of this cathelicidin immunomodulatory function in host defence and highlights an avenue for future therapeutic development.

Cathelicidin can also influence the outcomes of TLR signalling more indirectly; it has been reported that in monocytes and macrophages, cathelicidin can promote the expression of IL-10 (476,477). In the context of viral infection, cathelicidin has been shown to interact with PIC and promote enhanced PIC-mediated TLR3 signalling in bronchial epithelial cell line, kidney cell line, and PBMC models (478) Enhanced TLR3 signalling resulted in increased detection of double-stranded RNA, and cytokine production, in response to viral infection (478) Further studies have demonstrated that low concentrations of cathelicidin can synergise with pro-inflammatory stimuli targeting various TLRs, for example TLRs 1/2, 3 and 5, to enhance the expression of chemoattractants such as IL-8, and pro-inflammatory cytokines such as IL-6 (479).It is also apparent that although lipid TLR agonists can

reduce pro-inflammatory responses in the presence of exogenous cathelicidin, nucleic acid TLR agonists show enhanced pro-inflammatory responses in the presence of mCRAMP (476,477). Therefore, it is evident that cytokine expression mediated by cathelicidin is context dependent.

As indicated above, cathelicidin possesses potent chemotactic properties to recruit a variety of immune cells to aid in pathogen clearance (463,468). Cathelicidin chemotactic abilities are two-fold: cathelicidin can induce the production of chemokine molecules to indirectly recruit immune cells, and cathelicidin itself can act as a chemoattractant directly (18). In addition, cathelicidin-mediated degranulation of mast cells releases a number of components that promote the infiltration of immune cells from the vasculature to infection sites, therefore providing an additional indirect chemotactic effect (431). The pathways and receptors involved in cathelicidin-mediated chemotactic mechanisms are distinct for different cell types. In some cell types, modulation of chemokine expression primarily occurs via MAPK-mediated signalling which can be activated by binding of cathelicidin to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which acts as a direct intracellular receptor for cathelicidin. This interaction can lead to the activation of the p38-MAPK pathway which can activate the production of chemokines (480). In human monocytes, cathelicidin signals through the ERK1/2 and p38 kinase pathways to induce the expression of chemokines such as the chemoattractant IL-8 (481). Direct chemotactic effects of cathelicidin have been demonstrated for a variety of immune cells and occur via signalling through several receptors such as FPRs and formyl peptide receptor like 1 and 2 (FPRL1 and 2) to attract monocytes, macrophages, eosinophils and neutrophils (482–484), CXC chemokine receptor 2 (CXCR2) on

neutrophils (485), and Mas-related G protein-coupled receptor X2 (MrgX2) on mast cells (486). In addition, exogenous cathelicidin has been shown to promote enhanced early neutrophil influx and antimicrobial responses in a mouse model *of Pseudomonas aeruginosa (P. aeruginosa)* lung infection, which did not involve peptide modulation of chemokine levels. This suggests that cathelicidin can influence the influx of neutrophils into the lung during infection distinctly from modulating chemotactic mediators (487).

In keeping with cathelicidin's ability to modulate macrophage inflammatory responses, cathelicidin can also modulate macrophage differentiation of macrophages, DCs, and T cells. Cathelicidin promotes the differentiation of monocytes to the more pro-inflammatory classically activated phenotype, thereby enhancing macrophage pro-inflammatory responses (488); this will be discussed in more detail later in this section. In regard to activation of DCs, studies have demonstrated that cathelicidin can form complexes with self-DNA or RNA and enhance DC maturation and production of interferons, which has been implicated in the mechanisms driving auto-inflammatory responses in conditions such as psoriasis (489,490). Furthermore, cathelicidin-mediated DC differentiation results in mature cells with enhanced capacity for antigen ingestion and presentation, expression of chemokine receptors, and production of cytokines (491,492). Subsequently these cells are well equipped to promote the differentiation and activity of T cells and drive adaptive immune responses (491–493).

Cathelicidin can influence apoptosis in eukaryotic cells; it can both induce apoptosis and protect cells from apoptosis by different mechanisms in different cell types. Apoptosis protection mediated by cathelicidin has been reported in

keratinocytes, which occurs via cathelicidin-mediated upregulation of cyclooxygenase-2 and inhibitor of apoptosis-2 (IAP-2) expression, and production of prostaglandin E-2; all of these are associated with protective, anti-apoptotic pathways (494). Cathelicidin apoptosis protection has also been described in neutrophils; cathelicidin-mediated activation of FPRL1 and P2X purinoceptor 7 (P2X7) receptor signalling can result in expression of an anti-apoptotic Bcl-2 family protein B-cell lymphoma-extra large (Bcl-xL), and caspase-3 inhibition (495). Bacterial infection can increase eukaryotic cell sensitivity to cathelicidin-mediated cell death induction effects and reduce apoptosis resistance (496). Conversely, high concentrations of cathelicidin can induce apoptosis in murine airway epithelial cells; one study demonstrated that this process was dependent on mitochondrial outer membrane permeabilisation by Bax (496), while physiologically relevant concentrations had a minimal apoptotic effect. However during *P. aeruginosa* infection of airway epithelial cells, these low concentrations of cathelicidin could synergise with *P. aeruginosa* to enhance cell death in bacterial-infected cells via increased loss of $\Delta\psi_m$, cytochrome c release, caspase 9 activation and fragmentation of DNA (496), thereby providing another link between cathelicidin and mitochondrial responses in host defence. To expand upon the mechanisms behind cathelicidin-induced apoptosis, cathelicidin has been shown to rapidly accumulate in mitochondria and promote enhanced release of apoptosis-inducing factor (AIF) and cytochrome c, which further supports modulation of mitochondrial membrane structure as a mechanism behind cathelicidin's influence on apoptosis (497). Another study has also investigated cathelicidin-induced apoptosis of epithelial cells and highlighted activation of caspase activity as another particularly important mechanism by which cathelicidin induces apoptosis (498). However, this study also

showed that the presence of serum could inhibit cathelicidin-induced apoptosis and exert a protective effect, demonstrating that cathelicidin's influence on apoptosis would be dependent on the serum levels at the site of infection (498).

Cathelicidin has important roles in wound healing and angiogenesis to restore and maintain tissue health and homeostasis (499). It can induce the re-epithelialisation of skin at sites of wounds by promoting the growth, proliferation, and migration of epithelial cells to the wound sites (499)k. Cathelicidin can promote angiogenesis by interacting with FPRL1 present on endothelial cells, thereby promoting the re-vascularisation of damaged tissue (499,500). Furthermore, cathelicidin has been reported to influence keratinocyte migration by inducing epidermal growth factor receptor activation and subsequent signalling via, for example, the MAPK, NF-κB and PI3K pathways to promote keratinocyte migration and skin wound healing (463,501).

1.15.4 Cathelicidin interactions with host defence responses, macrophage intracellular killing mechanisms, and mitochondrial metabolism

Several studies have now been carried out in a variety of models and contexts to better understand mechanisms of interaction between cathelicidin and other intracellular host defence responses.

As mentioned above, vitamin D metabolism is a key mechanism for induction of CAMP gene expression, and a recent study has demonstrated that 25OHD3 enhances intracellular *CAMP* expression and hCAP18 production in human PBMC with and without stimulation with LPS or double-stranded RNA Poly (I:C) (PIC), to mimic detection of bacterial or viral pathogens respectively (502). However, 25OHD3

was also shown to enhance the secretion of cathelicidin (LL-37) into the extracellular environment, but only from PBMC following LPS or PIC exposure (502), suggesting that there is some synergy between vitamin D and pathogen exposure that further increases cathelicidin expression and secretion to enhance PBMC antimicrobial responses. This study also suggests that 25OHD3-mediated cathelicidin secretion from PBMC only after stimulation with LPS or PIC allows for regulated secretion when pathogens are detected, to prevent damage to host cells or killing of host microbiota when not required (502).

In keeping with the knowledge that vitamin D3 can upregulate *CAMP* gene expression, a mechanism has been described by which 1,25-dihydroxyvitamin D3-induced cathelicidin was essential for the induction of autophagy and clearance of intracellular Mtb in human macrophages, a process termed xenophagy (503). 1,25-dihydroxyvitamin D3 treatment could initiate the formation of autophagosomes in human macrophages and cathelicidin was required for 1,25-dihydroxyvitamin D3-induced, suggesting that autophagosome formation was mediated by cathelicidin induced by 1,25-dihydroxyvitamin D3 (503). Cathelicidin could mediate autophagosome formation by activating enhanced expression of the autophagy-associated genes Beclin-1 and autophagy-related 5 (Atg5), therefore illustrating an immunomodulatory role for cathelicidin in this system (503). Cathelicidin was also recruited to autophagosomes via the Ca/calmodulin-dependent protein kinase kinase β (CaMKK-β) and 5' adenosine monophosphate-activated protein kinase (AMPK) pathways, and 1,25-dihydroxyvitamin D3 was found to enhance the co-localisation of phagosomes containing Mtb with autophagosomes to enhance clearance of the pathogens, therefore it is possible that the enhanced recruitment of cathelicidin to

autophagosomes mediates the co-localisation of these organelles and the subsequent clearance of Mtb (503). This study illustrates a direct link between vitamin-D3-induction of *CAMP* gene expression, some immunomodulatory functions of cathelicidin, and enhancement of intracellular bacterial killing in macrophages.

The presence of cathelicidin during macrophage colony-stimulating factor (M-CSF)-mediated macrophage differentiation from monocytes promoted enhanced differentiation of monocytes into the pro-inflammatory classical activated macrophage type, while monocytes incubated with M-CSF alone favoured differentiation into the anti-inflammatory alternatively activated macrophage type (488). Furthermore, cathelicidin enhanced the expression of pro-inflammatory cytokines from macrophages, as cathelicidin-differentiated macrophages expressed less IL-10 (a characteristically anti-inflammatory cytokine) and more interleukin-12 subunit beta (IL-12p40) (a characteristically pro-inflammatory cytokine) when macrophages were also stimulated with various bacterial stimuli such as LPS or LTA, which is consistent with the enhanced promotion of the classically activated macrophage phenotype (488). Therefore, it is possible that the influence of cathelicidin on macrophage differentiation is driven by its ability to modulate cytokine expression. In addition, cathelicidin had the ability to modulate cytokine production even in the absence of stimulation with bacterial PAMPs; for example, incubation of alternatively-activated macrophages with cathelicidin resulted in less IL-10 expression and more IL-12p40, therefore cathelicidin immunomodulatory function was not constrained to classical activation (488). In terms of mechanism, it was essential that cathelicidin was internalised by monocytes and macrophages to exert these effects; F-actin polymerisation plays an important role in initiating the

internalisation of extracellular material, and cytochalasin D inhibition of F-actin polymerisation abolished cathelicidin effects on macrophage activation and cytokine production (488). The proposed biological context for these findings is that under steady state conditions, M-CSF derived from tissues keeps macrophages in an anti-inflammatory alternatively activated state, but when a pathogen or other inflammatory stimulus is detected, inflammatory mediators such as cathelicidin are released from inflammatory cells such as neutrophils and this can drive enhanced differentiation of monocytes into pro-inflammatory classically activated macrophages or re-model alternatively activated tissue macrophages to exhibit a more pro-inflammatory phenotype to combat the infection (488).

P2X7 purinergic receptor is a ligand-gated ion channel that is widely expressed on the surface of haematopoietic-derived cells including monocytes, macrophages, and DCs, and is known to play roles in cell death, pathogen killing and regulation of inflammation. The primary ligand for this receptor is ATP, however cathelicidin can also act as a direct ligand for the P2X7 receptor (504). Cathelicidin-mediated P2X7 receptor activation led to enhanced caspase-1 activation and processing of the pro-inflammatory cytokine IL-1β in monocytes pre-treated with LPS. Prolonged P2X7 receptor-ATP interaction can lead to increased cell permeabilisation and cell death, however, cathelicidin-mediated P2X7 receptor activation was not shown to have cellular cytotoxic effects (504). This illustrates the potent inflammatory signalling ability of cathelicidin, and that cathelicidin can directly interact with immune cell surface receptors to enhance host responses as a result of inflammatory stimuli detection.

In keeping with P2X7 receptor-cathelicidin interactions, a mechanism of cathelicidin internalisation by human macrophages has been characterised, via two endocytic pathways and mediated by the P2X7 surface receptor (505). It was shown that P2X7 receptor-mediated cathelicidin internalisation by monocyte-derived macrophages (MDM) and THP-1 monocytic cell lines was primarily associated with the clathrin-mediated endocytic pathway, but was also associated with the clathrin-independent, lipid raft-dependent pathway (505). Furthermore, internalisation of cathelicidin enhanced bacterial killing by THP-1 cells, as cathelicidin was found to co-localise with intracellular *S. aureus* and intracellular *S. aureus* viability was reduced in THP-1 cells following cathelicidin treatment (505). Internalisation of cathelicidin by THP-1 cells led to an increase in ROS activity, which was suppressed when cathelicidin internalisation was inhibited. Inhibition of ROS production was also found to decrease cathelicidin-mediated bacterial killing, suggesting that the enhancement of bacterial killing by cathelicidin-treated THP-1 cells required a synergy between internalised cathelicidin and the induction of ROS production (505). Cathelicidin internalisation also resulted in increased formation of intracellular lysosomes, which was also suppressed by inhibiting cathelicidin internalisation. In all of the above cases (enhanced bacterial killing, ROS production and lysosome formation), the effects of cathelicidin treatment on P2X7 receptor knockout cells were significantly reduced, providing further evidence of the important role the P2X7 receptor plays in cathelicidin internalisation and cathelicidin-mediated enhancement of bacterial killing (505). To provide some biological context to these results, the study also demonstrated that in addition to experimentally-added cathelicidin, THP-1 cells were able to internalise cathelicidin released from nearby neutrophils, and this

was also shown to involve the established P2X7 receptor-mediated mechanism (505).

Cathelicidin can enhance the phagocytic response of monocytes and macrophages, therefore providing another mechanism for direct modulation of immune cell antimicrobial responses by cathelicidin. Mechanisms by which cathelicidin can enhance the phagocytosis of *S. aureus* and *E. coli* opsonised by IgG, and the phagocytosis of non-opsonised *E. coli* by macrophages have been described (506). Cathelicidin upregulated the expression of CD32 and CD64 FcγRs on macrophage surfaces, both of which are key phagocytic receptors mediating uptake of IgG-opsonised bacteria. In addition, cathelicidin enhanced the expression of both TLR4 and CD14 on the macrophage surface, enhancing responses to LPS, and indirectly enabling cathelicidin-mediated enhancement of non-opsonised *E. coli* since TLR4 and other TLRs have been shown to enhance a gene programme that promotes phagocytic uptake of bacteria (507)**.** Finally, the authors identified that interaction of cathelicidin with FPR2, which influences a variety of inflammatory responses upon activation including phagocytosis activation and chemotaxis, was important for cathelicidin-mediated enhancement of phagocytosis (506). The importance of cathelicidin in regulating phagocytic function has been demonstrated in a murine peritoneal macrophage model of *E. coli* infection, as in mice lacking CRAMP, fewer macrophages were recruited to sites of infection and those that were recruited exhibited impaired phagocytosis (508).

A strong immunomodulatory link between cathelicidin and mitochondria has been demonstrated in a chronic skin inflammatory model (509). Cathelicidin had the ability to reduce the expression of a number of LPS-induced pro-inflammatory

cytokines, such as IL-8, IL-6 and TNF-α, demonstrating an important anti-inflammatory effect of cathelicidin. This anti-inflammatory effect required interactions between cathelicidin and mitochondria to enhance mitochondrial generation and preserve mitochondrial homeostasis (509). The presence of cathelicidin inhibited the loss of electron transport chain Complexes II, III and V that was observed with LPS induction. These findings demonstrate that cathelicidin has the ability to modulate mitochondrial metabolism and promote homeostasis to regulate inflammatory responses. Furthermore, cathelicidin was shown to prevent LPS-induced loss of inner mitochondrial transmembrane potential by enhancing the phosphorylation and activation of ERK1/2 and mTOR (509), suggesting that these may be important mediators for the anti-inflammatory effects of cathelicidin and protection of mitochondrial homeostasis.

In addition, a recent study has highlighted lipid droplets as a novel mechanism for mediating macrophage intracellular host defence responses, including cathelicidin and mitochondrial-associated responses (510). LPS-induced lipid droplets were shown to accumulate cathelicidin and other antimicrobial proteins and could promote bacterial killing by associating with bacterial cells. In particular, lipid droplets enriched with cathelicidin were shown to be effective in the clearance of *E. coli*, MRSA and *L. monocytogenes* (510). Furthermore, in steady-state conditions lipid droplets provide mitochondria with fatty acid substrates to drive oxidative phosphorylation. However, LPS-induced lipid droplets reduce contacts with mitochondria and it has been suggested that this uncoupling contributes to the reduction in oxidative phosphorylation associated with pro-inflammatory macrophage responses (510).

All of the above studies demonstrate that cathelicidin can interact with different antimicrobial mechanisms in a variety of human cell types including macrophages, and have a profound impact on bacterial clearance, either by mediating direct bacterial killing or the modulation of other host defence responses, including through mROS-dependent mechanisms. Therefore, this concept provides the rationale to study possible interactions between cathelicidin, mROS production and mitochondrial antimicrobial responses in macrophages.

1.16 Project hypothesis and aims

The project aims to investigate the functional roles of cathelicidin, mitochondrial-generated mROS and mitochondrial homeostasis alone and in combination for antibacterial responses in macrophages, and how these can be augmented to more effectively clear gram-positive bacterial infections. The hypothesis underpinning the project is that macrophages use multiple microbicidal mechanisms to efficiently clear bacterial infections, and the antimicrobial effects of cathelicidin, altered mitochondrial dynamics and mROS production, combine to enhance macrophage killing of bacteria. Three main aims were identified with which to address this:

1. Which factors influence the expression of cathelicidin in differentiated macrophages and does cathelicidin contribute to bacterial killing?

2. How are mitochondrial dynamics adapted in association with macrophage antibacterial responses?

3. How do alterations in mROS production influence macrophage antibacterial host defence responses, and are these altered by cathelicidin?

Chapter 2

Materials and methods

Details of reagents used can be found in Methods Appendix 2.1 at the end of this chapter.

2.1 Isolation and culture of human monocyte-derived macrophages

Human PBMC were isolated from whole blood donated by healthy volunteers using a Percoll density gradient. All centrifugation steps were carried out using a Thermo Scientific Multifuge X3 FR centrifuge. Blood was collected from volunteers and added to 50 ml Falcon tubes containing 4.4 ml 3.8% sodium citrate solution to prevent coagulation. Platelet-rich plasma was separated from whole blood by centrifuging blood at 350 x g, 20°C, acceleration and deceleration rate of 9/9, for 20 minutes. Autologous serum was prepared by placing 10 ml platelet-rich plasma into a glass bijou tube and adding 200 µl 1 millimolar calcium chloride ($CaCl_2$), then incubating plasma in a 37°C water bath for 1 hour to aggregate the platelet cells. 6 ml of pre-warmed 6% dextran solution was added to the blood tubes after plasma was removed, and the tubes were topped up to 50 ml with pre-warmed 0.9% sodium chloride solution (NaCl). Blood tubes were incubated at 37°C for 30 minutes to precipitate the red blood cells. After incubation, the translucent top layer of the sample was transferred into a new 50 ml Falcon tube and topped up to 50 ml with 0.9% NaCl. Samples were centrifuged at 350 x g, 20°C for 6 minutes to pellet blood cells. The Percoll density gradient was prepared by mixing a 90% Percoll stock solution (27 ml Percoll + 3 ml 10x phosphate buffered saline (PBS)). This stock

solution was then used with 1x PBS (-Ca, -magnesium (Mg)) to prepare solutions of different Percoll densities; an 81% solution, a 68% solution, and a 55% solution. The density gradient was layered by placing 3 ml 81% solution into a 15 ml Falcon tube, then carefully placing 3 ml 68% solution on top. Once the cells had spun down, the supernatant was discarded, and cells were re-suspended in 3 ml 55% solution which was placed on top of the 68% solution. The different cell types were separated by centrifuging the gradient at 720 x g, 20°C, acceleration rate of 9/9 and deceleration rate of 0/9, for 20 minutes. The separated cells appeared as cloudy bands between the different density solutions; the PBMC layer between the 55% and 68% layers, and the polymorphonuclear leukocyte (PMN) layer between the 68% and 81% layers. The PBMC and PMN bands were carefully removed from the gradients and placed into new 50ml Falcon tubes. Cells were washed by topping tubes up to 40 ml with 1x PBS (-Ca, -Mg) and centrifuging at 230 x g, 20°C, acceleration and deceleration rate of 9, for 6 minutes. The supernatants were discarded, and the cell pellets were re-suspended in 1 ml 1x PBS (-Ca, -Mg). The tubes were topped up to 50 ml with 1x PBS (-Ca, -Mg). 10 µl of each cell culture was placed on a haemocytometer and cells were counted by visualising cells with a 10x lens on a light microscope. Cells in two haemocytometer quadrants were counted, and this allowed for the number of cells obtained from the preparation to be calculated as follows:

Average cell count= (quadrant 1 count + quadrant 2 count)/2

Average cell count x 10^4= number of cells (million/ml)

Number of cells (million/ml) x 50 ml resuspension volume= Number of cells (million)

PBMC were seeded into 24-well plates at a concentration of 2.5 x 10^5 PBMC/well by centrifuging the PBMC culture at 300 x g, 20°C for 6 minutes, discarding the supernatant, and re-suspending the cell pellet in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) + 2 millimolar L-glutamine + 10% autologous serum to a concentration of 5 x 10^6 cells/ml. 500 µl of cell culture (corresponding to 2.5 x 10^5 PBMC/well) was added per well and the plates were incubated at 37°C and 5% carbon dioxide (CO_2) for 4 hours to promote cell adherence to the wells. After this time, the media was removed from wells and replaced with 1 ml RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum (FBS) (termed complete media). PBMC were incubated for 14 days at 37°C and 5% CO_2, with media changes twice a week, to differentiate them into monocyte-derived macrophages (MDM). Following the two week culture period, MDM were visualised by light microscopy to inspect MDM morphology and density prior to use, and representative wells were periodically scraped, and MDM counted to confirm a cell density of approximately 2 x 10^5 MDM.

2.1.1 Preparation of coverslips for microscopy

If MDM were required on coverslips for microscopy, 13 mm circular glass coverslips were prepared. These were stored in a 50 ml Falcon tube containing ethanol, so the required number of coverslips were removed with tweezers and left to dry completely prior to PBMC plating. Once dry, one coverslip was placed into a well of a 24-well plate. PBMC were seeded onto coverslips at a concentration of 2.5 x 10^6 PBMC/well in 100 µl rather than 500 µl, so after PBMC were counted, the number of cells required for all coverslips was calculated and the volume of culture required was added to a separate 50 ml Falcon tube. The PBMC culture was

centrifuged at 300 x g, 20°C for 6 minutes, the supernatant was discarded, and the cell pellet was re-suspended in RPMI 1640 RPMI 1640 + 2 millimolar L-glutamine + 10% autologous serum to a concentration of 25 x 10^6 cells/ml. 100 µl of cell culture was added per coverslip. Incubation, addition of complete media and subsequent media changes were carried out as for regular plated MDM.

2.1.2 Accutase dissociation of mature MDM and re-plating onto chamber slides

In the latter stages of this project, chamber slides were used more frequently for preparing samples for microscopy than coverslips, due to the convenience of preparing up to eight different samples on one slide and the samples did not require mounting and overnight drying. PBMC were seeded into 24-well plated and cultured into mature MDM as usual. To re-seed into chamber slide wells, media was removed from MDM, and they were washed with 1 ml PBS. 400 µl of Accutase solution was added per well, and MDM were incubated at 37 °C for 40 minutes to allow dissociation of adherent MDM from the plate. Morphologically, adherent MDM appear flat and slightly branched when visualised by light microscopy while dissociated MDM appear spherical; therefore, MDM were visualised by light microscopy following accutase treatment to confirm the presence of this morphology. MDM were re-suspended further by careful pipetting and all MDM/accutase suspensions from one plate were pooled into a 50 ml Falcon tube. To ensure maximum retrieval of MDM from the plate, wells were washed with 1ml PBS, carefully pipetted, and pooled into the same 50ml Falcon tube. MDM were centrifuged at 300 x g, 20°C for 6 minutes, the supernatant was discarded, and the cell pellet was resuspended in 10 ml PBS. MDM counts were carried out using a

haemocytometer as described above. MDM were centrifuged again at 300 x g, 20°C for 6 minutes and re-suspended in complete media at a concentration of 6 x 10^5 cells/ml. 250 µl was plated per chamber slide well, therefore re-seeding MDM at a final concentration of 1.5 x 10^5 cells/well. Chamber slides were incubated at 37°C and 5% CO_2 at least overnight to promote MDM re-adherence; therefore, this process was carried out at least one day in advance of sample preparation.

2.1.3 Treatment of MDM with compounds

In a number of experiments, MDM were treated with the following compounds prior to further treatment, bacterial challenge, or analysis. These compounds were added to MDM and incubated at 37°C and 5% CO_2 for 1 hour before further experimentation: 10 micromolar Mitoparaquat (MitoPQ), a mitochondrial-targeted redox cycler to induce mROS production (321); 10 micromolar Mitoquinone (MitoQ), a mitochondrial-targeted scavenger antioxidant to inhibit mROS production (511); 10 micromolar diphenyleneiodonium chloride (DPI), a NADPH oxidase inhibitor to inhibit cellular ROS production (512); 2 micromolar rotenone, a mitochondrial Complex I inhibitor that can have contrasting roles on mROS production in different contexts, by inducing mROS when forward electron flow is occurring but inhibiting mROS when RET is occurring (329,403); or 100 nanomolar carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation to induce mitochondrial inner membrane depolarisation (513,514). In some experiments, combinations of inhibitor and inducer compounds were required, for example MitoQ and MitoPQ. In these cases, the inhibitor compound was incubated with MDM for 1 hour, then the inducer was added for a subsequent hour.

2.2 Isolation and culture of mouse bone marrow-derived macrophages (BMDM)

BMDM were extracted from 3-month old male and female C57BL/6 mouse femurs and tibias and cultured at 37°C and 5% CO_2 in complete media (Dulbecco's Modified Eagle Medium (DMEM) containing 10% endotoxin-free foetal calf serum (FCS), 1:100 Glutamax, and 5000 U/ml penicillin/streptomycin) supplemented with 100 ng/ml colony stimulating factor-1 (CSF-1) for seven days prior to use. BMDM were initially cultured in 100 mm square Sterilin plates, then re-seeded into 24-well plates after seven days at a concentration of 5 x 10^5 BMDM/well. For experiments using the cathepsin B inhibitor CA074-methyl ester (CA074-me), BMDM were incubated at 37°C and 5% CO_2 with 25 micromolar CA074-me for 1 hour before bacterial challenge.

2.3 Bacterial strains and challenge of MDM

The serotype 2 *S. pneumoniae* D39 or the *S. aureus* SH1000 lab strains were used in all challenge experiments unless otherwise stated, and 14 day old MDM were at a concentration of 2 x 10^5 MDM/well. Bacterial stocks were prepared by plating the relevant bacterial strain, stored on beads in microbanks at -80°C, onto blood agar and incubating plates at 37°C and 5% CO_2 overnight. The next day, bacterial colonies were inoculated into brain heart infusion (BHI) media and cultures were grown at 37°C and 5% CO_2 to the mid log phase of bacterial growth; this was confirmed via optical density measurements of 0.6 for *S. pneumoniae* D39 and 0.8-1.0 for *S. aureus* SH1000. 1 ml aliquots of cultures were prepared as working stocks and stored at -80°C. To confirm that the expected bacteria had grown, morphology

was confirmed via Gram staining and appearance of colonies grown on blood agar plates. Furthermore, to calculate the working stock concentrations, one aliquot of each culture following storage at -80^{o}C was thawed, washed three times in PBS and serial dilutions prepared and plated onto blood agar overnight. Colonies were counted the next day and bacterial concentrations in colony forming units (CFU)/ml were calculated as described below for the killing assay experiments; this concentration represented the working concentration for the relevant stock in regard to calculating the multiplicity of infection (MOI) required in each experiment.

Before challenge, *S. pneumoniae* were washed three times with 900 µl PBS and opsonised using 500 µl RPMI 1640 + 2 millimolar L-glutamine + 10% human immune serum from healthy donors vaccinated against *S. pneumoniae*. Opsonisation was carried out by incubating at 37°C and 5% CO_2 for 30 minutes while placed on a shaking plate within the incubator. At this stage both *S. pneumoniae* and *S. aureus* were washed twice with 900 µl PBS and added to MDM at a MOI of 10, unless otherwise stated, in complete media. Where MDM were challenged with *S. pneumoniae*, MDM were incubated on ice for 1 hour to enhance adherence, then incubated at 37°C and 5% CO_2 for 3 hours to allow internalisation. MDM challenged with *S. aureus* were incubated at 37°C and 5% CO_2 for 4 hours. Following incubation, MDM were washed three times with PBS, and if a longer incubation time was required, fresh complete media was added to MDM before subsequent incubation at 37°C and 5% CO_2 for the remaining time.

2.4 siRNA knockdown of Drp1 and Mff genes in MDM

In a number of experiments, the roles of the mitochondrial fission components Drp1 and Mff were investigated by treating MDM with Drp1- or Mff-targeted siRNA prior to use in experiments; this was carried out with Dr Brian McHugh in the Dockrell group. MDM were washed with 500 µl pre-warmed OptiMEM minimal transfection media (OptiMEM), then incubated at 37°C and 5% CO_2 in 210 µl OptiMEM until required. siRNA transfection reagent was prepared by combining 20 µl OptiMEM and 0.6 µl Lipofectamine RNAiMAX reagent (Lipofectamine) per well of MDM to be treated. For each well to be treated,1.6 µl of 20 micromolar smartPOOL siRNA oligos for the gene of interest, or control siRNA, were diluted in 20 µl OptiMEM, then mixed with 20.6 µl of siRNA transfection reagent to produce siRNA/lipid complexes. These mixes were incubated at room temperature for 5 minutes, then the 40.6 µl siRNA/lipid mix was added to the 210 µl OptiMEM in the relevant MDM wells for each treatment condition. MDM were incubated at 37°C and 5% CO_2 for 72 hours, then the above process was repeated with fresh siRNA oligos and reagents. MDM were incubated at 37°C and 5% CO_2 for a further 48 hours before use in further experiments. In addition, successful knockdown of target genes in MDM was confirmed by real-time/reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression in the early stages of these experiments.

2.5 Macrophage killing assay

After incubation with bacteria at 37°C and 5% CO_2, MDM were washed three times with PBS and antibiotic treatment was added. For *S. pneumoniae* challenge, this was 40 units of benzyl-penicillin and 20 µg gentamicin per well in RPMI. For *S.*

aureus challenge, this was 20 µg gentamicin and 0.75 µg vancomycin per well in RPMI. MDM were incubated at 37°C and 5% CO_2 for 30 minutes. MDM were washed three times with PBS, and the final 1 ml wash was diluted and plated to count extracellular bacterial numbers- these represented the pre-lysis bacterial counts. MDM were incubated at 37°C and 5% CO_2 with 250 µl 2% saponin (1g of saponin powder dissolved in 50 ml PBS and filtered) per well, for 12 minutes. After incubation, 750 µl PBS was added per well to dilute the saponin. MDM were lysed by scraping and vigorous pipetting. Serial dilutions were prepared (neat, 10^{-1}, 10^{-2}), and three 10 µl drops of each dilution were plated onto blood agar- these represented the post-lysis bacterial counts. Plates were incubated at 37°C and 5% CO_2 overnight, after which pre-lysis and post-lysis CFU were counted and intracellular bacterial counts (CFU/ml) calculated to represent MDM phagocytosis efficiency using the surface viability counting method described by Miles and Misra (515). Intracellular counts were calculated by subtracting the pre-lysis counts from the post-lysis counts.

Where exogenous LL-37 peptides were used in the killing assay, 25 µg/ml of the peptide was added to MDM in 250 µl RPMI 1640 + 2 millimolar L-glutamine after bacterial incubations and antibiotic treatment. MDM were incubated at 37°C and 5% CO_2 for 1 hour prior to final washes, pre-lysis dilutions and saponin lysis.

2.5.1 Bacterial challenge of BMDM and killing assay

The method was identical for BMDM as for human MDM, however BMDM required DMEM and complete DMEM (DMEM supplemented with 10% low endotoxin FCS) instead of RPMI and complete media.

2.6 Bacteria and cathelicidin susceptibility assay

Susceptibility of *S. pneumoniae* and *S. aureus* to direct cathelicidin exposure was measured by adapting the protocol described by Habets et al (119). 3 x 10^6 CFU S. *pneumoniae* or *S. aureus* was added to 100 µl complete media or RPMI 1640 + 2 millimolar L-glutamine in a well of a 96 well plate. 25 µg/ml cathelicidin or scrambled peptide control was added to the wells, and samples were incubated at 37°C and 5% CO_2 for 1 or 4 hours as required. Following incubation, serial dilutions were prepared (10^{-1} to 10^{-6}), and three 10 µl drops of 10^{-4}, 10^{-5} and 10^{-6} dilutions were plated onto blood agar. Plates were incubated at 37°C and 5% CO_2 overnight, after which CFU were counted, and CFU/ml calculated to represent bacterial viability following cathelicidin exposure.

2.7 Confocal microscopy

Mitochondrial morphology, uptake of carboxytetramethylrhodamine (TAMRA)-labelled cathelicidin, internalised bacteria, and mROS production were all visualised by confocal microscopy. If required, MDM were treated with compounds, siRNA, and/or challenged with bacteria for the necessary time before staining. All incubations steps were carried out at room temperature unless otherwise stated.

2.7.1 General protocol

MDM were washed three times with pre-warmed Hank's Balanced Salt Solution (HBSS) and fixed in 2% paraformaldehyde for 20 minutes. MDM were permeabilised with 50 millimolar NH_4Cl permeabilisation solution (0.27% NH_4Cl and 0.1% Triton X-100 in 500 ml PBS) for 15 minutes, washed once with PBS, and

blocked with PGAT blocking solution (0.2% gelatine, 0.02% sodium azide, 0.01% Triton X-100 in 1 L PBC) for 15 minutes. The required primary antibody was added to MDM for 1 hour, or alternatively overnight at 4°C. MDM were washed three times with PGAT, and the required fluorescent secondary antibody was added to MDM for 1 hour. MDMs were washed three times with PGAT, and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000 dilution) for 12 minutes. MDM were washed three times with PGAT and twice with PBS. If chamber slides were used, MDM were kept in 250 µl PBS, slides were wrapped in foil and stored at 4°C until imaging. If coverslips were used, these were carefully removed from wells, quickly washed in sterile water to remove excess PBS, mounted in 6 µl Prolong Diamond, and allowed to dry in the dark overnight. Z-stacks were obtained using the Leica TCS SP8 confocal laser scanning microscope and Leica LAS X software, the Andor Revolution XDi spinning disk microscope and iQ3 software, or latterly the Andor Revolution Xdi spinning disk microscope and Fusion software. For all images, the 63 x and 60 x oil objective lenses were used on the Leica SP8 and Andor spinning disk microscopes, respectively. For all imaging data presented herein, details of the particular microscope used will be provided in the figure legend.

2.7.2 Mitochondrial morphology staining

Changes in mitochondrial morphology were visualised by staining with the primary antibody targeting the mitochondrial outer membrane protein translocase of outer mitochondrial membrane 20 (TOMM20) (1:500 dilution), followed by the fluorescent secondary antibody AlexaFluor 488 (1:1000 dilution). AlexaFluor 488 has an absorption maximum of 490 nm, and an emission maximum of 525 nm so was visualised with the 488 nm laser.

2.7.3 TAMRA-labelled cathelicidin

In order to visualise the uptake of TAMRA-labelled cathelicidin, 25 µg/ml of peptide was added to MDM and incubated at 37°C and 5% CO_2 for 1 hour prior to paraformaldehyde (PFA) fixation. TAMRA has an absorption maximum of 546 nm, and an emission maximum of 579 nm so was visualised with the 561 nm laser.

2.7.4 mROS staining

mROS production by MDM after bacterial challenge was analysed using MitoSOX Red Mitochondrial Superoxide Indicator. This is designed to localise to mitochondria and produces a red fluorescent signal when oxidised by superoxide, therefore is designed to be specific for mitochondrial-derived ROS. 2 micromolar MitoSOX Red was added per well to stain for mROS and incubated at 37°C and 5% CO_2 for 30 minutes prior to PFA fixation. MitoSOX Red has an absorption maximum of 510 nm, and an emission maximum of 580 nm so was visualised with the 561 nm laser.

2.7.5 Staining of bacteria

Any internalised bacteria present within MDM were stained with DAPI during the nuclear staining step. DAPI has an absorption maximum of 435 nm and an emission maximum of 470nm so was visualised with the 405 nm laser. In one experiment, heat-killed planktonic *S. aureus* was stained with DAPI and MitoSOX (see above), to overcome potential issues with imaging live bacteria using communal microscopy equipment. One vial of *S. aureus* bacteria was thawed and heated in a 60^{o}C heat block for 30 mins, bacteria were pelleted and resuspended in HBSS

containing MitoSOX and incubated as described above. Bacteria were pelleted and washed, then stained with DAPI solution as described above. After staining, 250 µl of culture was placed into wells of a chamber slide, then visualised with the appropriate lasers for DAPI and MitoSOX.

2.8 BMDM mROS production measurements by confocal microscopy

mROS production after challenge of BMDM was analysed by confocal microscopy. BMDM were stained with 2 µM MitoSOX Red as for MDM, then BMDM were fixed in 2% paraformaldehyde as described before. Images were taken using the Leica TCS SP8 laser scanning confocal microscope using the 63x oil objective lens, and the Andor Revolution XDi spinning disk microscope using the 60x oil objective lens. Fluorescence intensity was analysed in Fiji/ImageJ 1.52i.

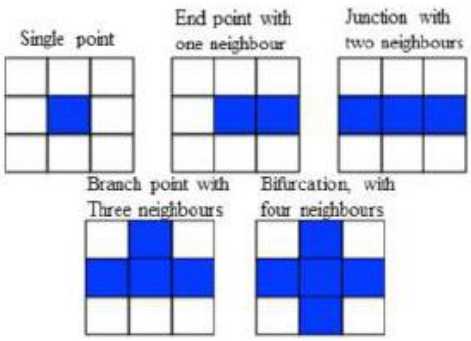
2.9 Image analysis

Z-stacks of confocal images were viewed and processed using Fiji/ImageJ 1.52i. Different coloured channels were split, and maximum intensity Z projections obtained for each channel.

2.9.1 Mitochondrial network complexity analysis

The AlexaFluor 488 channel maximum Z projected images were analysed to calculate mitochondrial network complexity. The individual macrophage of interest was selected from the image and duplicated. Brightness and contrast were adjusted to reduce background signal, and the macrophage of interest was freehand selected to ensure that only that macrophage was included in the analysis. A 13 x 13 Hat filter, developed by Kurt De Vos University of Sheffield, was applied to improve

resolution (516). The threshold was adjusted, the image was made binary and then skeletonised. The Binary Connectivity plugin was applied, and a histogram list generated. The mitochondrial network complexity score was calculated from the histogram data. The figure below from Mohasin et al. 2019 (pre-print in BioArchive (BioRxiv) (387)) explains the histogram data and calculation (Figure 2.1):



Value (WT-Mock infected)	Count	Description	Network complexity
0	62232	Background	Ratio of total number of Branch points(value ≥4) and End points(value =2) (187+33+3)/322= 0.69
1	4	Single pixels	
2	322	End points	
3	1708	Junction with two neighbours (more count higher the length)	
4	187	Triple point with three neighbour (Branch point)	
5	33	Bifurcation with four neighbours (Branch point)	
6	3	Five neighbours (branch point)	

Figure 2.1: Calculation of mitochondrial network complexity from histogram list values in Fiji/ImageJ, Mohasin et al. 2019 (pre-print in BioRxiv (387)**).** Each value and count in the histogram list correspond to the branching complexity of the point measured and the number of those points calculated, for example value 1 has a count of 4, indicating that there are 4 single points within the mitochondrial network of the analysed macrophage. The mitochondrial network complexity score is calculated as the sum of all counts at values ≥4, divided by the count at value 2. This calculation allows for mitochondrial network complexity of individual macrophages to be numerically determined and quantified.

2.9.2 Analysis of corrected total cell fluorescence (CTCF)

The following analysis was identical for measuring the fluorescence intensity of TAMRA-LL-37 and MitoSOX Red signals. As all of these are fluorescent by red wavelengths of light and for the purposes of describing the analysis process, these signals will be described generally as the red channel.

In order to accurately determine the cell boundaries so that only cell-associated red fluorescent signal was analysed, the maximum Z projected mitochondrial and red channel images were merged. The red channel was selected on the sliding scale to ensure that only this channel was included in the analysis. The individual macrophage of interest was selected from the image and duplicated. The macrophage of interest was freehand selected to ensure that only that macrophage was included in the analysis. The following measurements for the selected area were generated by Fiji, and these were used to calculate the corrected total cell fluorescence: the integrated density (IntDen) measurement for the red channel, which is the sum of intensities from all pixels in the selected cells; the area of the selected cell; and the mean background fluorescence of the red channel which was measured by selecting regions of the image without cells. The corrected total cell fluorescence (CTCF) was calculated using the following formula, where a higher CTCF score correlated with higher fluorescence:

CTCF = Integrated Density – (area of cell x background fluorescence)

2.9.3 Image analysis macro production

As this protocol was required to analyse every cell in an image, a Fiji macro was produced for the fluorescence intensity and mitochondrial network complexity protocols in order to speed up the analysis process. The individual cells in an image were selected as described above, then the macro was run to quickly skeletonise cells, generate histogram lists, and calculate measurements for CTCF analysis for multiple cells simultaneously.

2.10 *CAMP* gene expression in MDM

CAMP gene expression and factors that may induce this expression were investigated using qRT-PCR. 1,25 dihydroxyvitamin-D3 (termed calcitriol) is a known inducer of HDP gene expression, particularly *CAMP*, and was used in most of the qRT-PCR experiments. 100 nM of calcitriol was used to induce *CAMP* gene expression in MDM, and MDM were incubated at 37°C and 5% CO_2 for 24 hours prior to bacterial challenge or other treatments in all cases unless otherwise stated. In the experiments investigating the influence of PBA, MDM were treated with 2 micromolar PBA for 24 hours as described above for calcitriol, unless otherwise stated.

2.10.1 *S. pneumoniae* influence on *CAMP* gene expression

After calcitriol incubation, *S. pneumoniae* was washed and opsonised as described above and added to MDM at a MOI of 10. Conditions were prepared in at least duplicate to obtain sufficient RNA. MDM were incubated on ice for 1 hour then at 37°C and 5% CO_2 for 3 hours as described above. MDM were washed 3 times by

filling wells with PBS, then MDM were lysed using 600 µl of Buffer RLT from the RNeasy Plus Mini Kit, supplemented with 2% β-mercaptoethanol. The buffer was added to one well for each condition and MDM lysed by scraping and careful pipetting. The buffer was transferred to the next well of the same condition and the process repeated. The final lysate was placed into a 1.5 ml tube and vortexed to ensure thorough homogenisation. RNA was extracted from MDM using the RNeasy Plus Mini Kit, following the manufacturer's instructions. After extraction, RNA concentrations were measured using 1 µl on the Nanodrop 1000 spectrophotometer (Thermo Fisher) where concentrations were given in ng/µl. cDNA was synthesised using the Quantitect Reverse Transcription Kit, following the manufacturer's instructions. The volume of RNA included in each reaction was dependent on the original RNA concentration. The relative gene expression levels were measured using quantitative PCR. Reactions were prepared using the TaqMan Gene Expression Master Mix and the TaqMan gene expression assays for the following: *CAMP* (LL-37), and *18S* rRNA as the housekeeping gene. *18S* rRNA was used in this system as a validated TaqMan probe was easily available for this gene and other TaqMan users had used this probe successfully in other models. Samples were run on the Applied Biosystems StepOne Plus system (Thermo Fisher). Since gene assays used fluorescein amidite (FAM) dye and the *18S* assay used VIC dye, the two fluorescence outputs could be measured simultaneously within a sample. Data was analysed and graphs generated in GraphPad Prism (version 8.0.1).

2.10.2 *S. aureus* influence on *CAMP* gene expression

MDM were treated with calcitriol, *S. aureus* was washed and added to MDM at a MOI of 10, plates were incubated at 37°C and 5% CO_2 for 4 hours, and RNA

was extracted as for *S. pneumoniae*-treated MDM. However, these experiments were carried out after the *S. pneumoniae* experiments, and as a more cost effective system than TaqMan, the Promega GoTaq 1-Step RT-qPCR system was used according to the manufacturer's instructions. This system is primer-based rather than probe-based and combines cDNA synthesis and qRT-PCR (the SYBR principle), so only RNA and primers are required to add to reactions. Therefore, it was necessary to design primers for the required genes rather than use a commercially available probe. Primer oligos for the *CAMP* gene and the housekeeping genes for β-actin and GAPDH were designed using the human gene sequences in the Snapgene Viewer software (version 5.2.4), purchased from Integrated DNA Technologies, Inc. (IDT), and primer efficiencies were optimised for this kit. β-actin and GAPDH were selected as the housekeeping genes in this case as prior to undertaking this project, I previously used homologues of these genes successfully in qPCR experiments in honeybee and bumblebee models. Furthermore, it was beneficial to measure two housekeeping genes rather than one in this case; as the primers are not commercially validated like the TaqMan probes, these extra measurements minimise potential variations or discrepancies in primer efficiency between samples or experiments. Primer sequences can be found in Table 2.1. To analyse gene expression, 100 ng of RNA was added to each reaction, along with 5 millimolar of forward and reverse primers for the gene to be analysed. Samples were also run on the Applied Biosystems StepOne Plus system, and data was also analysed, and graphs generated in GraphPad Prism (version 8.0.1).

PRIMER NAME	SEQUENCE (5'-3')	ORIGINAL PUBLISHED SEQUENCE FROM WHICH PRIMERS WERE DESIGNED
CAMP F	TGCTGGGTGATTTCTTCCGGA	*Homo sapiens* cathelicidin antimicrobial peptide (*CAMP*), mRNA. NCBI Reference Sequence: NM_004345.5
CAMP R	CCTGAGGAAATTGCTCTCATAGT	
β-actin F	TATTTTGAATGATGAGCCTTCG	*Homo sapiens* chromosome 7, GRCh38.p13 Primary Assembly. NCBI Reference Sequence: NC_000007.14
β-actin R	TCATTTTTAAGGTGTGCACTTTTAT	
GAPDH F	AGCAAGAGCACAAGAGGAAGA	*Homo sapiens* glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RefSeqGene on chromosome 12. NCBI Reference Sequence: NG_007073.2
GAPDH R	TAACTGGTTGAGCACAGGGTACT	

Table 2.1: Details of the IDT primer oligos used in the gene expression qPCR experiments of *S. aureus*-challenged MDM.

2.10.3 Pro-inflammatory cytokine influence on *CAMP* gene expression

The pro-inflammatory cytokines TNF-α and IFN-γ were also used in *CAMP* gene expression experiments to determine if their presence altered the expression of *CAMP* in MDM, as there are common pro-inflammatory cytokines that a macrophage would encounter in an inflammatory infection environment. Cytokines were exogenously applied to MDM, and two variations of this experiment were carried out- simultaneous incubation of MDM with calcitriol and the relevant cytokine at 37°C and 5% CO_2 for 24 hours, and 24 hour calcitriol incubation followed by 4 hour cytokine incubation at 37°C and 5% CO_2. In both cases, all cytokines were added to MDM at a final concentration of 20 ng/ml in complete media. RNA was extracted after the

required incubation times as for bacterial challenged MDM, and *CAMP* gene expression analysed as for *S. aureus*-treated MDM. Data was also analysed and graphs generated in GraphPad Prism (version 8.0.1).

2.11 Inner mitochondrial transmembrane potential ($\Delta\psi_m$) measurements in MDM by flow cytometry- general protocol

After bacterial challenge and/or compound treatment, MDM were washed three times with PBS and three times with pre-warmed HBSS. The required fluorescent dye was added to MDM and samples were incubated at 37°C and 5% CO_2 for 30 minutes. Samples were washed twice with 1 ml pre-warmed HBSS and resuspended in 300 µl PBS per well by gentle scraping to dislodge adherent cells. Samples were placed in flow cytometry (FACS) tubes and analysed on the Attune NxT acoustic focusing cytometer. 10,000 events were analysed per sample, and forward and side scatter were used to determine the cell population. Flow cytometry data was analysed using the FCS Express 6 Flow Research Edition software, and graphs generated using GraphPad Prism (version 8.0.1).

2.11.1 Dyes for detecting changes in inner mitochondrial transmembrane potential ($\Delta\psi_m$)

The following dyes are widely used to detect changes in $\Delta\psi_m$- tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and tetramethylrhodamine methyl ester (TMRM). JC-1 is a cationic carbocyanide dye that accumulates in mitochondria and changes its form and fluorescence in response to changes in $\Delta\psi_m$. While $\Delta\psi_m$ is high, JC-1 accumulates in mitochondria and aggregates; these aggregations produce red fluorescence. When $\Delta\psi_m$ is diminished, less JC-1

accumulates and does not aggregate; these monomers produce green fluorescence. This shift in fluorescence can be detected as a marker of changes in $\Delta\psi_m$. As mentioned above, TMRM dye also accumulates in mitochondria and produces a bright red fluorescence when $\Delta\psi_m$ is high, which is reduced when $\Delta\psi_m$ is lost. Therefore, rather than a shift in the fluorescence produced, TMRM indicates changes in $\Delta\psi_m$ by the intensity of the fluorescent signal and may therefore be more sensitive to more subtle changes (517). 10 micromolar JC-1 was added per well. JC-1 red aggregates have an absorption maximum of 585 nm and an emission maximum of 590 nm, so fluorescence was detected on the flow cytometer using the BL1 channel. As MDM have high green autofluorescence properties, changes in JC-1 fluorescence were measured as gain or loss of red fluorescence. 1x TMRM was added per well. TMRM has an absorption maximum of 548 nm and an emission maximum of 574 nm, so fluorescence was detected on the flow cytometer also using the BL1 channel.

2.12 Statistics

All data is represented as mean and standard error of the mean (SEM) unless otherwise indicated. Statistical significance was determined using the tests in GraphPad Prism (version 8.0.1). Details of the particular statistical tests used can be found in individual figure legends. Statistical tests were carried out using the average values per donor in each condition for each independent experiment, even where individual cell values are plotted. Statistical significance was determined as $p<0.05$. Multiple comparisons tests were conducted as appropriate; test selection was based on the recommendations provided by GraphPad Prism depending on the statistical test being conducted and the type of comparison. Examples of statistical

tests/comparison types and the recommended multiple comparisons tests are shown in Table 2.2.

STATISTICAL TEST	TYPE OF COMPARISON	RECOMMENDED MULTIPLE COMPARISONS TEST
Kruskal-Wallis test	Any	Dunn's multiple comparisons
One-way ANOVA	Independent or pre-selected group comparisons	Sidak's multiple comparisons
Two-way ANOVA	Every row mean with every other row mean Or Every column mean with every other column mean	Tukey's multiple comparisons
	Independent or pre-selected group comparisons	Sidak's multiple comparisons

Table 2.2: Details of the multiple comparison tests conducted in this thesis, depending on the original statistical test and type of comparison.

REAGENT	MANUFACTURER	CAT. NUMBER	ADDITIONAL DETAILS
0.9% NaCl	Baxter	UKF7124	Used for isolation of PBMC and PMN from blood
8-well chamber slides	Ibidi	80806	-
100 mm square Sterilin plates	Thermo Fisher	11349273	-
13 mm round glass coverslips	Scientific Laboratory Supplies	NPC1613	1.5 mm thickness
24-well tissue culture plates	Thermo Fisher	10732552	Corning CoStar brand, flat bottom, for MDM culture
48-well tissue culture plates	Thermo Fisher	10065370	Corning CoStar brand, flat bottom
96-well tissue culture plates	Thermo Fisher	10687551	Corning brand, flat bottom
Accutase	BioLegend	423210	Cell dissociation solution used for re-plating MDM onto chamber slides
Alexa Fluor 488	ABCAM	ab150077	Goat anti-rabbit polyclonal IgG, fluorescent secondary antibody for mitochondrial staining, stock concentration of 2 mg/ml, 1:1000 dilution used
Ammonium chloride	Fluka	09711	NH_4Cl, used to prepare permeabilisation solution (0.27% in 500 ml PBS) for mitochondrial staining.
Anti-TOMM20 antibody	ABCAM	Ab78547	Rabbit polyclonal IgG, primary antibody for mitochondrial staining, stock concentration of 1 mg/ml, 1:500 dilution used
Baxter's water	Baxter	UKF7114	Sterile water

β-mercaptoethanol	Sigma	M3148-100mL	10 µl added per 1ml of RLT lysis buffer for RNA extraction
Benzylpenicillin	Sigma	P0389-5KU	Penicillinase from *Bacillus cereus* lyophilised powder, 1500-3000 units/mg protein
CA074-methyl ester	Sigma	C5857	Cathepsin B inhibitor, stock concentration prepared to 25 millimolar in DMSO, working concentration of 25 micromolar
Calcitriol	ABCAM	ab141456-50µg	1,25-dihydroxyvitamin D3, stock solution prepared to 100 micromolar, working concentration of 100 nanomolar
Calcium chloride dihydrate ($CaCl_2$)	Scientific Laboratory Supplies	C3881-500g	>99% purity, stock solution prepared to 1 millimolar, used to prepare autologous serum at concentration of 20 micromolar
Cell scrapers	Thermo Fisher	10702192	-
4′,6-diamidino-2-phenylindole (DAPI)	Thermo Fisher	D1306	Used for nuclear and bacterial staining, stock concentration prepared to 5 mg/ml in dH_20, 1:1000 dilution used
Dextran	Pharmacosmos	5510 0500 8007	Dextran 500 Pharmaceutical Quality, 6% solution prepared in Baxter's NaCl and filtered
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	21969	+ D-glucose, + sodium pyruvate, - L-glutamine, used for BMDM culture
DMSO	Sigma	276855	Anhydrous, used to prepare stock solutions of carious compounds and as compound negative controls

Diphenyleneiodonium chloride (DPI)	Sigma	D2926	NADPH oxidase inhibitor, stock concentration prepared to 10 millimolar in DMSO, working concentration of 10 micromolar
Dulbecco's phosphate buffered saline (PBS), 10x	Sigma	D1408-500ML	-$CaCl_2$, -$MgCl_2$, used in preparation of Percoll density gradient
Dulbecco's phosphate buffered saline (PBS), 1x	Gibco	14190-094	-$CaCl_2$, -$MgCl_2$, used in preparation of Percoll density gradient
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)	ABCAM	ab120081	Mitochondrial oxidative phosphorylation uncoupler, stock concentration prepared to 100 micromolar in DMSO, working concentration of 100 nanomolar
Foetal bovine serum (FBS) Good Forte	Pan Biotech Ltd	P40-47500HI	Heat inactivated, low endotoxin, lot number P160706, for MDM culture
Gelatine from bovine skin	Sigma	G9391-500G	Used to prepare PGAT solution for mitochondrial staining (0.2% gelatine in 1 L PBS)
Gentamicin	Scientific Laboratory Supplies	LZ17-519Z	Lonza, stock solution of 10 mg/ml
GoTaq 1-Step RT-qPCR System	Promega	A6020	Used to analyse *CAMP* gene expression in MDM challenged with *S. aureus*
Hank's Balanced Salt Solution (HBSS)	Gibco	14025-050	+$CaCl_2$, +$MgCl_2$
Human recombinant IFN-γ cytokine	STEMCELL Technologies Inc.	78020.1	Stock solution prepared to 50 µg/ml, working concentration of 20 ng/ml

Human recombinant TNFα cytokine	STEMCELL Technologies Inc.	78068.1	Stock solution prepared to 10 µg/ml, working concentration of 20 ng/ml
Image-iT TMRM Reagent	Life Technologies	I34361	Mitochondrial membrane potential indicator, 1000x stock concentration, 1x working concentration
Tetraethylbenzimidazolylcarbocyanine iodide (JC-1)	Cambridge BioScience	15003-1mg-CAY	Mitochondrial membrane potential indicator, stock prepared to a concentration of 1 mg/ml (1.53 millimolar) in DMSO, working concentration of 10 micromolar
L-glutamine	Life Technologies	25030-024	Stock concentration of 200 millimolar, working concentration of 2 millimolar in RPMI 1640
Lipofectamine RNAiMAX reagent	Life Technologies	13778-075	Used in siRNA transfection of MDM
LL-37 peptide	Almac	Batch number: 3131P01	Stock concentration prepared to 5 mg/ml in endotoxin-free water, working concentration of 25 µg/ml
MicroAmp Fast optical 96-well plate	Life Technologies	4346906	Used in preparation of RT-qPCR reactions
MicroAmp optical adhesive film	Life Technologies	4311971	Used in preparation of RT-qPCR reactions
Microscope slides	Thermo Fisher	12362098	Twin Frosted Super Premium
Mitoparaquat (MitoPQ)	ABCAM	ab146819	Mitochondrial-targeted redox cycler, stock concentration of 1 millimolar in DMSO, working concentration of 10 micromolar

Mitoquinone (MitoQ)	Cambridge BioScience	89950-5mg-CAY	Mitochondrial-targeted O_2 scavenger, stock concentration of 5 mg/ml (7.37 millimolar) in DMSO, working concentration of 300 nanomolar
MitoSOX Red	Life Technologies	M36008	For detection of mitochondrial superoxide, stock concentration prepared to 2 millimolar in DMSO, working concentration of 2 micromolar
MitoTEMPO	Enzo Life Sciences	ALX-430-150-M005	Mitochondrial-targeted antioxidant, stock concentration prepared to 10 millimolar in DMSO, working concentration of 100 micromolar
OptiMEM minimal transfection media	Life Technologies	31985-062	Used in siRNA transfection of MDM
Paraformaldehyde (PFA)	Life Technologies	28908	4% stock concentration prepared, 2% working concentration used
Penicillin-streptomycin	Life Technologies	15070063	100ml containing 5000 units/ml penicillin and 5000µg/ml streptomycin
Percoll	GE Healthcare	17-0891-01	Density: 1.129 g/ml, used in preparation of Percoll density gradient
Phenol red-free RPMI 1640	Sigma	R7509	For staining with fluorescent dyes (MitoSOX, JC-1, TMRM)
Prolong Diamond	Life Technologies	P36965	Fade-resistant mounting media for preparation of microscopy slides, 6 µl used to mount each coverslip
Quantitect Reverse Transcription Kit	Qiagen	205311	Used to prepare cDNA from RNA extracted from *S. pneumoniae*-challenged MDM

RNeasy Plus Mini Kit	Qiagen	74134	For RNA extraction
Rotenone	Sigma	R8875-1G	Provided by the Walmsley group, stock concentration prepared to 2 millimolar, working concentration of 10 micromolar
RPMI 1640	Sigma	R8758	Used for MDM culture
Saponin	Fluka	47036	2% stock solution prepared in Baxter's water and filtered
scLL-37 scrambled peptide	Almac	Batch number: 3131p02	Stock concentration prepared to 5 mg/ml in endotoxin-free water, working concentration of 25 µg/ml
smartPOOL siRNA oligos	Horizon Discovery/ Dharmacon	Drp1: L-012092-00-0005 Mff: L-018261-00-005	Drp1 and Mff siRNA oligos used on MDM, stock concentration prepared to 20 micromolar, 1.6 µl per MDM well. Drp1 siRNA is listed by manufacturer as DNML1, dynamin-like protein.
Sodium azide	Sigma	S2002-25G	5% stock solution used to prepare PGAT solution for mitochondrial staining (0.02% sodium azide in 1 L PBS)
TAMRA-LL-37 peptide	Almac	Batch number: 2017p01	Stock concentration prepared to 5 mg/ml in endotoxin-free water, working concentration of 25 µg/ml
TaqMan Gene Expression Master Mix	Life Technologies	4369016	Used to analyse *CAMP* gene expression in MDM challenged with *S. pneumoniae*
TaqMan Gene Expression Assay: *CAMP*	Life Technologies	4331182	Assay ID: Hs00189038_m1, used to analyse *CAMP* gene expression in MDM challenged with *S. pneumoniae*

TaqMan Gene Expression Assay: 18S	Life Technologies	448489	Assay ID: Hs99999901_s1, used to analyse *CAMP* gene expression in MDM challenged with *S. pneumoniae*
Trisodium citrate dihydrate	Sigma	S1804-500G	3.8% stock solution prepared in Baxter's water and filtered
Triton-X 100	Sigma	X100-500mL	Used to prepare PGAT solution (0.01% in 1 L PBS) and permeabilisation solution (0.1% in 500 ml PBS) for mitochondrial staining
Vancomycin	Sigma	V2002	Stock solution prepared to 750 µg/ml in H_20, working concentration of 0.75 µg/ml

Methods Appendix 2.1: Equipment and reagents used throughout the project.

Chapter 3

Cathelicidin expression in human macrophages and microbicidal functions against *Streptococcus pneumoniae* and *Staphylococcus aureus*

3.1 Introduction

The cationic host defence peptide cathelicidin is well established as an important component of the host immune system (425). The *CAMP* gene, encoding cathelicidin, is regulated by a range of mechanisms in different cell types, for example vitamin D metabolism, the action of butyrate, and pro-inflammatory cytokine signalling (432–434,436). Many studies have been carried out to investigate mechanisms of *CAMP* expression in depth in epithelial cell models, while mechanisms of *CAMP* expression in macrophage models are less well established. Vitamin D has been shown to upregulate *CAMP* expression in primary human PBMC and monocytes (444,502), and vitamin D and PBA have been reported to upregulate *CAMP* expression the THP-1 macrophage-like cell line and in human MDM infected with the gram-negative bacterium *Klebsiella pneumoniae* (518). However, the influence of infection with the gram-positive bacteria *S. pneumoniae* and *S. aureus* on vitamin D-mediated *CAMP* expression in human MDM is unclear. Furthermore, pro-inflammatory cytokines have been shown to impair vitamin D-mediated *CAMP* expression in airway epithelial cells (436), but the influence of pro-inflammatory cytokines on vitamin D-mediated *CAMP* expression in human MDM has not been established. Therefore, these were the focus of the first part of this chapter.

I hypothesised that vitamin D and PBA inducers would upregulate *CAMP* expression in human MDM and that *S. pneumoniae* or *S. aureus* infection, or pro-inflammatory cytokines would further modulate *CAMP* expression. I therefore investigated the influence of vitamin D and PBA on *CAMP* gene expression in MDM and how bacterial infection and inflammatory cytokines modified these responses, examining both expression data and the kinetics of responses.

Macrophages possess a variety of microbicidal responses to clear pathogens, however there are limitations to these responses. They are less effective at killing intracellular bacteria than other immune cells such as monocytes or neutrophils (289,290), and pathogens possess adaptations to these responses to resist or evade macrophage killing (20,21).Therefore, a multi-layered immune response with combined microbicidal responses for optimal macrophage killing of bacteria are required. *S. pneumoniae* and *S. aureus* present contrasting challenges to the macrophage in regard to intracellular burden; *S. pneumoniae* is more difficult to phagocytose due to the polysaccharide capsule (108), but this results in a lower intracellular burden that macrophages can clear more easily. *S. aureus* is easily internalised by macrophages but is more adapted to resist intracellular killing (72), thereby representing a greater stress to the macrophage. Therefore, comparing and contrasting macrophage responses against these pathogens provided a greater insight into how the macrophage responds to different challenges. Cathelicidin is known to have bactericidal activity against many different bacteria, including *S. pneumoniae* and *S. aureus* (463), and the importance of vitamin D-mediated enhancement of cathelicidin expression in mesenchymal stem cell antibacterial defence has been demonstrated for *S. aureus* and *E. coli* (519). Cathelicidin also

contributes to enhanced human macrophage clearance of intracellular bacteria, such as Mtb and *Klebsiella pneumoniae* (503,518), and promotes phagocytosis of both gram-positive and gram-negative bacteria by human macrophages (506,508). However, the influence of cathelicidin on MDM killing of *S. pneumoniae* and *S. aureus* is not established, therefore this was the focus of the second part of this chapter. I hypothesised that in addition to direct bactericidal functionality on extracellular bacteria, the presence of cathelicidin would enhance MDM killing of internalised *S. pneumoniae* and *S. aureus*. The addition of exogenous cathelicidin to MDM during the bacterial killing assays allowed me to investigate this.

3.2 Results

3.2.1 *CAMP* gene expression in MDM is upregulated after calcitriol treatment, and further upregulated by bacterial challenge following calcitriol treatment

As described above, 1,25-dihhydroxyvitamin D3, or calcitriol, is a well-characterised inducer of *CAMP* expression in various cell types, including macrophages (432), therefore I began by using this to investigate changes in *CAMP* expression in MDM in response to vitamin D stimulation and bacterial challenge. MDM were incubated with calcitriol for 24 hours followed by 4 hour mock infection, *S. pneumoniae* or *S. aureus* challenge. *CAMP* expression was determined by RT-qPCR. In both cases, relative *CAMP* expression levels were calculated relative to the expression of the housekeeping genes. As the *S. pneumoniae* experiments used the TaqMan probe system, relative *CAMP* expression levels were calculated using the 18S rRNA housekeeping gene expression levels. As the *S. aureus* experiments used

the One-Step RT-qPCR (SYBR) primer-based system, relative *CAMP* expression levels were calculated using the average β-actin and GAPDH gene expression levels for each condition. Figure 3.1 shows the relative *CAMP* expression levels in response to calcitriol and/or *S. pneumoniae*, and Figure 3.2 shows the relative *CAMP* expression levels in response to calcitriol and/or *S. aureus*.

In both cases, calcitriol treatment alone significantly upregulated *CAMP* expression, as expected. In contrast, bacterial challenge alone did not upregulate *CAMP* expression, suggesting that the presence of bacteria alone was insufficient to enhance *CAMP* expression in MDM. However, there was a non-significant trend toward a reduction in *CAMP* expression following *S. pneumoniae* challenge in MDM compared to mock infection. This suggests that *S. pneumoniae* might be able to subtly suppress baseline *CAMP* expression in MDM, a phenomenon that has previously been demonstrated in a rabbit lung and intestinal epithelial tissue model, where *Shigella* infection resulted in reduced cathelicidin expression (520) Interestingly, calcitriol treatment followed by bacterial challenge significantly upregulated *CAMP* expression over calcitriol alone in both cases. This shows that, although bacterial challenge did not directly alter *CAMP* expression in MDM, it significantly enhanced the induction of *CAMP* in the presence of calcitriol. Therefore, there was a synergistic effect of these stimuli on the induction of *CAMP* expression, which is greater than each stimulus alone, or the additive effect of both stimuli.

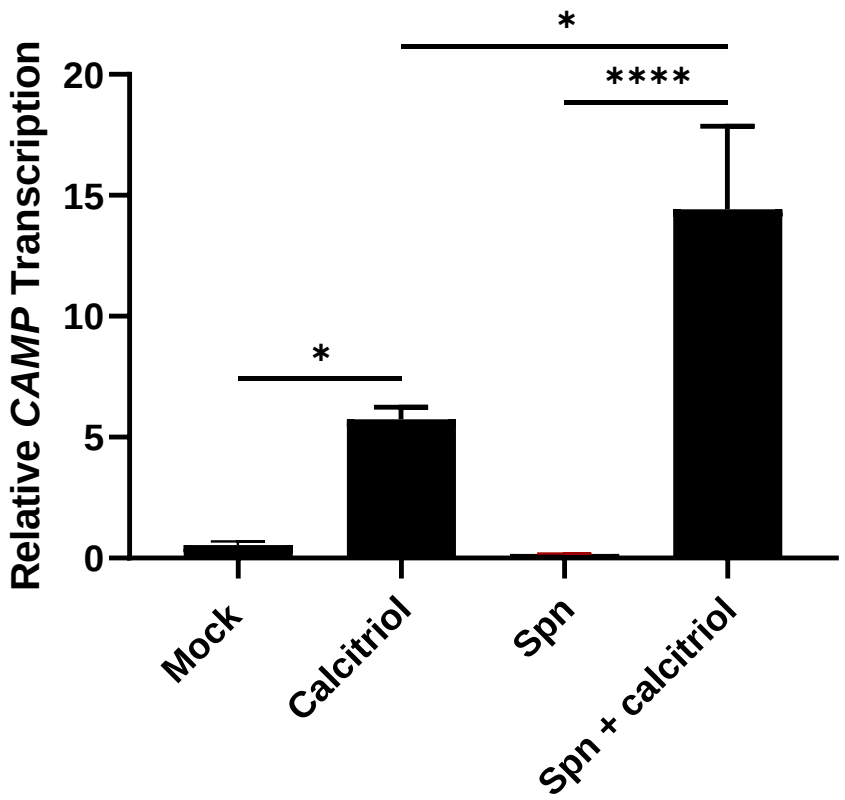


Figure 3.1: Upregulation of *CAMP* gene expression in response to calcitriol induction and *S. pneumoniae* challenge. Relative gene expression of *CAMP* in MDM after calcitriol treatment or vehicle control, with mock infection or bacterial challenge with opsonised *S. pneumoniae* at a multiplicity of infection (MOI) of 10. Experiments run using the TaqMan probe system, therefore used 18S rRNA as the housekeeping gene. Error bars represent the standard error of the mean (SEM). n=4 independent experiments. Two-way ANOVA with Sidak's multiple comparisons test carried out. *p<0.05, ****p<0.0001. Overall ANOVA results: ****presence of calcitriol, **presence of bacteria, **interaction between bacterial challenge and calcitriol.

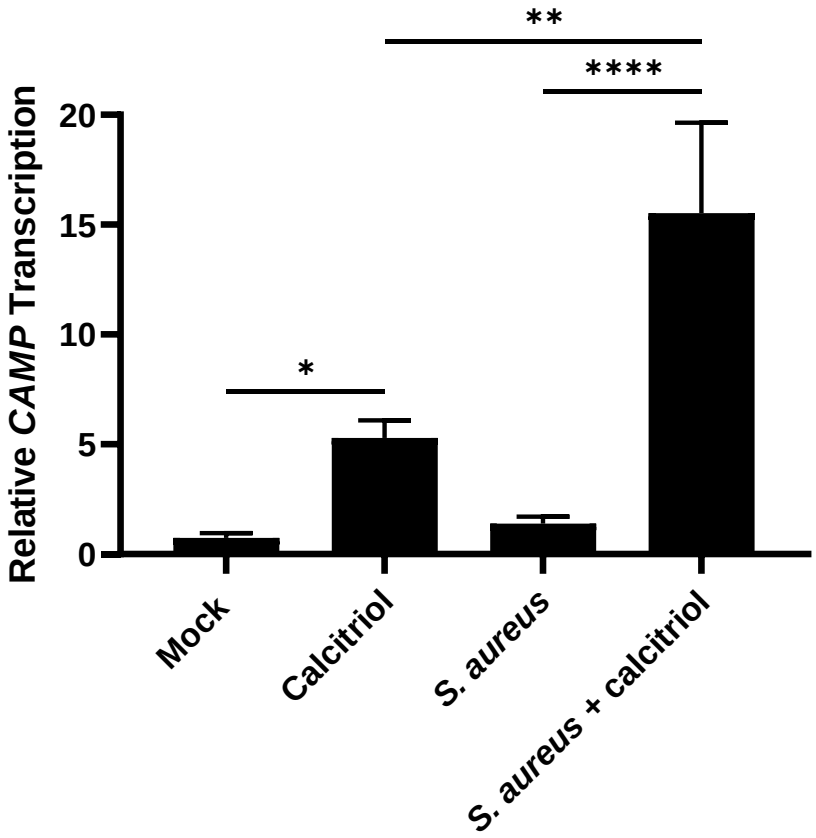


Figure 3.2: Upregulation of *CAMP* expression in response to calcitriol induction and *S. aureus* challenge. Relative gene expression of *CAMP* in MDM after mock infection or bacterial challenge with *S. aureus,* MOI=10, combined with sequential calcitriol or vehicle treatment. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=4 independent experiments. Two-way ANOVA with Sidak's multiple comparisons test carried out. *p<0.05, **p<0.01, ****p<0.0001. Overall ANOVA results: ****presence of calcitriol, ***presence of bacteria, **interaction between bacterial challenge and calcitriol.

3.2.2 The pro-inflammatory cytokines TNF-α and IFN-γ dampen calcitriol-mediated induction of *CAMP* gene expression in MDM

Next it was important to establish how induction of *CAMP* expression in macrophages may be altered within an inflammatory environment, as these are the conditions in which macrophages most commonly encounter and clear bacterial infections. This was represented experimentally by the addition of the pro-inflammatory cytokines TNF-α and IFN-γ. MDM were treated with calcitriol and/or either TNF-α or IFN-γ pro-inflammatory cytokine, either sequentially (24 hour calcitriol followed by 4 hour cytokine incubation), or simultaneously for 24 hours. RT-qPCR was carried out using the One-Step RT-qPCR (SYBR) primer based system, and β-actin and GAPDH as the housekeeping genes.

As shown in Figure 3.3, calcitriol treatment significantly upregulated *CAMP* expression as expected. Neither 4 hour (Figure 3.3A) nor 24 hour (Figure 3.3B) cytokine treatment alone upregulated *CAMP* expression, again suggesting that this single stimulus is insufficient to induce *CAMP* expression in MDM. Sequential calcitriol and cytokine treatment (Figure 3.3A) did not significantly impact *CAMP* expression levels compared to calcitriol treatment alone, suggesting that once MDM are primed with calcitriol, the presence of pro-inflammatory cytokines has little effect on calcitriol-induced *CAMP* expression. However, simultaneous calcitriol and cytokine treatment (Figure 3.3B) significantly reduced *CAMP* expression levels compared to calcitriol treatment alone. These results demonstrate that the pro-inflammatory cytokines TNF-α and IFN-γ impair calcitriol-induced upregulation of *CAMP* expression in MDM when these factors are present simultaneously.

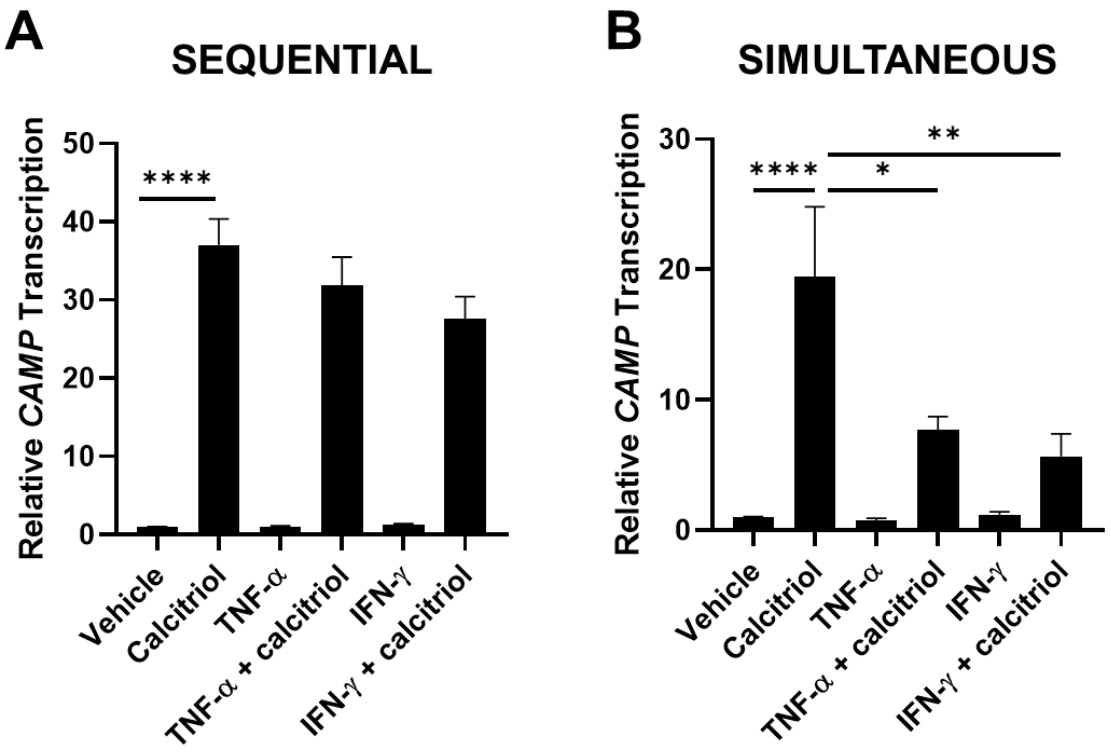


Figure 3.3: Influence of calcitriol and pro-inflammatory cytokines on *CAMP* gene expression in MDM. Relative gene expression of *CAMP* in MDM following 24 hour vehicle or calcitriol treatment, and either A) sequential treatment with TNF-α or IFN-γ for 4 hours following 24 hour mock or calcitriol treatment, or B) simultaneous calcitriol treatment with TNF-α or IFN-γ for 24 hours. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=3 independent experiments. Two-way ANOVA with Tukey's multiple comparisons test carried out. *p<0.05, **p<0.01, ****p<0.0001. Overall ANOVA results for sequential calcitriol and cytokine treatment: ****presence of calcitriol, *presence of cytokine, *interaction between cytokine and calcitriol treatment.

3.2.3 The influence of TNF-α and IFN-γ on calcitriol-mediated induction of *CAMP* gene expression in MDM over time

The inhibitory effects of TNF-α and IFN-γ on calcitriol-mediated induction of *CAMP* expression were intriguing. Therefore, to understand the kinetics of this response in more depth, I investigated how these cytokines influence calcitriol-mediated *CAMP* expression over time. MDM were vehicle treated or treated with calcitriol and either TNF-α or IFN-γ pro-inflammatory cytokine, either alone or simultaneously for 2, 4, 8, or 24 hours. RT-qPCR was carried out using the One-Step RT-qPCR (SYBR) primer based system, and β-actin and GAPDH as the housekeeping genes.

As shown in Figures 3.4 and 3.5, calcitriol treatment alone modestly upregulated *CAMP* expression after 2 hours and this effect increased over time, with significant upregulation observed after 24 hours. This suggested that calcitriol optimally upregulates *CAMP* expression at later time points. Figure 3.4 shows TNF-α treatment alone modestly, but not significantly, upregulated *CAMP* expression after 2 hours, and upregulation increased further after 4 hours. However, TNF-α-induced *CAMP* expression levels began to wane after this 4 hour point, which suggested that TNF-α effects on *CAMP* expression are transient but not significant. As shown in Figure 3.5, IFN-γ alone did not upregulate *CAMP* expression at any time point, therefore IFN-γ as a single stimulus is insufficient to induce *CAMP* expression in MDM. Interestingly, in both cases, simultaneous treatment of calcitriol and cytokine resulted in a reduction of *CAMP* expression levels to some extent from the 4 hour time point, although this only reached significance at 24 hours. Therefore, these results show that in MDM, TNF-α only transiently and not significantly induced *CAMP*

expression and this effect was not sustained, while IFN-γ did not upregulate *CAMP* expression. Vitamin D-mediated *CAMP* upregulation was a slower response but resulted in significant later upregulation of *CAMP* expression. However, the presence of these pro-inflammatory cytokines alongside calcitriol significantly dampened vitamin D-mediated induction of *CAMP* expression by 24 hours. Therefore, a sustained pro-inflammatory environment dampens the upregulation of *CAMP* expression in MDM.

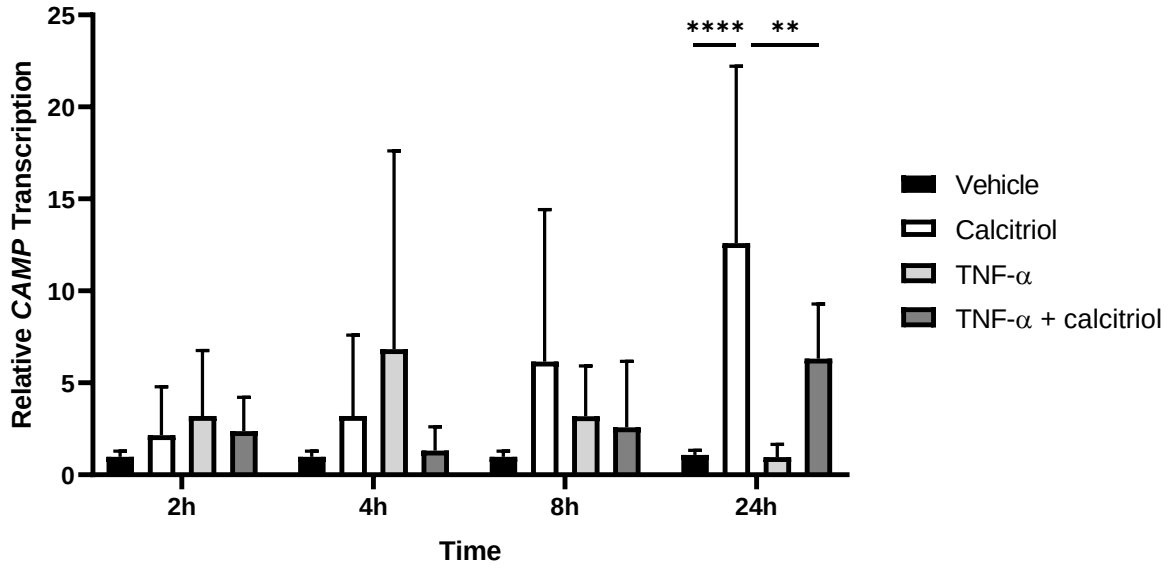


Figure 3.4: Influence of TNF-α on the calcitriol-mediated induction of *CAMP* gene expression in MDM over time. Relative gene expression of *CAMP* in MDM over time following treatment with vehicle, calcitriol or TNF-α alone, or calcitriol and TNF-α simultaneously. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=4 independent experiments. Two-way ANOVA with Tukey's multiple comparisons test carried out. **p<0.01, ****p<0.0001. Overall ANOVA results for 24 hour time point: ****presence of calcitriol, *presence of TNF-α, ****interaction between calcitriol and TNF-α.

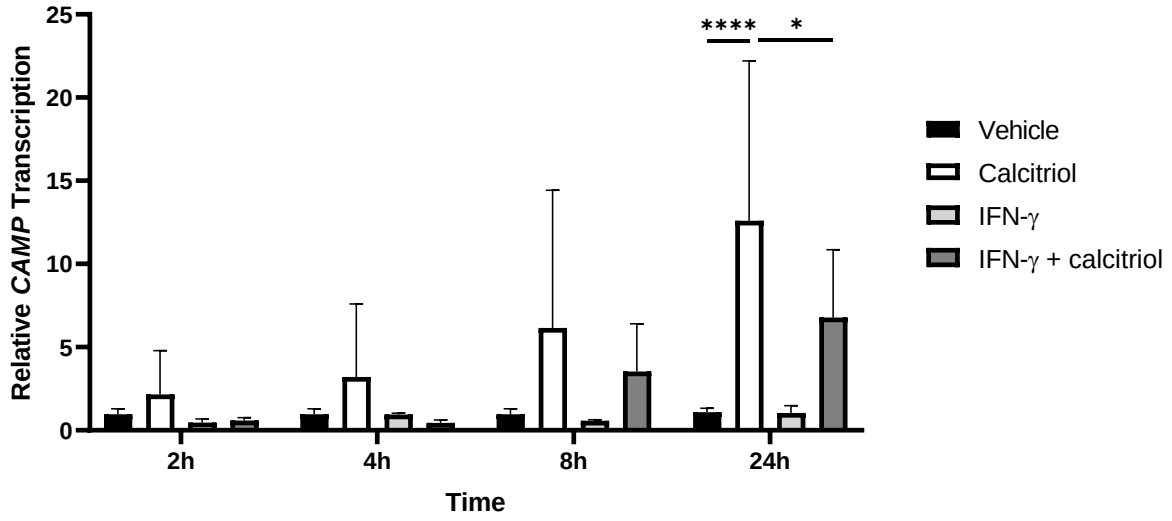


Figure 3.5: Influence of IFN-γ on the calcitriol-mediated induction of *CAMP* gene expression in MDM over time. Relative gene expression of *CAMP* in MDM over time following treatment with vehicle, calcitriol or IFN-γ alone, or calcitriol and IFN-γ simultaneously. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=3 independent experiments. Two-way ANOVA with Tukey's multiple comparisons test carried out. **p<0.01, ****p<0.0001. Overall ANOVA results for 24 hour time point: ****presence of calcitriol, ***presence of IFN-γ, **interaction between calcitriol and IFN-γ.

3.2.4 Phenylbutyrate (PBA) upregulates *CAMP* gene expression in MDM and synergises with calcitriol to enhance expression further

Another well-characterised stimulus for *CAMP* expression is butyrate, which is known to regulate *CAMP* expression by epigenetic mechanisms as a HDAC inhibitor in cell types such as epithelial and endothelial cells (447), but also possesses signalling and transcriptional modulation abilities to regulate *CAMP* gene expression in other cell types, including macrophages (434,450). These effects have been shown in BMDM and THP-1 macrophage-like cells (434,452), but the effects in primary human MDM are not well established. Reports have also highlighted that butyrate and vitamin D can function synergistically to enhance *CAMP* (451), therefore I investigated the effect of the clinically available derivative PBA on *CAMP* expression in MDM and its influence on *CAMP* expression in combination with calcitriol. MDM were vehicle treated or treated with calcitriol or PBA, either alone or simultaneously for 4 or 24 hours. RT-qPCR was carried out using the One-Step RT-qPCR (SYBR) primer based system, and β-actin and GAPDH as the housekeeping genes.

Calcitriol and PBA did not significantly upregulate *CAMP* expression, either alone or together, after 4 hours (Figure 3.6A). In contrast, both calcitriol and PBA alone significantly upregulated *CAMP* expression after 24 hours (Figure 3.6B). Interestingly, the simultaneous treatment with calcitriol and PBA on MDM for 24 hours resulted in a significant and dramatic upregulation of *CAMP* expression over either stimulus alone, demonstrating that there was a synergistic effect of these stimuli on the upregulation of *CAMP* expression in MDM.

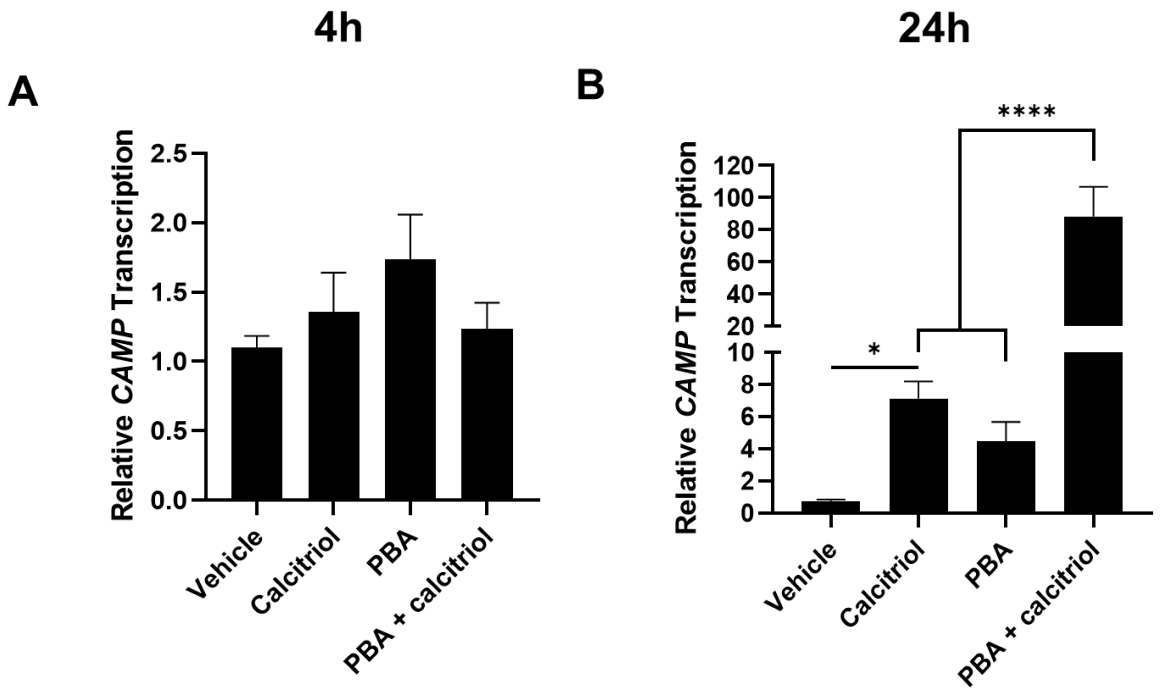


Figure 3.6: Upregulation of *CAMP* gene expression in response to calcitriol and PBA induction. Relative gene expression of *CAMP* in MDM following mock infection and treatment with vehicle, calcitriol and/or PBA treatment for A) 4 hours or B) 24 hours. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=4 independent experiments. Two-way ANOVA with Sidak's multiple comparisons test carried out. *p<0.05, **p<0.01, ****p<0.0001. Overall ANOVA results for 24 hour time point: ****presence of calcitriol, ****presence of PBA, ****interaction between calcitriol and PBA treatment.

3.2.5 TNF-α dampens PBA-mediated induction of *CAMP* gene expression in MDM, but IFN-γ does not

Next, as for calcitriol-induced *CAMP* expression, I wanted to investigate the effects of pro-inflammatory cytokines on PBA-induced *CAMP* expression. MDM were mock treated or treated with PBA and either TNF-α or IFN-γ, either alone or simultaneously for 4 or 24 hours. RT-qPCR was carried out using the One-Step RT-qPCR (SYBR) primer based system, and β-actin and GAPDH as the housekeeping genes.

None of the stimuli significantly upregulated *CAMP* expression after 4 hours (Figure 3.7A). 24 hour PBA treatment upregulated *CAMP* expression as expected (Figure 3.7B). 24 hour cytokine treatment alone did not upregulate *CAMP* expression, as observed previously. However, simultaneous PBA and TNF-α treatment significantly reduced *CAMP* expression levels compared to PBA treatment alone, while simultaneous PBA and IFN-γ treatment did not. These results demonstrate that, as with calcitriol-induced upregulation, TNF-α dampens PBA-induced upregulation of *CAMP* expression, but in contrast IFN-γ only dampens calcitriol-induced upregulation of *CAMP* expression.

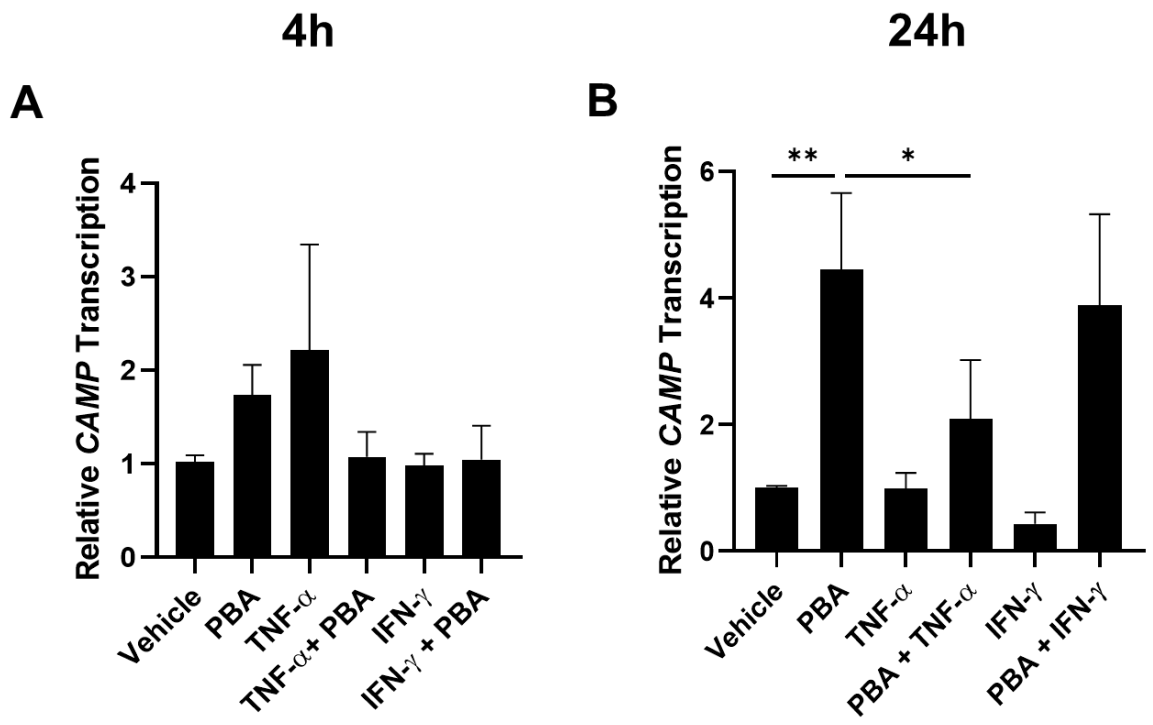


Figure 3.7: Influence of PBA and pro-inflammatory cytokines on *CAMP* gene expression in MDM. Relative gene expression of *CAMP* in MDM following vehicle, PBA and/or the indicated cytokine treatment for A) 4 hours or B) 24 hours. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n=3 independent experiments. Two-way ANOVA with Tukey's multiple comparisons test carried out. *p<0.05, **p<0.01. Overall ANOVA results for 24 hour time point: ****presence of PBA.

3.2.6 TNF-α modestly dampens induction of *CAMP* gene expression in MDM following PBA and calcitriol co-stimulation, but IFN-γ does not

Next, I wanted to investigate the effect of pro-inflammatory cytokines on the synergistic upregulation of *CAMP* expression by the combination of calcitriol and PBA. MDM were mock treated or treated with a combination of calcitriol and PBA, and either TNF-α or IFN-γ, for 4 or 24 hours. RT-qPCR was carried out using the One-Step RT-qPCR (SYBR) primer based system, and β-actin and GAPDH as the housekeeping genes.

As observed for PBA and cytokine treatment, none of the stimuli significantly upregulated *CAMP* expression after 4 hours (Figure 3.8A). 24 hour calcitriol and PBA treatment significantly upregulated *CAMP* expression, as expected (Figure 3.8B). Simultaneous calcitriol and PBA combined with TNF-α treatment also significantly upregulated *CAMP* expression compared to vehicle This was to a lesser extent than calcitriol and PBA co-stimulation, but the reduction was not significant. In contrast, simultaneous calcitriol and PBA with IFN-γ treatment showed no alteration versus calcitriol and PBA alone. These results demonstrate that although TNF-α modestly dampened the synergistic calcitriol/PBA-induced upregulation of *CAMP* expression, the synergy of these inducers prevented a significant reduction.

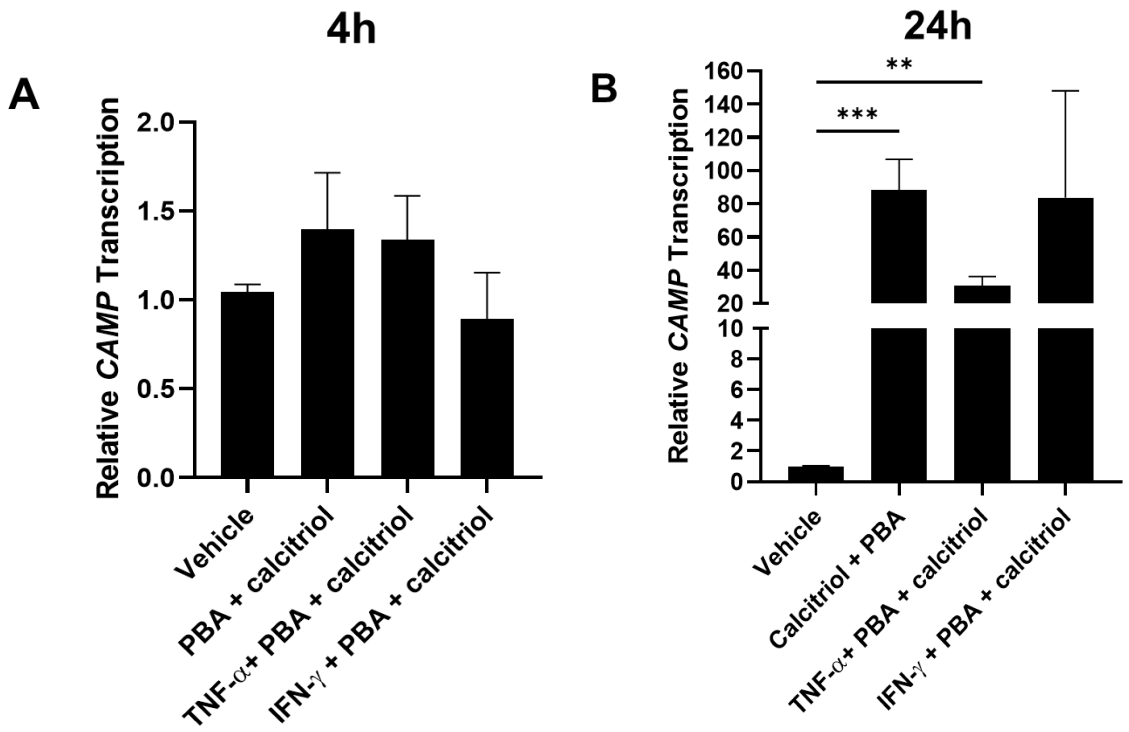


Figure 3.8: Influence of pro-inflammatory cytokines on simultaneous calcitriol and PBA-mediated *CAMP* gene expression in MDM. Relative gene expression of *CAMP* in MDM following vehicle treatment, or simultaneous PBA and calcitriol treatment with or without the indicated cytokines for 4 or 24 hours. Data represented as mean values. Experiments run using the One-Step RT-qPCR (SYBR) primer-based system, therefore used β-actin and GAPDH as the housekeeping genes. Error bars represent the SEM. n= 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons carried out. ***p<0.001.

3.2.7 Exogenous cathelicidin has a direct antibacterial effect on *S. pneumoniae* and *S. aureus* in the absence of serum

After establishing the factors and kinetics involved in regulating *CAMP* gene expression in MDM, I began investigating the functional consequences of exogenous cathelicidin on *S. pneumoniae* and *S. aureus* directly and on macrophage killing of these bacteria. It was important to study both contexts as cathelicidin encounters both extracellular bacteria and macrophage-internalised bacteria in the infection microenvironment. For example, cathelicidin is released from innate immune cells such as PBMC and neutrophils into the extracellular environment (429,502), and is found on extracellular structures such as NETs where it will encounter extracellular bacteria, but it has also been shown that macrophages can take up neutrophil-derived cathelicidin from the extracellular environment (505). Therefore, it is possible that exogenous cathelicidin could enhance macrophage killing of internalised bacteria by direct interactions with the bacteria or by modulating other macrophage responses to enhance killing, while endogenous production by the stimuli I have described above could also enhance killing.

To first examine the direct effect of cathelicidin on *S. pneumoniae* or *S. aureus* alone, bacteria in serum-free RPMI 1640 + 2 millimolar L-glutamine were incubated with exogenous cathelicidin or scrambled peptide control for 1 or 4 hours. Exogenous cathelicidin significantly reduced viable *S. pneumoniae* counts after both 1 hour and 4 hours (Figure 3.9A), which showed that cathelicidin has a direct bactericidal effect against *S. pneumoniae* in these conditions. Cathelicidin did not significantly alter viable *S. aureus* counts after 1 hour (Figure 3.9B), suggesting that cathelicidin's bactericidal effect was observed against *S. pneumoniae* but not *S.*

aureus at this early time point. Cathelicidin did significantly reduce viable *S. aureus* growth after 4 hours. This effect was also observed for *S. aureus* cultures incubated with the scrambled peptide control, to a lesser extent, but cathelicidin was significantly more effective than the scrambled control (Figure 3.9B). This suggests that the reduction in *S. aureus* viability is possibly due to non-specific charge-based interactions of the peptides with the bacteria rather than the bactericidal effect of cathelicidin. However, the significant reduction in *S. aureus* viability with cathelicidin over scrambled peptide treatment, suggests that cathelicidin does have some bactericidal effect on *S. aureus*.

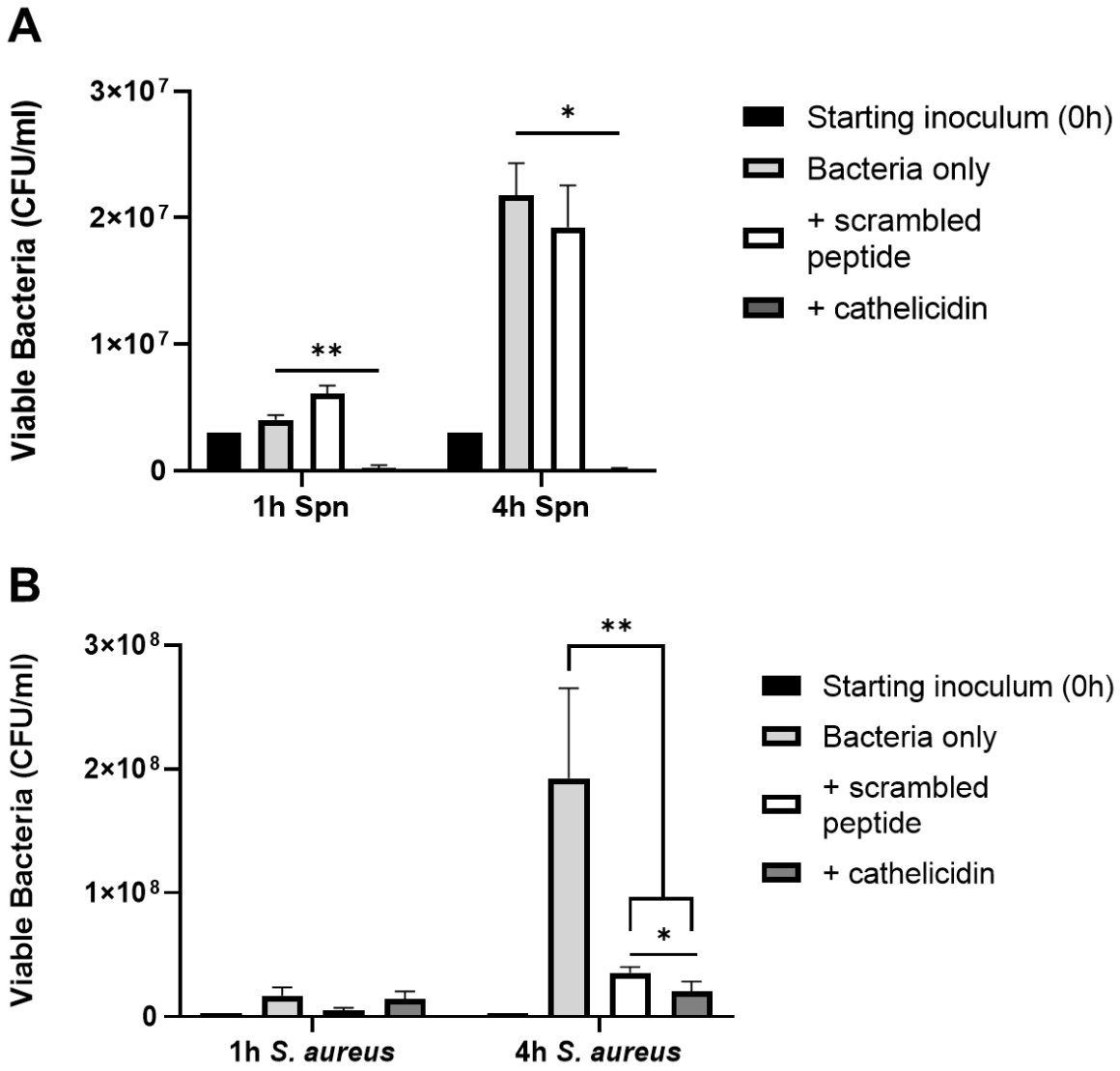


Figure 3.9: Influence of exogenously applied cathelicidin on the viable counts of planktonic bacterial cultures in serum-free media. The mean viable counts obtained from planktonic A) *S. pneumoniae* and B) *S. aureus* cultures in serum-free media incubated with exogenous cathelicidin or scrambled peptide control for 1 hour or 4 hours. Starting inoculum = 3 x 10^6 CFU. Growth medium = RPMI 1640 + 2 millimolar L-glutamine. Error bars represent the SEM. n=3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons carried out. *p<0.05, **p<0.01.

3.2.8 Exogenous cathelicidin does not affect the viability of *S. pneumoniae* and *S. aureus* in the presence of serum

It has been widely reported that the bactericidal effects of cathelicidin are inhibited by serum, due to the binding and neutralisation of cathelicidin by serum proteins (521). To confirm if the bactericidal effect of cathelicidin on *S. pneumoniae* and *S. aureus* was altered by the presence of serum, the 4 hour peptide incubation experiment in Figure 9 was repeated in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum), and cultures were diluted and plated as described previously.

In both cases, the bactericidal effect of cathelicidin on *S. pneumoniae* (Figure 3.10A) and *S. aureus* (Figure 3.10B) observed in the serum-free cultures after 4 hours was lost and there were no alterations in bacterial viability. This confirmed that the presence of serum inhibits the antibacterial effect of cathelicidin on *S. pneumoniae* and *S. aureus*. Therefore, for the MDM bacterial killing experiments, the cathelicidin incubation step was carried out in serum-free media to prevent inhibition of cathelicidin activity.

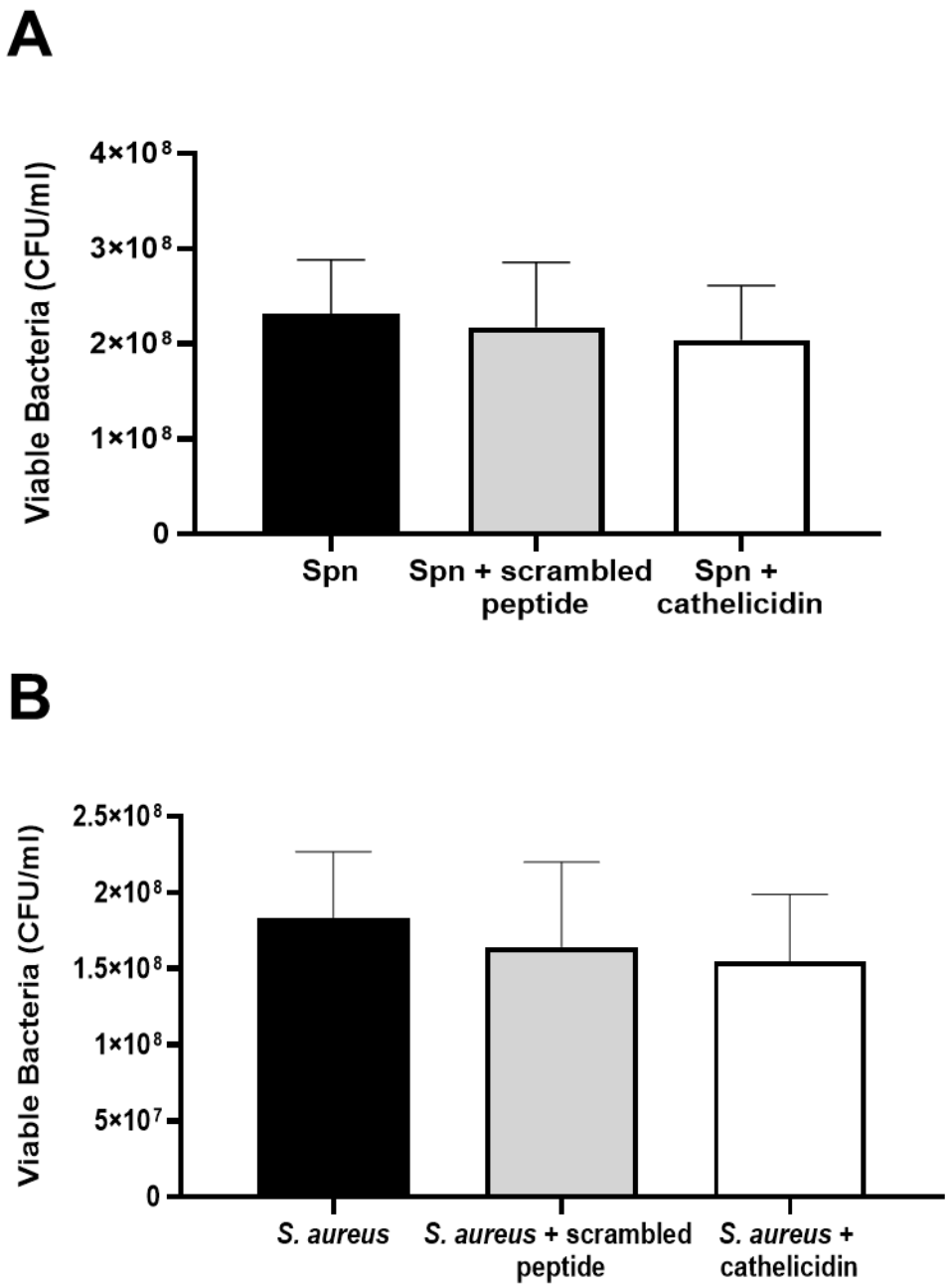


Figure 3.10: Influence of exogenously applied cathelicidin on the viable counts of planktonic bacterial cultures in complete media. The mean viable counts obtained from planktonic A) *S. pneumoniae* and B) *S. aureus* cultures in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum) and incubated with exogenous cathelicidin or scrambled peptide control for 4 hours. Error bars represent the SEM. n=4 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons carried out.

3.2.9 Exogenous cathelicidin may increase MDM killing of intracellular *S. aureus*

Next, the effect of cathelicidin on macrophage killing of internalised *S. pneumoniae* and *S. aureus* was investigated using the MDM bacterial killing assay. Following challenge of MDM with opsonised *S. pneumoniae* or *S. aureus* for 4 hours, MDM were treated with antimicrobials to kill remaining extracellular bacteria, then treated with exogenous cathelicidin for 1 hour. The supernatant from the MDM culture prior to MDM lysis, and the lysate following MDM lysis, were diluted, and plated to detect extracellular and intracellular bacteria and calculate viable intracellular bacterial counts.

Cathelicidin treatment did not alter the viable counts of internalised *S. pneumoniae* obtained from MDM (Figure 3.11A), therefore cathelicidin did not contribute to macrophage killing of internalised *S. pneumoniae* under these conditions. Interestingly, there was a trend towards a significant reduction in viable counts of internalised *S. aureus* obtained from MDM following cathelicidin treatment (Figure 3.11B), when compared to mock-treated MDM, as well as a significant reduction in viable bacterial counts with cathelicidin treatment compared with scrambled peptide treatment. These results suggest that exogenous cathelicidin can contribute to macrophage killing of internalised *S. aureus* under these conditions.

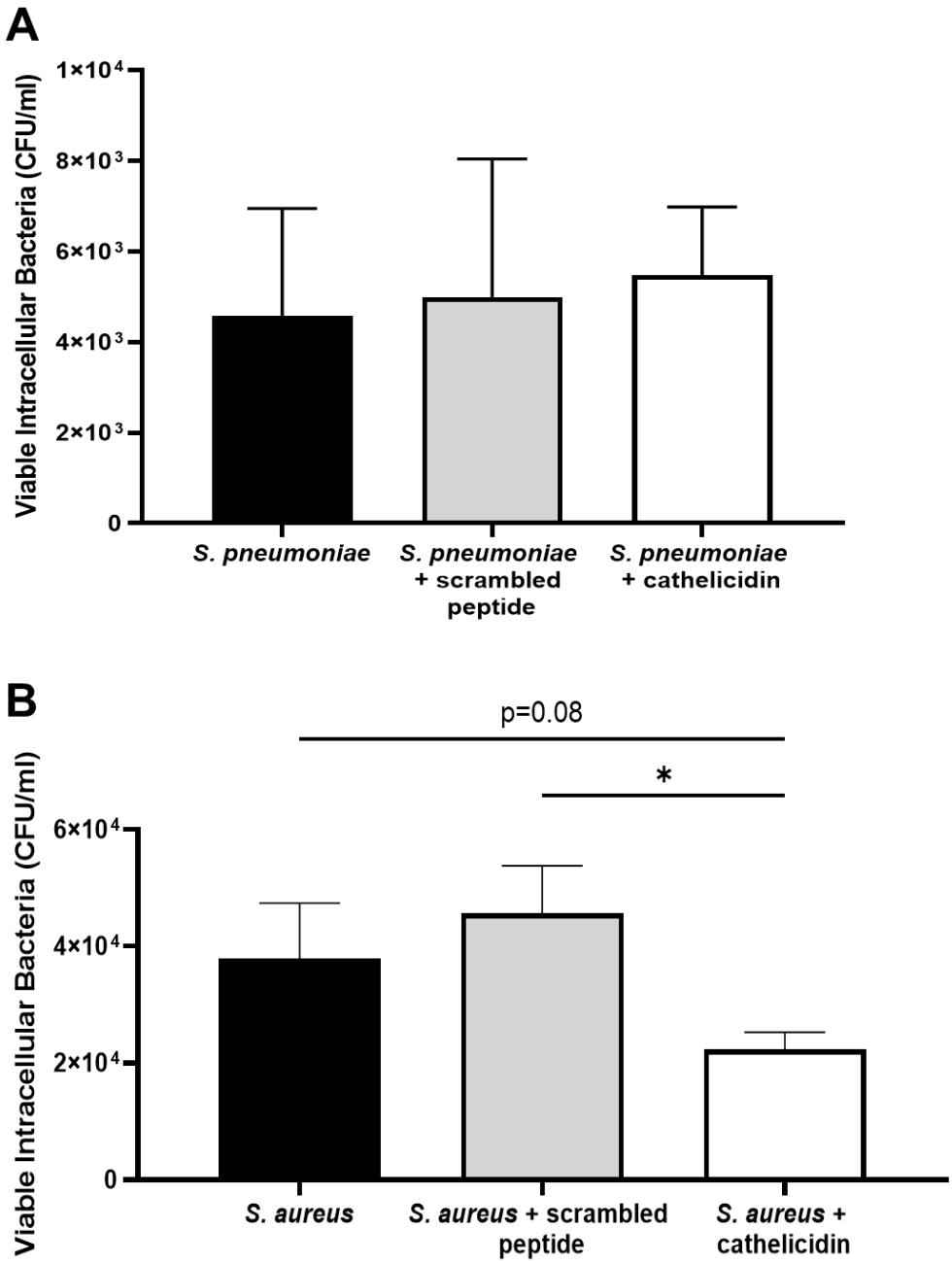


Figure 3.11: Influence of exogenously applied cathelicidin on MDM killing of internalised bacteria. The mean viable counts obtained from A) opsonised *S. pneumoniae* and B) *S. aureus*, both at MOI=10, following incubation with MDM for 4 hours, then 1 hour of exogenous cathelicidin or scrambled peptide treatment on MDM. Assays carried out in serum-free media (RPMI 1640 + 2 millimolar L-glutamine). Error bars represent the SEM. n=3 independent experiments for *S. pneumoniae* and 6 independent experiments for *S. aureus*. Kruskal-Wallis test with Dunn's multiple comparisons carried out. *p<0.05.

3.2.10 Exogenous cathelicidin may increase MDM killing of high intracellular *S. aureus* burdens

I wanted to probe the effect of cathelicidin on MDM killing of *S. aureus* further, since I reasoned that if there was a role for cathelicidin in intracellular killing it might be most apparent when intracellular bacterial burden was high and early macrophage phagolysosomal killing mechanisms were overwhelmed. This could explain why I observed no role with *S. pneumoniae*, as the intracellular burden in this case would not be high enough to overwhelm canonical phagolysosomal killing mechanisms. To further test this I explored the role of cathelicidin in higher dose *S. aureus* challenge. Therefore, I investigated how cathelicidin treatment altered MDM killing of low and high burdens of *S. aureus* challenge. The 4 hour macrophage bacterial killing assay was carried out using different MOIs of *S. aureus*- 0.1 or 0.5 to represent a low bacterial burden, and 20 to represent a very high bacterial burden. Viable intracellular counts were calculated and represented alongside the counts obtained with *S. aureus* at an MOI of 10.

Cathelicidin treatment did not alter viable counts of internalised *S. aureus* from MDM challenged with the low MOIs of 0.1 and 0.5 (Figure 3.12A and 3.12B), suggesting that cathelicidin did not influence macrophage bacterial killing responses under these conditions and when bacterial burdens were low. It is likely that these bacterial burdens were low enough to not overwhelm macrophage intracellular killing responses. However, in macrophages challenged with *S. aureus* at MOI 20 (Figure 3.12D), a slight trend towards a reduction in viable internalised bacterial counts was observed following cathelicidin treatment, however the greatest effect was still observed with bacterial challenge at MOI 10 (Figure 3.12C). This suggests that

although exogenous cathelicidin treatment might contribute to enhanced macrophage killing of *S. aureus* when bacterial burdens are high, there may be a bacterial burden threshold where, if exceeded, the cathelicidin effects on enhanced MDM killing are reduced.

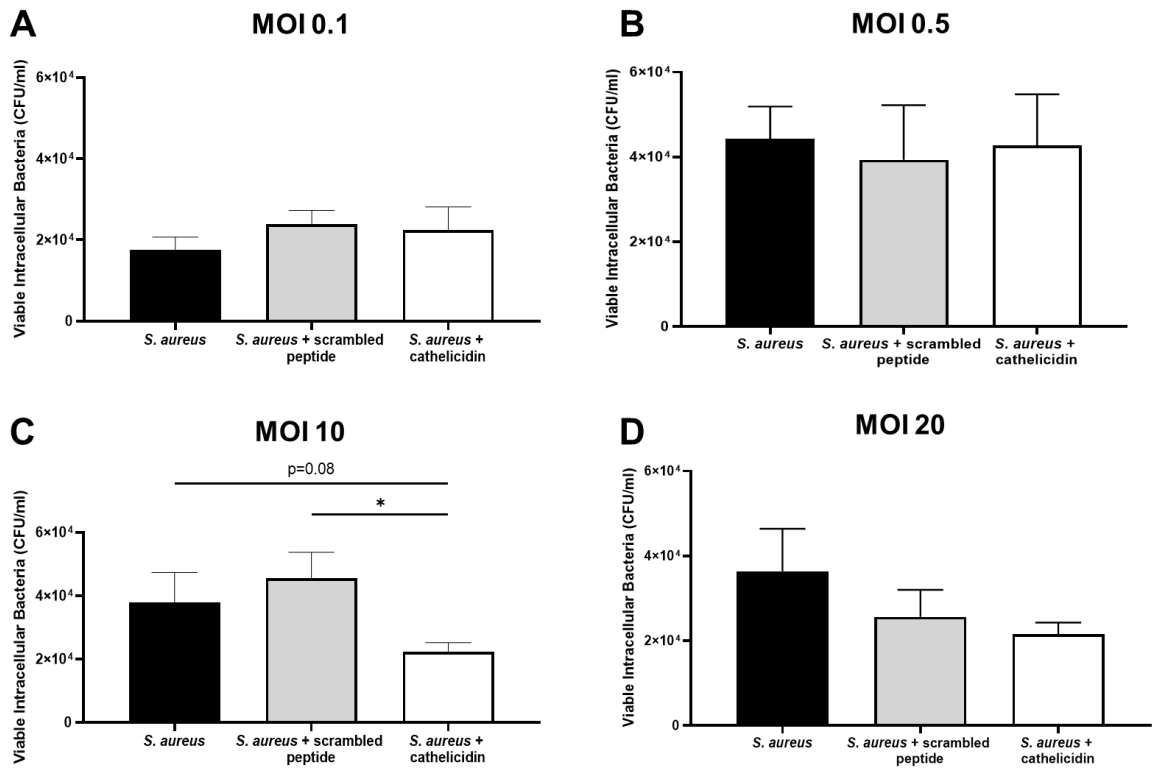


Figure 3.12: Influence of exogenously applied cathelicidin on MDM killing of low and high *S. aureus* burdens. The mean intracellular viable counts obtained from *S. aureus* at A) MOI 0.1, B) MOI 0.5, C) MOI 10, or D) MOI 20 following incubation with MDM for 4 hours, then 1 hour of exogenous cathelicidin or scrambled peptide treatment on MDM. Assays carried out in serum-free media (RPMI 1640 + 2 millimolar L-glutamine). Error bars represent the SEM. n=3 independent experiments for MOI 0.1, MOI 0.5, and MOI 20, and 4 independent experiments for MOI 10. Kruskal-Wallis test with Dunn's multiple comparisons carried out for each data set. p=0.24 for *S. aureus* MOI 10.

3.2.11 TAMRA-labelled cathelicidin uptake into MDM is increased in response to early *S. pneumoniae* challenge, but not in response to late *S. pneumoniae* or early *S. aureus* challenge

Following the observations made on the effect of exogenous cathelicidin on macrophage killing of intracellular bacteria, I investigated the uptake of exogenous cathelicidin by macrophages by using TAMRA-labelled cathelicidin (TAMRA-LL-37). As mentioned previously, macrophages have been shown to take up extracellular cathelicidin originating from neutrophil granules (505), therefore I wanted to establish if cathelicidin uptake by macrophages was also observed in cultures containing intracellular bacteria. MDM were challenged with *S. pneumoniae* for 4 hours or 14 hours, or *S. aureus* for 4 hours, then treated with exogenous TAMRA-labelled cathelicidin for 1 hour. MDM were imaged by confocal microscopy and the mean fluorescence intensity for TAMRA-labelled cathelicidin in each MDM was calculated.

The mean fluorescence intensity scores showed that TAMRA-labelled cathelicidin uptake by MDM challenged with *S. pneumoniae* for 4 hours was modestly increased compared to 4 hour mock-infected MDM, but there was no alteration in cathelicidin uptake following 14 hour *S. pneumoniae* challenge compared to mock-infection (Figure 3.13). Cathelicidin uptake by MDM was not altered by *S. aureus* challenge for 4 hours (Figure 3.14). These results suggested that an increase in cathelicidin uptake by MDM may occur early in response to *S. pneumoniae* challenge, but this is not functionally significant for MDM killing of bacteria. Morphologically, cathelicidin taken up by mock-infected MDM was localised to punctate, endosome-like structures, while cathelicidin appeared more diffuse throughout bacterial-challenged MDM as shown in the example images. However, it

should be noted that while the mean fluorescence intensity scores provide a global indication of cathelicidin uptake by MDM in each condition, the level of uptake by individual MDM in a population is highly variable; some MDM take up more cathelicidin than others, as shown in the example images. It is possible that the variation in uptake may be associated with the internalisation capacity of individual MDM, as some MDM may be able to internalise more than others. This means global scores may not capture all the variation between conditions and may miss more subtle changes in distribution.

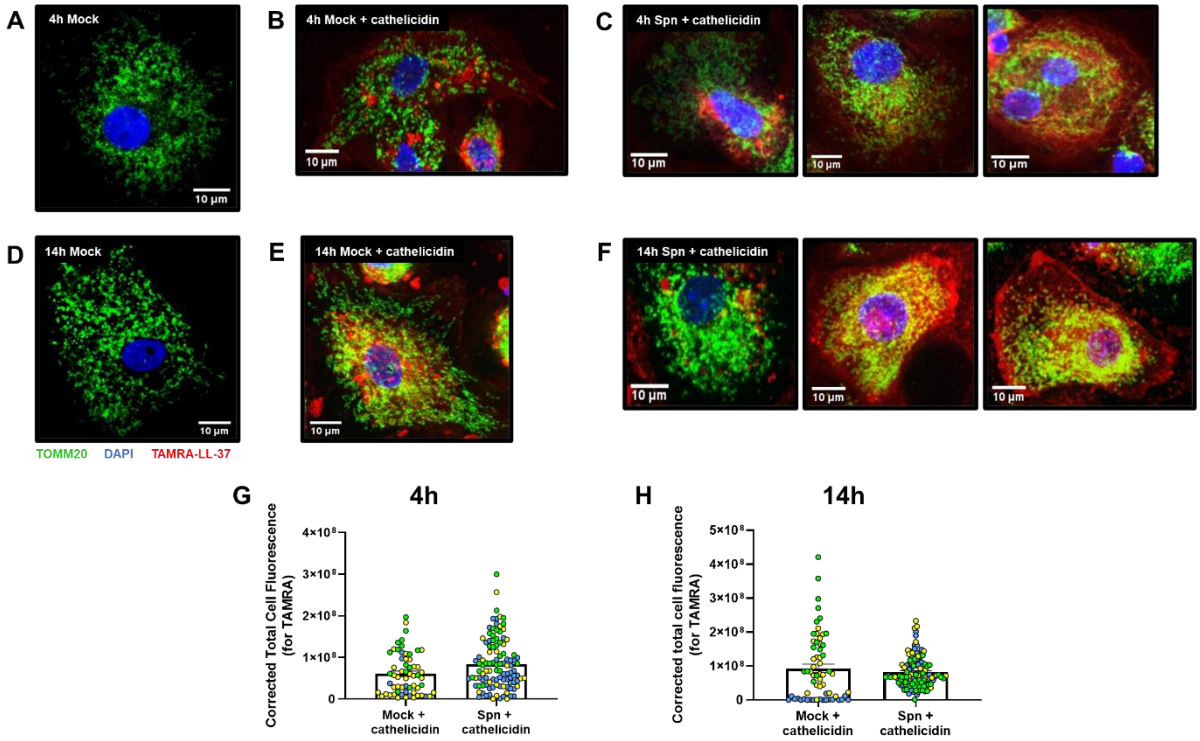


Figure 3.13: Uptake of exogenous TAMRA-labelled cathelicidin (TAMRA-LL-37) by MDM following *S. pneumoniae* challenge. MDM were mock infected or challenged with opsonised *S. pneumoniae* for 4 or 14 hours then exposed to TAMRA-labelled cathelicidin for 1 hour. MDM were stained with DAPI (blue) to visualise nuclei and TOMM20 (green) to stain mitochondria. Images were taken on the Leica SP8 at 60x magnification. A-F) Example images illustrating the localisation and variation of TAMRA-LL-37 (shown in red) uptake by individual MDM within the same condition. G and H) Corrected total cell fluorescence intensity scores for TAMRA-LL-37 in MDM following mock infection or opsonised *S. pneumoniae* challenge for G) 4 hours and H) 14 hours, then exogenous TAMRA-LL-37 treatment for 1 hour. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean fluorescence intensity. Error bars represent the SEM. n = 3 independent experiments. Mann-Whitney test carried out.

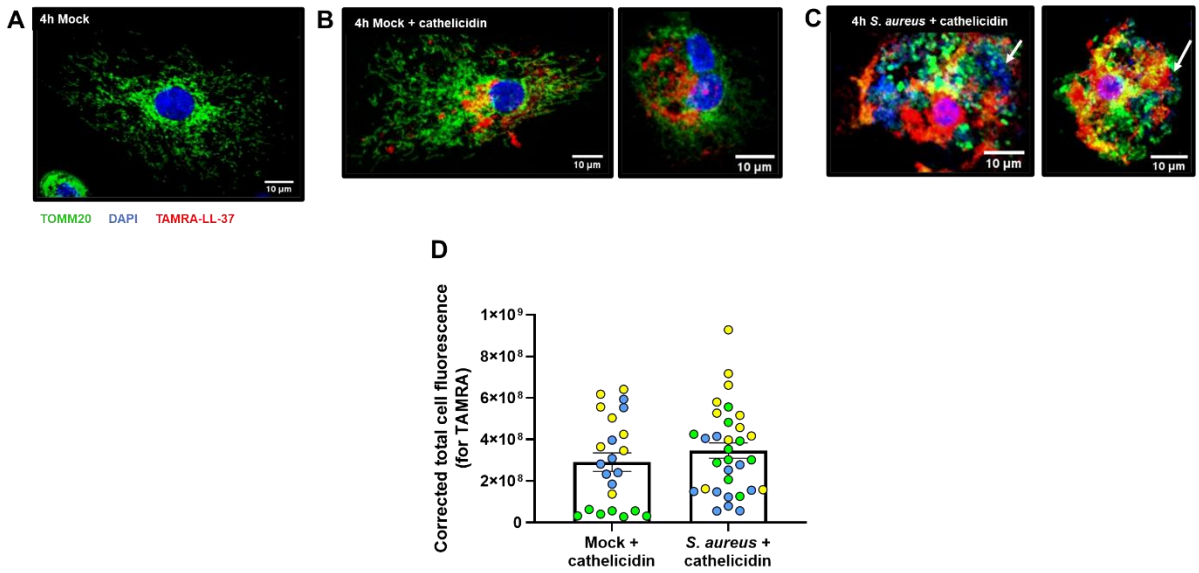


Figure 3.14: Uptake of exogenous TAMRA-labelled cathelicidin (TAMRA-LL-37) by MDM following *S. aureus* challenge. MDM were mock infected or challenged with opsonised S. aureus for 4 hours then exposed to TAMRA-labelled cathelicidin for 1 hour. MDM were stained with DAPI (blue) to visualise nuclei and bacteria (see representative examples indicated with white arrows) and TOMM20 (green) to stain mitochondria. Images were taken on the Leica SP8 at 60x magnification. A) Example images illustrating mock-infected MDM, B) TAMRA-LL-37 uptake by mock-infected MDM and C) TAMRA-LL-37 uptake following 4 hour *S. aureus* challenge. D) Corrected total cell fluorescence intensity scores for TAMRA-LL-37 in MDM following mock infection or *S. aureus* challenge for 4 hours, then exogenous TAMRA-LL-37 treatment for 1 hour. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean fluorescence intensity. Error bars represent the SEM. n = 3 independent experiments. Mann-Whitney test carried out.

3.3 Discussion

In this chapter, I have described how vitamin D, PBA, *S. pneumoniae* or *S. aureus* infection and the pro-inflammatory cytokines TNF-α and IFN-γ interact to regulate *CAMP* expression in human MDM. I have also described the influence of cathelicidin on *S. pneumoniae* and *S. aureus* directly, and on MDM killing of internalised bacteria. My findings are summarised schematically in Figures 3.15 and 3.16.

The roles of vitamin D and butyrate on upregulation of *CAMP* expression have been characterised in epithelial cells, PBMC and monocytes, the THP-1 cell line, and in the context of *Klebsiella pneumoniae* infection in human MDM (444,502,518), but their influence in the context of gram-positive infection in MDM is unclear. I have shown that vitamin D induces *CAMP* expression in MDM, as expected, and *CAMP* upregulation increased over time with exposure to vitamin D, with significant upregulation occurring after 24 hours. This suggests that optimal vitamin D-induced *CAMP* upregulation is a later response in macrophages. The presence of *S. pneumoniae* or *S. aureus* following 24 hour vitamin D stimulation resulted in further *CAMP* upregulation in a synergistic manner, which suggests that upon detection by macrophages, these bacterial stimuli function through the vitamin D metabolic pathway as a mechanism for enhancing macrophage immune responses. Since the presence of bacteria synergises with calcitriol to dramatically upregulate *CAMP* expression, it suggests that vitamin D priming of macrophages facilitates bacterial-induced cathelicidin expression. This effect of vitamin D priming therefore highlights the implication of environmental and lifestyle factors in the modulation of host defence responses. Seasonal changes and lower levels of sunlight in parts of the

world, for example in Scotland, can lead to vitamin D deficiency in many members of these populations (522). Furthermore, as vitamin D can also be obtained via diet, individuals could become vitamin D deficient as a result of, for example dietary restrictions or limitations on access to food (522). Studies have demonstrated that vitamin D deficiency results in enhanced susceptibility to infection (523), and several studies and clinical trials have investigated the benefits of vitamin D supplementation on cathelicidin levels and susceptibility to infection with mixed, but generally positive results (524). Therefore, it is possible that the impact of vitamin D deficiency on host immune responses to infection could be associated with lower levels of cathelicidin in deficient individuals; this would be a very interesting avenue for further research into the impact of environmental and socioeconomic factors on host immunity and responses to infection.

PBA also induced *CAMP* expression after 24 hour exposure as expected, and also synergised with vitamin D to further enhance *CAMP* expression. This shows that combinations of stimuli are important for maximal upregulation of *CAMP* expression in MDM. Several studies and clinical trials have demonstrated the benefit of therapeutically-administered vitamin D and PBA synergy on enhanced Mtb clearance by macrophages in tuberculosis patients (525–527), so it is possible that such combinations of inducers could be used therapeutically to enhance MDM responses to other bacterial infections as a method to combat antimicrobial resistance.

Interestingly, the presence of the pro-inflammatory cytokines TNF-α or IFN-γ modulated vitamin D-mediated *CAMP* expression, while only TNF-α modulated PBA-mediated *CAMP* expression. Pro-inflammatory cytokines have been shown to reduce

vitamin D-mediated *CAMP* expression in airway epithelial cells (436), but not in MDM, and these results suggest that cytokines also function in this manner in MDM. This suggests that pro-inflammatory cytokines modulate vitamin D- or PBA-mediated cathelicidin production in MDM by dampening *CAMP* expression. This could have both positive and negative consequences for the host in different contexts; the dampening of *CAMP* expression could regulate cathelicidin production and prevent excessive cathelicidin-induced inflammatory responses. For example, cathelicidin-mediated pore formation leads to leakage of bacterial cell contents and subsequent bacterial killing (462); this could result in the release of bacterial PAMPs which could subsequently enhance further pro-inflammatory responses, similar to the action of other lytic factors (111). In addition, elevated cathelicidin levels have been associated with a pathological enhancement of inflammation in chronic conditions such as rosacea or COPD (528,529). In the case of the latter, elevated cathelicidin levels was also associated with increased bacterial colonisation and exacerbation risk (528). Therefore, dampening *CAMP* expression could act as a brake on these processes, and this brake functionality could be beneficial in regulating inflammation mediated by cathelicidin production. However, it also suggests that cathelicidin production would not be optimally induced in macrophages in a sustained pro-inflammatory environment, for example in chronic inflammatory conditions, therefore this macrophage antimicrobial response would be less effective in these contexts. Furthermore, dampening of *CAMP* expression could also lead to a reduction in macrophage killing capacity or could impact responses where the immunomodulatory functions of cathelicidin play an important role. Examples of such responses include the induction of chemotaxis of other immune cells such as neutrophils, monocytes and eosinophils (483,484), or the differentiation of cells such

as macrophages or DCs to promote pro-inflammatory responses or enhance T cell activation, respectively (488,491).

It is known that cathelicidin has direct bactericidal functionality, including against *S. pneumoniae* and *S. aureus* (459). Other studies have determined that cathelicidin has a minimum inhibitory concentration (MIC) of 14 µg/ml against *S. pneumoniae* (530), while the MIC reported for S. aureus is <10 µg/ml (531). Cathelicidin also contributes to enhanced human macrophage clearance of intracellular bacteria, such as Mtb and *Klebsiella pneumoniae* (503,518) However, the influence of cathelicidin on MDM killing of *S. pneumoniae* and *S. aureus*, which are not typically associated with prolonged intracellular survival, is not established. I have shown that cathelicidin had direct bactericidal functionality on *S. pneumoniae* after 1 and 4 hour incubations, while cathelicidin only exerted a bactericidal effect on *S. aureus* after 4 hours. However, this bactericidal effect was only observed in the absence of serum in the media; multiple factors present in serum have been shown to bind to cathelicidin and inhibit its antibacterial activity, such as apolipoprotein A-I and F-actin (521,532,533). Therefore, my susceptibility results in the absence of serum suggested that cathelicidin activity would not be impaired when added to the MDM bacterial killing assay under serum-free conditions.

Cathelicidin did not influence MDM killing of 4 hour internalised *S. pneumoniae*. This could reflect the lower intracellular burden of *S. pneumoniae* in macrophages. *S. aureus* stresses macrophage microbicidal responses more than S. *pneumoniae* due to a higher intracellular burden and well-adapted mechanisms to reduce intracellular killing. My results showed that cathelicidin may enhance MDM killing of 4 hour internalised *S. aureus* when intracellular bacterial burden was high.

This suggests that exogenous cathelicidin treatment might contribute to enhanced macrophage killing of *S. aureus* when the macrophage is stressed and early macrophage microbicidal responses become overwhelmed. However, my results also suggest that if the bacterial burden increases to extremely high levels, cathelicidin's effects are reduced, suggesting that the very high bacterial burden overwhelms cathelicidin's enhancement of macrophage killing. These results illustrate the importance of a multi-layered macrophage response to infection in order to efficiently respond to and clear different pathogens and different levels of bacterial burden. It is possible that macrophages are capable of killing *S. pneumoniae* at this early 4 hour time point without the contribution of cathelicidin; due to the lower intracellular burden, additional layers of immune responses may not be required (534). In contrast, the higher intracellular burden of *S. aureus* may require multiple layers of immune defence, for example phagolysosomal killing followed by cathelicidin-induced responses, to ensure efficient clearance of this pathogen (72).

Cathelicidin uptake by MDM was increased following 4 hour *S. pneumoniae* infection, but not 14 hour *S. pneumoniae* or 4 hour *S. aureus* infection, and uptake was variable among MDM under the same stimulation conditions. These results were particularly interesting as they contrast with the results that I had expected following the observations of cathelicidin on macrophage killing of intracellular *S. pneumoniae* and *S. aureus*. Cathelicidin uptake by macrophages was increased following 4 hour *S. pneumoniae* challenge but did not influence macrophage killing. Cathelicidin may contribute to enhanced macrophage killing of *S. aureus* following 4 hour challenge, but there was no increase in cathelicidin uptake. This suggested that cathelicidin's potential influence on macrophage killing of *S. aureus* was not

associated with the global measure of cathelicidin taken up by the macrophage. Therefore, cathelicidin may exert its effect in more subtle ways and by inducing or combining with another intracellular mechanisms to promote macrophage killing of *S. aureus*. It is also possible that rather than the concentration of cathelicidin taken up, the location of bacteria within the macrophage and the timing of cathelicidin-mediated responses are the more important influences on cathelicidin-mediated macrophage killing of intracellular *S. aureus*. My killing assay experiments, as described above, focused on one time point which provided a snapshot of cathelicidin's influence on macrophage killing. Therefore, the assay could be carried out at multiple time points following bacterial challenge to determine when during *S. aureus* infection the macrophage requires additional factors to efficiently kill the bacteria and provide a more comprehensive overview of the kinetics of cathelicidin's influence on macrophage killing. Furthermore, the killing assay does not provide information on the potential co-localisation of cathelicidin and intracellular *S. aureus*, therefore further microscopy experiments would be beneficial to investigate this. Bacteria could be stained with, for example, DAPI to determine overall bacterial uptake, and pHrodo dyes that fluoresce in acidic conditions to detect bacteria within phagolysosomal compartments. TAMRA-labelled cathelicidin could then be included to determine co-localisation of cathelicidin with different subsets of bacteria within the macrophage, which would provide more details on the interactions of exogenous cathelicidin and bacteria within the macrophage,

These results were very interesting and provided a basis for investigating potential interactions of cathelicidin and other macrophage microbicidal responses to enhance bacterial killing, but there are further investigations that could be carried out

to build upon these findings. As described previously, the human MDM model was critical for my work and was used for almost all experiments. MDM were a good model for my work as they are primary human macrophages, so more relevant than a cell line or murine BMDM for studies on human innate immune responses to bacteria, they were fairly easily attainable, and the Dockrell group has well-established protocols for MDM culture (535). However, as MDM were derived from PBMC extracted from donated blood, variability in responses of MDM from different donors was to be expected. Many of my results are based on relatively small group sizes (n=3 or 4 donors) due to time constraints, therefore it would be very beneficial to increase these numbers in order to reduce effects of donor variability, increase statistical power and carry out more robust statistical analyses. It would also be interesting to investigate *CAMP* expression and cathelicidin's influence on macrophage killing in more specialised tissue-resident macrophage subtypes, such as alveolar macrophages. This model would be relevant in this context as *S. pneumoniae* and *S. aureus* can both cause respiratory diseases, therefore alveolar macrophages are one of the dominant macrophage subtypes that would be encountered. In addition, alveolar macrophages are uniquely adapted for optimal function in the lung environment compared to other macrophage subsets and exert their antimicrobial effects in consort with other cells such as neutrophils and lung epithelial cells, which all act to form a complex immune environment *in vivo* (536–539). The limitations of MDM culture exclude all of these other factors that would be present *in vivo*, therefore although MDM are a useful *in vitro* model, studies in specialised tissue macrophages would be very important to gain more physiologically relevant insight.

Typically, changes in gene expression levels correlate well with changes in the corresponding protein levels, but it would have been beneficial to have the opportunity to measure hCAP18 and active cathelicidin protein expression levels in MDM treated as for the gene expression studies. It would be important to measure both hCAP18 and cathelicidin protein levels as hCAP18 is the direct product of the *CAMP* gene, which is then processed into cathelcidin. Therefore, hCAP18 levels would determine if there were a good correlation between *CAMP* expression and protein production, and cathelicidin levels would show how much functionally active peptide is being generated in response to the stimuli. The localisation and timing of endogenous peptide production in relation to intracellular bacteria and other microbicidal responses would also be beneficial to investigate. The microscopy experiments described above visualising the location of DAPI- and pHrodo-stained *S. aureus* within the macrophage would also be very informative here, as endogenous cathelicidin could also be stained to determine co-localisation with bacteria. Additionally, the localisation of cathelicidin with other microbicidal factors, such as reactive species, could be investigated by microscopy. Furthermore, it would be very interesting to use live cell imaging to investigate the timing of cathelicidin and other microbicidal responses to *S. aureus* infection, which would provide insight into when the macrophage requires additional microbicidal factors and how these may combine in real time to clear intracellular bacteria.

The macrophage intracellular killing assays were carried out using exogenously applied cathelicidin peptide, therefore it would be informative to carry out the assays in MDM that had been induced to produce endogenous cathelicidin. The concentration of exogenous peptide used (25µg/ml) has been shown to be

physiologically relevant in the context of pulmonary infections (496). However, it is still fairly high, therefore endogenous cathelicidin induction via, for example vitamin D or PBA treatment, would provide a more relevant insight into the functional roles of cathelicidin in the MDM system directly. In addition, studies have shown that the hCAP18 precursor can be post-translationally modified into not only cathelicidin, but other cathelicidin variants with additional antimicrobial functionality (540). Therefore, it is possible that endogenous expression would lead to the generation of a more heterogeneous pool of peptides with antimicrobial capacity, while exogenously applied synthetic peptide is limited to one peptide variant. However, it is likely that vitamin D or PBA treatment would induce the expression of other genes as well as *CAMP*, so to confirm that any observed effect on macrophage killing involved cathelicidin induction, I would carry out the experiment in parallel using BMDM from wild-type and *Camp*-knockout mice, as the knockout mice are unable to produce cathelicidin. Firstly, it would be beneficial to simply conduct the macrophage killing assays with *S. pneumoniae* and *S. aureus*, without additional compound treatment, to determine if intracellular bacterial killing by the macrophages is altered by the absence of cathelicidin expression. Next, induction of cathelicidin expression could be introduced into the assay; it would be necessary to use PBA, rather than vitamin D, as the inducer in BMDM as the murine *Camp* gene does not have a VDRE in its promoter (541). Differences in bacterial counts from MDM and *CRAMP*-knockout BMDM treated with PBA, as well as wild-type and *CRAMP*- knockout BMDM with and without PBA treatment, could be compared to determine if endogenous cathelicidin production contributes to macrophage killing of bacteria, and if vitamin D exerts cathelicidin-independent effects on macrophage killing

To summarise, this chapter describes the mechanisms of regulating cathelicidin expression in MDM and the influence of cathelicidin on bacterial killing by macrophages. I have shown that increased cathelicidin expression in human MDM is dependent on vitamin D and is synergistically enhanced in combination with bacteria or PBA. This demonstrates the importance of vitamin D in the regulation of macrophage antimicrobial responses and emphasises the importance of combinatory responses for optimal macrophage antimicrobial activity. Cathelicidin production is also heavily modulated by pro-inflammatory cytokines, highlighting the crucial role that inflammation plays in regulating macrophage responses. Cathelicidin has direct antibacterial activity on the gram-positive bacteria *S. pneumoniae* and *S. aureus* and may contribute to macrophage killing of *S. aureus*. However, this only occurs with high *S. aureus* burdens and may depend on the timing and localisation of cathelicidin in relation to bacterial infection and other microbicidal responses; this emphasises the requirement of different responses with different spatial and temporal niches for generating a multi-layered immune response to efficiently clear pathogens. These results are very interesting and provide a basis for further study into the roles of cathelicidin in human MDM.

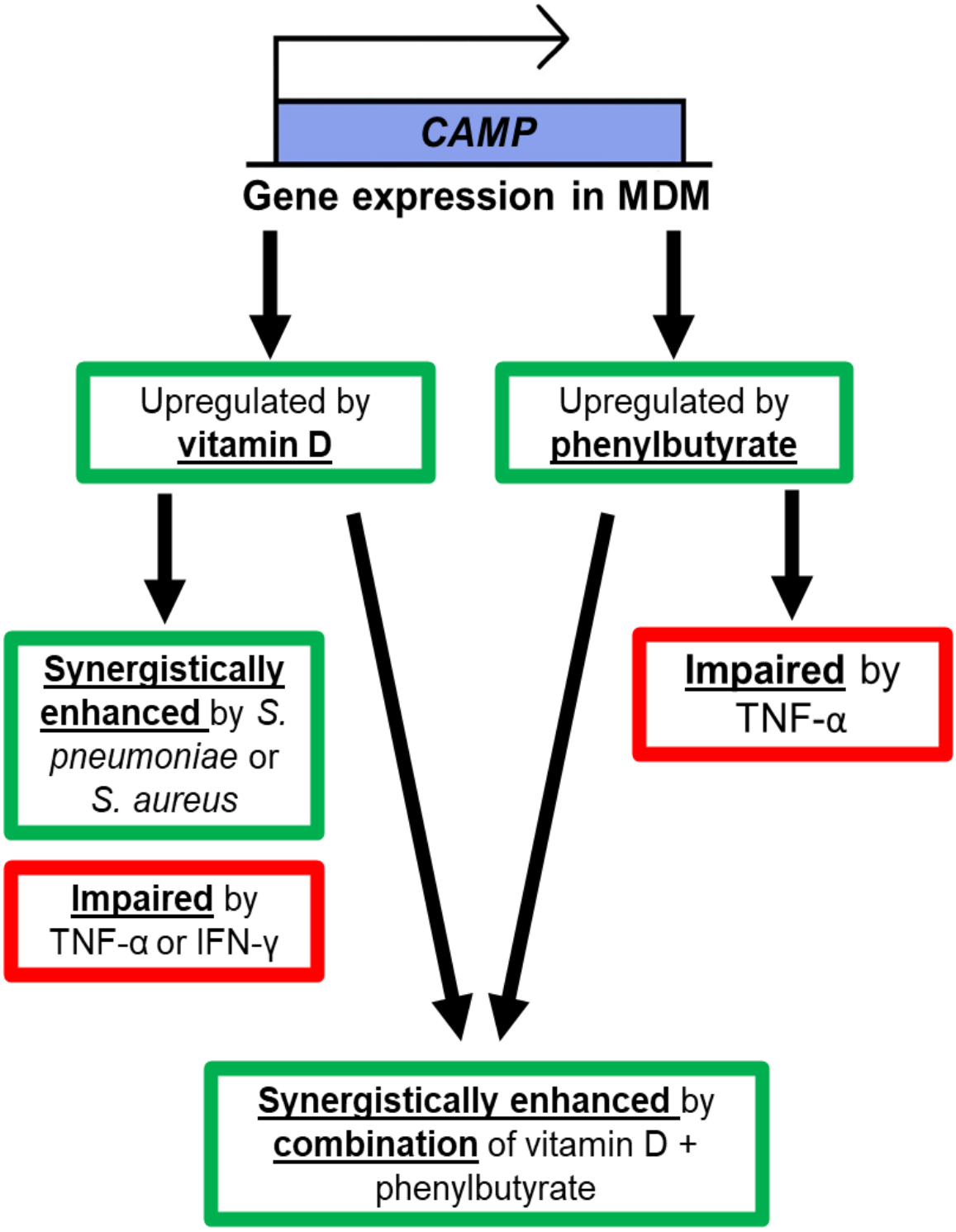


Figure 3.15: Schematic representation of the interactions and kinetics of vitamin D, phenylbutyrate (PBA), bacterial infection and the pro-inflammatory cytokines TNF-α and IFN-γ, on the regulation of *CAMP* gene expression in human MDM.

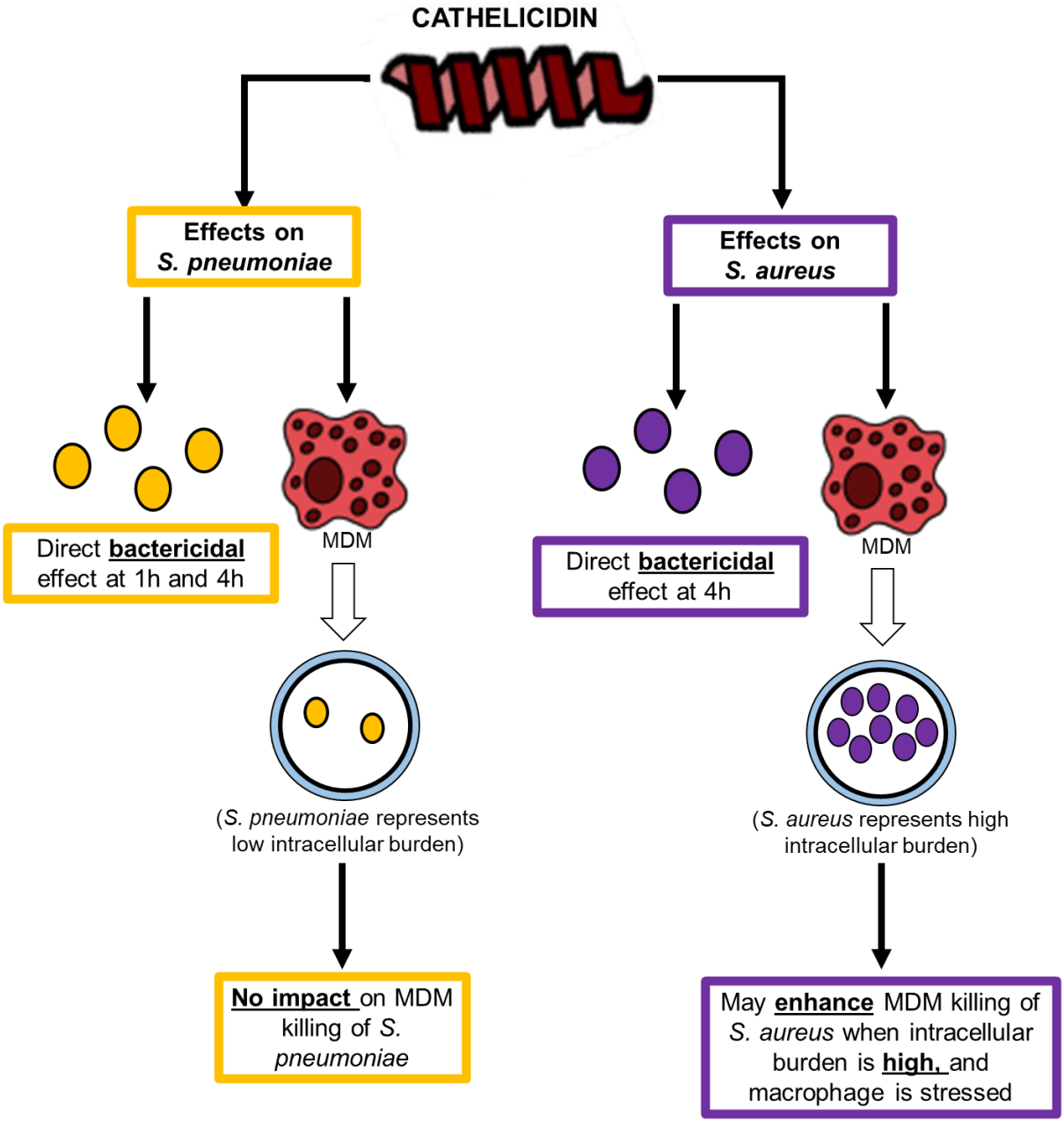


Figure 3.16: Schematic representation of the influence of cathelicidin on *S. pneumoniae* and *S. aureus* directly, and on MDM killing of internalised bacteria in human MDM.

Chapter 4

Mitochondrial fission and macrophage antimicrobial responses to *Streptococcus pneumoniae* and *Staphylococcus aureus*

4.1 Introduction

Mitochondria are widely known for their key roles in regulating cellular metabolism and energy generation (308,309) but have now also been established as important regulators of macrophage immune responses during infection (325,327). Mitochondria are highly dynamic organelles which constantly balance states of fission and fusion in response to changing cellular needs (542), and alterations in mitochondrial dynamics, in particular enhanced levels of fission, have been shown to be induced in macrophages in association with pro-inflammatory and anti-microbicidal responses during infection such as increased mROS production (309,322). We have previously shown that mitochondria undergo fission in response to late-stage intracellular *S. pneumoniae* (387). This occurs upstream of the apoptosis-associated killing pathway; enhanced mitochondrial fission may contribute to the initiation of apoptosis-associated killing to efficiently clear persistent intracellular *S. pneumoniae* or may represent an independent microbicidal mechanism unrelated to apoptosis-associated killing (387). In contrast, it is known that *S. aureus* does not induce apoptosis-associated killing in macrophages (292), and *S. aureus* infection allows analysis of mitochondrial dynamics in macrophages in a system that does not progress to apoptosis. Furthermore, cathelicidin has been shown to induce the mitochondrial pathway of apoptosis in *P. aeruginosa*-infected airway epithelial cells (496), as well as modulate mitochondrial dynamics and

function in response to infection or to inflammatory stimuli (418,509). However, the influence of cathelicidin on mitochondrial morphology in human macrophages in the context of *S. pneumoniae* or *S. aureus* infection, remain unclear. Therefore, I questioned whether *S. aureus* infection, which stresses canonical killing and does not induce apoptosis-associated bacterial killing (72,543), may also provide a stimulus that induces mitochondrial fission in human MDM, as an alternative microbicidal strategy. In addition, I questioned whether the presence of cathelicidin would further alter mitochondrial dynamics in the context of bacterial infection.

In regard to mechanisms of mitochondrial fission, Drp1, Mff and Fis1 have been well characterised as the key factors facilitating fission. The mechanism of Drp1 recruitment to mitochondria via interactions with Mff and Fis1, Drp1 oligomerisation and constriction to split mitochondria has been described in detail (340,342). However, more recent studies have begun to describe non-canonical fission mechanisms that function independently of Drp1 in different contexts (368,369), suggesting that the mechanisms of mitochondrial fission could be more complex than initially understood. Despite the fact that mitochondrial fission occurs in response to bacterial infection and enhances macrophage pro-inflammatory responses and is associated with microbial killing (309,322,387), the mechanistic details, and roles of mitochondrial fission components are incompletely characterised particularly in the context of *S. pneumoniae* or *S. aureus* infection. I therefore investigated whether bacterial-induced mitochondrial fission involved Drp1 and Mff, and if this resulted in increased mROS production and enhanced intracellular bacterial killing by macrophages. siRNA targeting Drp1 and Mff allowed me to knock down these genes and investigate the roles of Drp1 and Mff on *S. pneumoniae*- or *S.*

aureus-induced mitochondrial fission, MDM intracellular bacterial killing, and *S. aureus*-mediated mROS production. In this chapter, the Drp1 and Mff siRNA experiments involving *S. pneumoniae* were conducted with Dr Brian McHugh of the Dockrell group, while all other experiments were conducted solely by me.

4.2 Results

4.2.1 Mitochondrial fission occurs after late stage, but not early stage, *S. pneumoniae* challenge of MDM

Mitochondrial fission has been shown to occur upstream of apoptosis-associated killing in macrophages, which allows for the clearance of persistent intracellular bacteria such as *S. pneumoniae*, during late stages of infection (387). To investigate the effect of *S. pneumoniae* challenge on mitochondrial morphology and the time span of this response in MDM bacterial infection models, MDM were mock infected or challenged with *S. pneumoniae* for 4 or 14 hours. 4 hours represents early stage bacterial challenge during which canonical phagolysosomal killing occurs, and 14 hours represents the late stage of bacterial challenge where phagolysosomal killing mechanisms are exhausted and early stages of apoptosis occur. Following bacterial challenge, mitochondria were stained and visualised by confocal microscopy and mitochondrial network complexity scores were calculated.

Mitochondria exhibited a complex branched network in steady-state conditions at both 4 and 14 hours (Figure 4.1A and 4.1B), as expected. Mitochondrial structure remained elongated and branched after 4 hour *S. pneumoniae* challenge (Figure 4.1C), showing that *S. pneumoniae* does not induce fission at this early time point. However, mitochondrial morphology became markedly

punctate and fragmented after 14 hour *S. pneumoniae* challenge (Figure 4.1D), showing that *S. pneumoniae* does indeed induce fission at this later time point. The mitochondrial network complexity scores calculated from the image analysis confirm these observations; Mitochondrial network complexity is significantly reduced following 14 hour *S. pneumoniae* challenge compared to 14 hour mock infection or 4 hour *S. pneumoniae* challenge (Figure 4.1E). These results show that late stage, but not early stage, *S. pneumoniae* challenge induces mitochondrial fission in MDM.

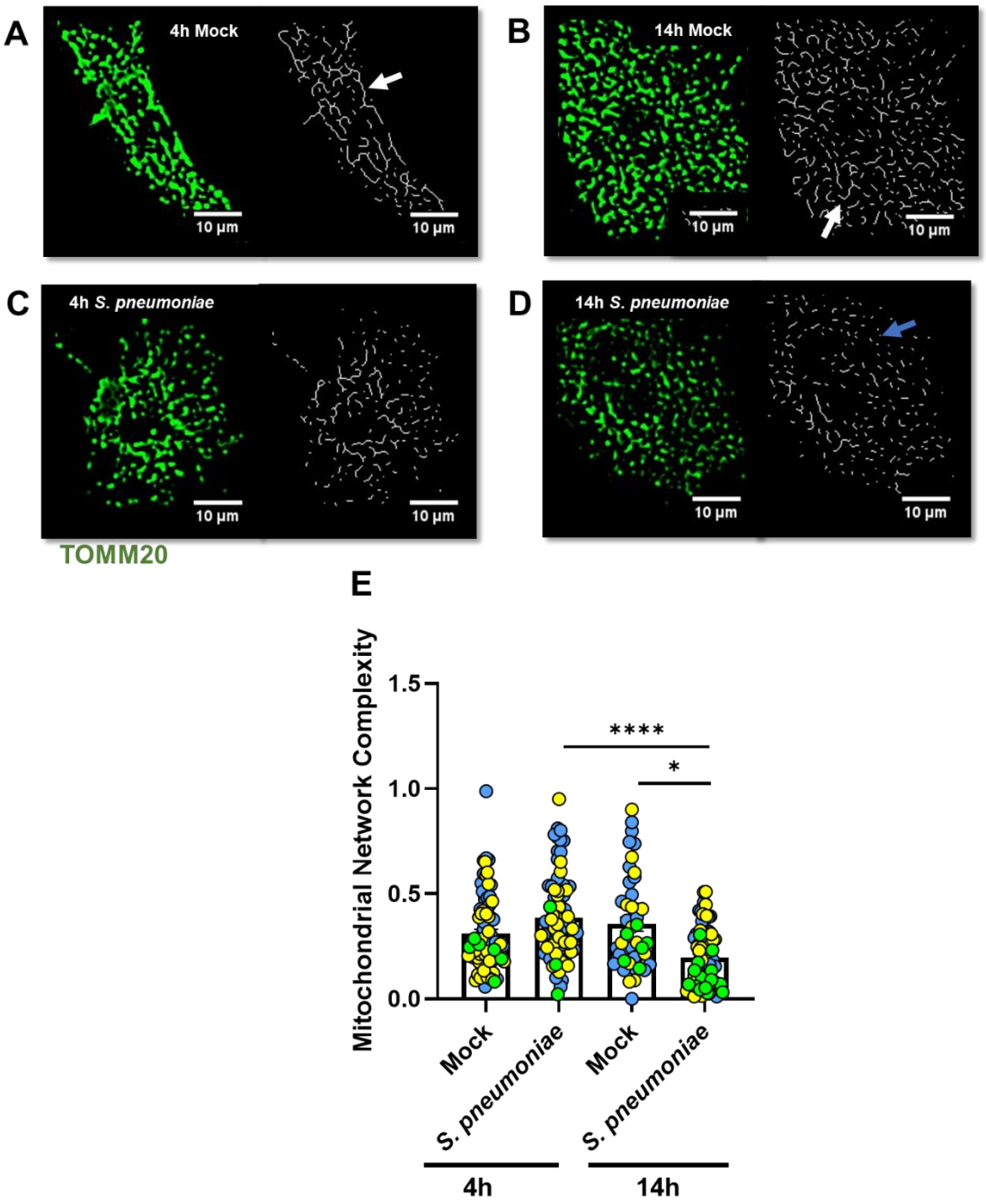


Figure 4.1: Changes in MDM mitochondrial morphology following early and late stage *S. pneumoniae* challenge. A-D) Example images of mitochondrial morphology in MDM following early and late stage *S. pneumoniae* challenge at MOI=10. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification. E) Mitochondrial network complexity scores per condition at 4 and 14 hours. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, ****p<0.0001.

4.2.2 Mitochondrial fission occurs after early and late stage *S. aureus* challenge of MDM

Next, I investigated the effect of early and late stage *S. aureus* challenge on MDM mitochondrial morphology as I was interested to discover if the greater intracellular burden and stress caused by *S. aureus* on the macrophage led to changes in mitochondrial morphology that were different from those observed with *S. pneumoniae*. MDM were mock infected or challenged with *S. aureus* for 4 or 14 hours as for *S. pneumoniae*, mitochondria were stained and visualised, and mitochondrial network complexity analysed as described above.

Mitochondria in mock-infected MDM at 4 and 14 hours were highly branched (Figure 4.2A and 4.2B) as observed previously. 14 hour *S. aureus* challenge induced a marked degree of fission (Figure 4.2D), suggesting that mitochondrial fission is induced during late stage infection with other bacteria in addition to *S. pneumoniae*. However, fission was also observed following 4 hour *S. aureus* challenge (Figure 4.2C), suggesting that *S. aureus* induces fission more rapidly than *S. pneumoniae*. The mitochondrial network complexity scores also show that following both 4 and 14 hour *S. aureus* challenge, there is a reduction in network complexity compared to mock infection (Figure 4.2E). This indicates that macrophage responses to *S. aureus* infection include an earlier change in mitochondrial morphology than those observed with *S. pneumoniae*.

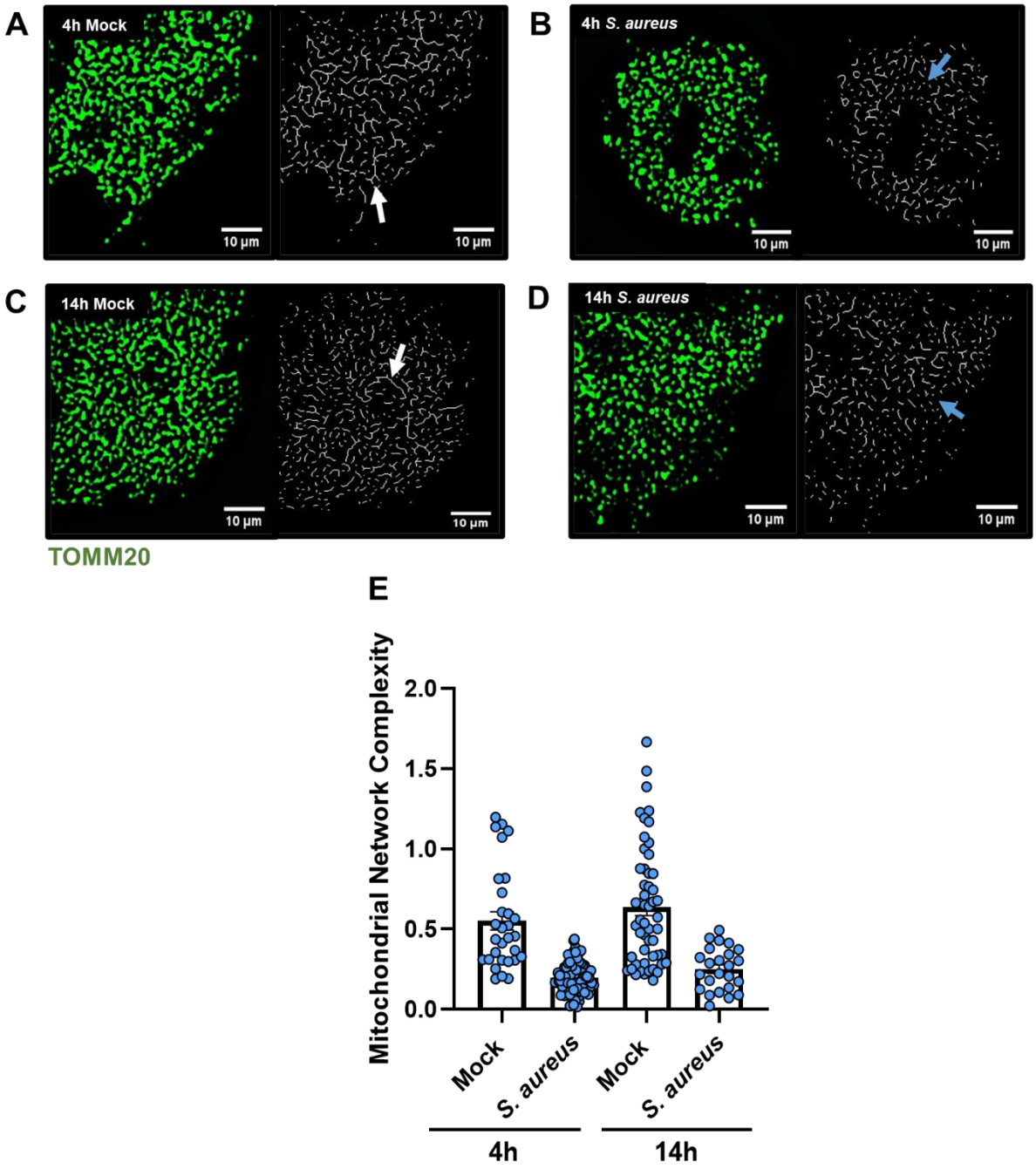


Figure 4.2: Changes in mitochondrial morphology following early and late stage *S. aureus* challenge of MDM. A-D) Example images of mitochondrial morphology in MDM following early and late stage *S. aureus* challenge at MOI=10. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification E) Mitochondrial network complexity scores per condition at 4 and 14 hours. Each point represents a single MDM. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 1 experiment.

4.2.3 Mitochondrial fission begins to occur as early as 2 hours following *S. aureus* challenge of MDM

As the above results indicated that *S. aureus* induces mitochondrial fission much earlier than *S. pneumoniae*, I was very interested to investigate the kinetics of this and determine at which time point mitochondrial fission in response to *S. aureus* begins. MDM were mock infected for 8 hours or challenged with *S. aureus* for various time points from 30 minutes to 8 hours. Mitochondrial were stained and visualised, and mitochondrial network complexity analysed as described above.

The branched mitochondrial morphology was observed following 8 hour mock infection (Figure 4.3A) as expected and remained branched at the earliest stages of *S. aureus* challenge (30 minutes to 1 hour) (Figure 4.3B and 4.3C), suggesting that *S. aureus* does not induce fission at these very early time points. Unexpectedly, mitochondrial morphology became more fragmented from 2 hour *S. aureus* challenge onward (Figure 4.3D-G), with significant fragmentation observed at 8 hours (Figure 4.3H). This was reflected in the mitochondrial network complexity scores (Figure 4.4). Complexity was high in mock infected, 30 min *S. aureus* and 1 hour *S. aureus* challenge conditions but was reduced with *S. aureus* challenge from 2 hours onward and significantly reduced with 8 hour *S. aureus* challenge. These results show that macrophage responses to *S. aureus* infection include a very rapid induction of mitochondrial fission, beginning as early as 2 hours post-infection, which contrasts with the much slower kinetics of fission observed in MDM in response to *S. pneumoniae*.

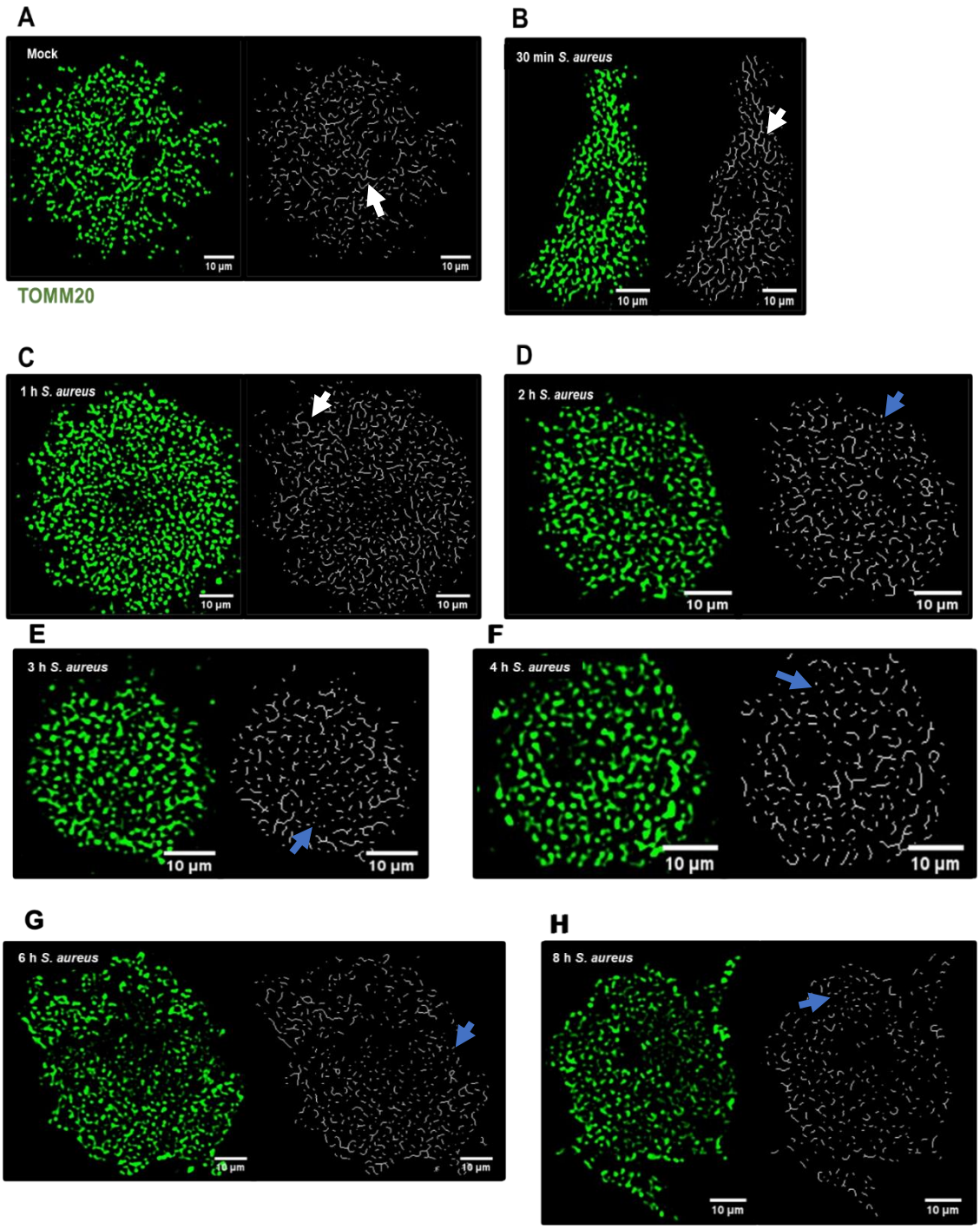


Figure 4.3: Changes in mitochondrial morphology following *S. aureus* challenge of MDM over time. A-H) Example images of the mitochondrial morphology in MDM after different time points of *S. aureus* challenge at MOI=10. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification. n = 3 independent experiments.

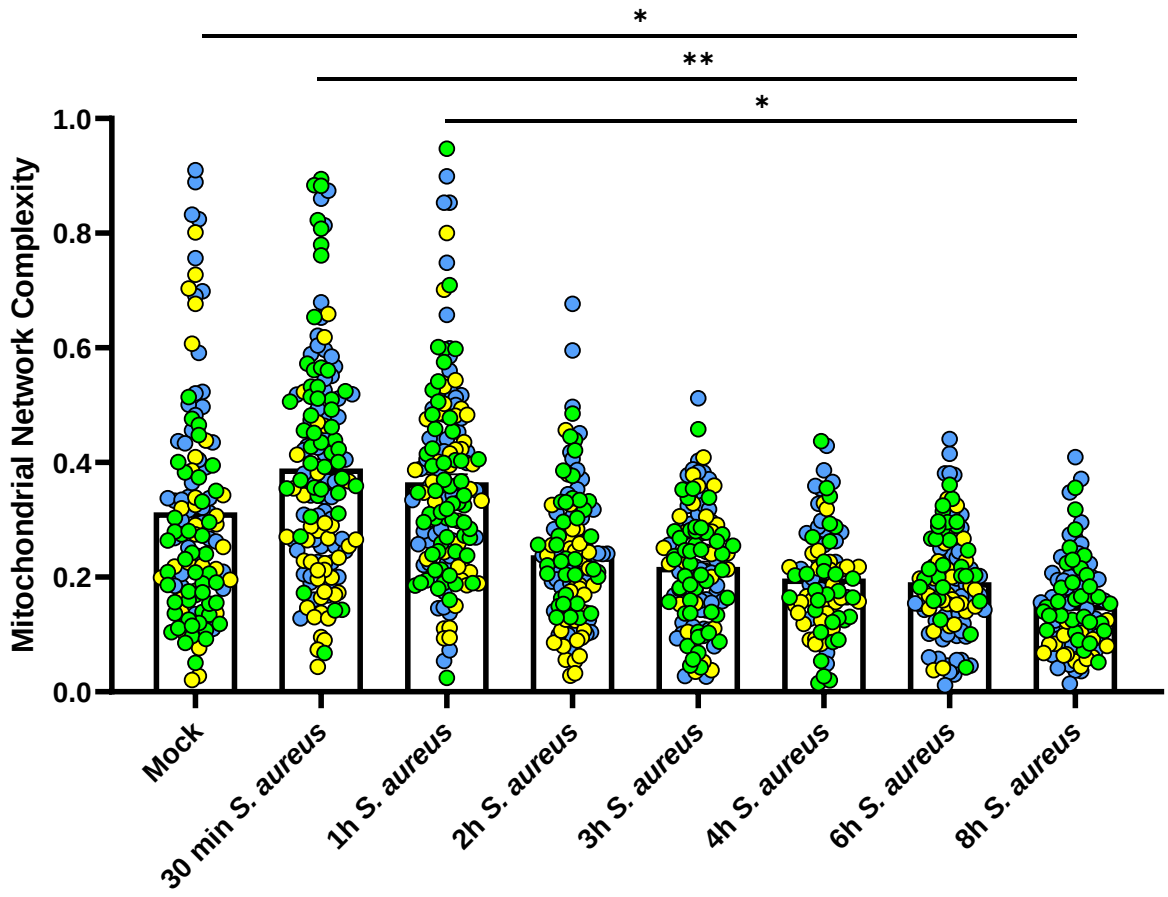


Figure 4.4: Quantification of changes in mitochondrial morphology following *S. aureus* challenge of MDM over time. Mitochondrial network complexity scores at each time point following *S. aureus* challenge at MOI=10. Mitochondrial network complexity scores decrease over time with *S. aureus* challenge, with significance observed at 8h. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, **p<0.01.

4.2.4 The influence of cathelicidin on mitochondrial morphology in bacterial-challenged MDM

It is evident that late-stage *S. pneumoniae* infection and early-stage *S. aureus* infection led to enhanced induction of mitochondrial fission in MDM, therefore I wanted to investigate the influence of cathelicidin on mitochondrial morphology during bacterial infection. In chapter three of this thesis, TAMRA-labelled cathelicidin was exogenously applied to MDM following 4 and 14 hour *S. pneumoniae* challenge, or 4 hour *S. aureus* challenge, before visualising MDM by confocal microscopy and calculating TAMRA fluorescence. The mitochondria of these MDM were also stained following treatment with TAMRA-labelled cathelicidin. Mitochondria were visualised and mitochondrial network complexity analysed as described previously.

I began by investigating the influence of cathelicidin on mitochondrial morphology during early stage *S. pneumoniae* challenge of MDM. Mitochondria exhibited the branched morphology expected under steady-state conditions (Figure 5A), but this was not significantly altered by cathelicidin treatment (Figure 4.5B). This suggests that exogenous cathelicidin does not induce mitochondrial fission in MDM at this time point under these conditions. The mitochondrial branching was also observed following 4 hour *S. pneumoniae* challenge as expected (Figure 4.5C), as high levels of fission are not induced by *S. pneumoniae* at this stage. However, cathelicidin treatment following 4 hour *S. pneumoniae* challenge resulted in significant levels of fission as shown by Figure 4.5D and by the reduction in mitochondrial network complexity in Figure 4.5E, suggesting that cathelicidin induces increased fission in MDM in the presence of early-stage *S. pneumoniae* infection

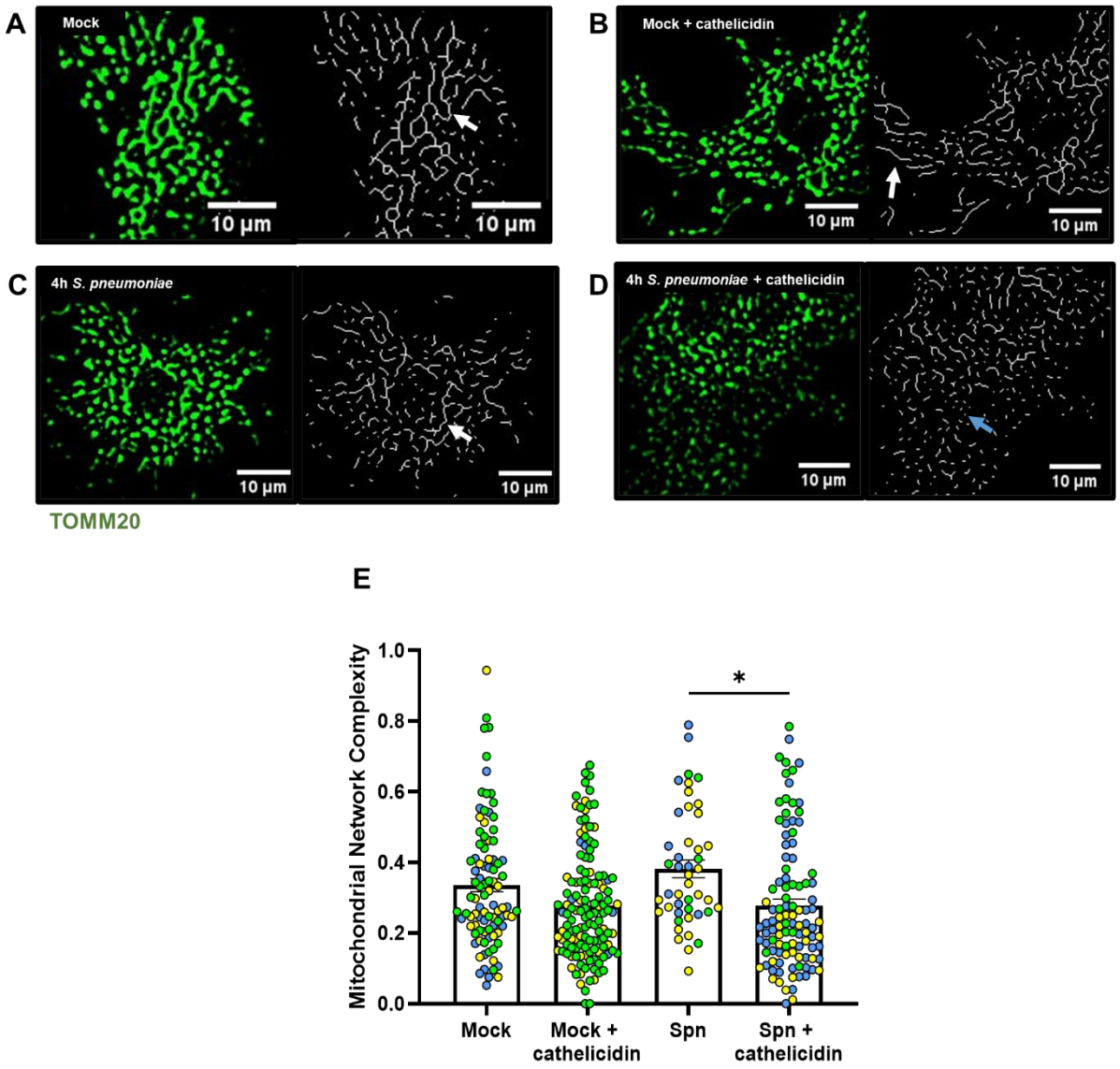


Figure 4.5: Influence of cathelicidin on changes in mitochondrial morphology in early stage *S. pneumoniae*-challenge MDM. A-D) Example images of mitochondrial morphology in MDM following *S. pneumoniae* challenge for 4 hours at MOI=10, and/or TAMRA-labelled cathelicidin treatment for 1 hour. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification. E) Mitochondrial network complexity scores per condition at 4 hours. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05.

Next, I investigated cathelicidin's effect on mitochondrial morphology during the later stages of *S. pneumoniae* challenge. As observed previously, a high degree of mitochondrial branching was evident with and without cathelicidin treatment in steady-state conditions (Figure 4.6A and 4.6B), and 14 hour *S. pneumoniae* challenge led to the more fragmented mitochondrial morphology (Figure 4.6C) and a significant reduction in network complexity (Figure 4.6E). However, cathelicidin treatment following 14 hour *S. pneumoniae* challenge rescued the more branched mitochondrial morphology as shown in Figure 4.6D, and increased network complexity compared to *S. pneumoniae* challenge alone (Figure 4.6E). Therefore, cathelicidin has contrasting effects on mitochondrial morphology in MDM during different stages of *S. pneumoniae* infection; cathelicidin promotes mitochondrial fission early during infection but prevents some of the fission induced by *S. pneumoniae* during late-stage infection.

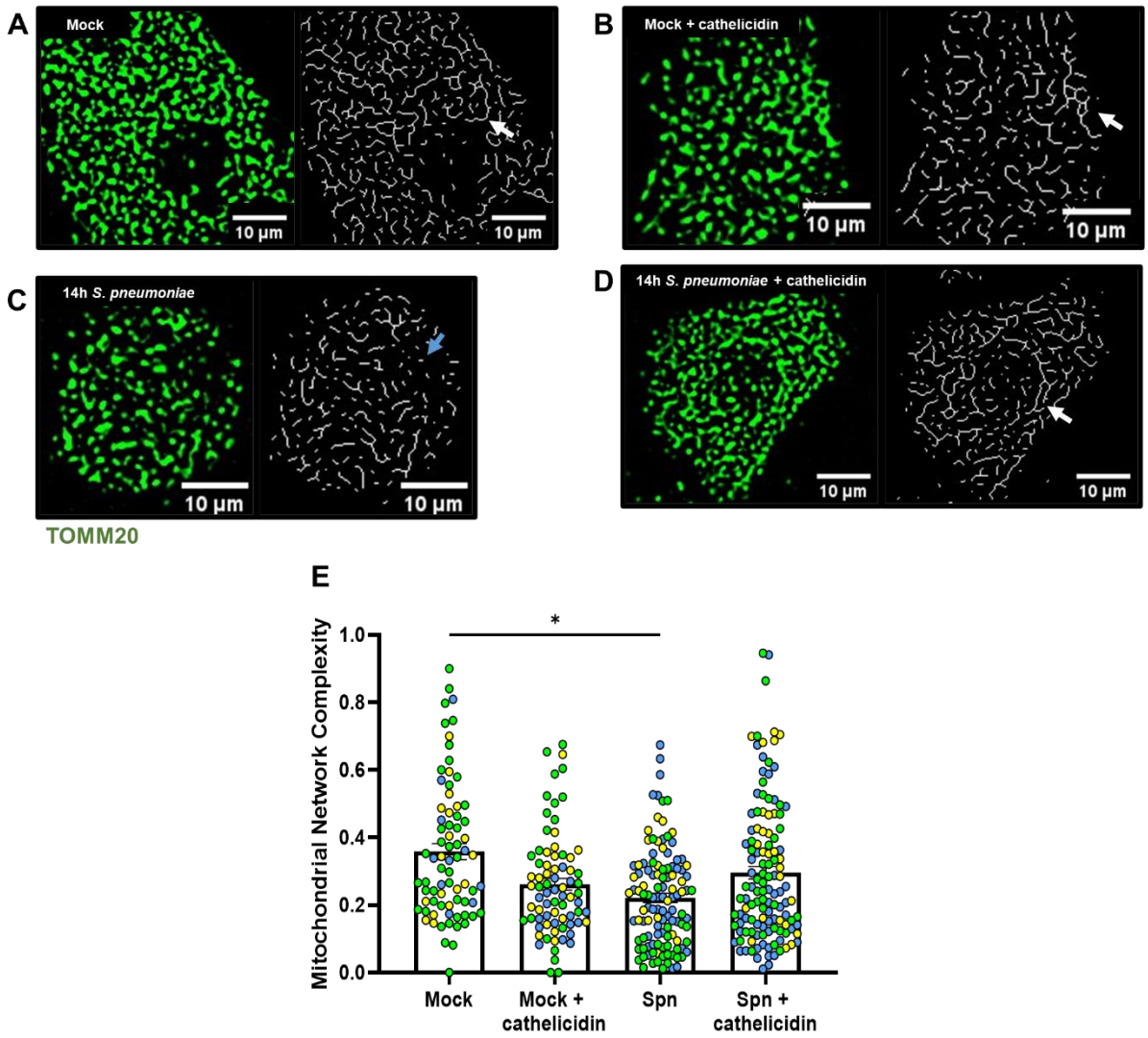


Figure 4.6: Influence of cathelicidin on changes in mitochondrial morphology in late stage *S. pneumoniae*-challenge MDM. A-D) Example images of mitochondrial morphology in MDM following *S. pneumoniae* challenge for 14 hours at MOI=10, and/or TAMRA-labelled cathelicidin treatment for 1 hour. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification. E) Mitochondrial network complexity scores per condition at 14 hours. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05.

Next, I studied how cathelicidin impacted early stages of mitochondrial morphology network adaption following *S. aureus* challenge. As observed previously, a high degree of mitochondrial branching was evident with and without cathelicidin treatment in steady-state conditions (Figure 4.7A and 4.7B). The significantly more fragmented mitochondrial morphology was observed following 4 hour *S. aureus* challenge (Figure 4.7C) and a significant reduction in network complexity (Figure 4.7E), as shown previously. However, cathelicidin treatment following 4 hour *S. aureus* challenge did not prevent *S. aureus*-induced mitochondrial fragmentation (Figure 4.7D and 4.7E). Therefore, cathelicidin does not influence changes in mitochondrial morphology following early stages of *S. aureus* challenge. It should be noted that effects during later stages of *S. aureus* challenge are not detailed here. The influence of cathelicidin on mitochondrial morphology following 14 hour *S. aureus* challenge of MDM was briefly investigated, however the MDM did not survive these conditions therefore I was unable to generate these data. In order to gain insight into cathelicidin's influence at these later stages, it would be beneficial to explore other *S. aureus* challenge time points between 4 and 14 hours that do not impact MDM survival to this extent.

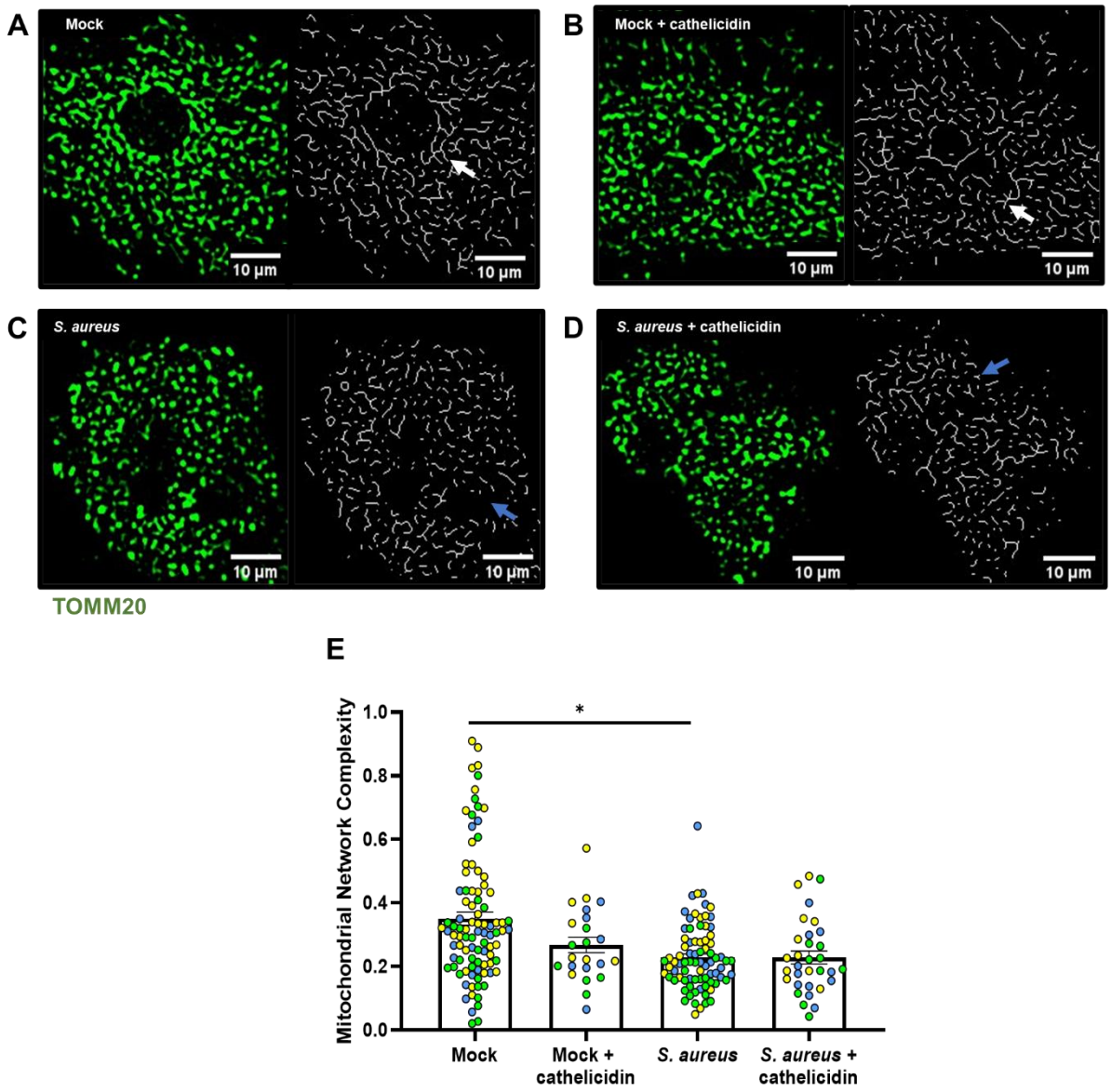


Figure 4.7: Influence of cathelicidin on changes in mitochondrial morphology in *S. aureus*-challenged MDM. A-D) Example images of mitochondrial morphology in MDM following *S. aureus* challenge for 4 hours at MOI=10, and/or TAMRA-labelled cathelicidin treatment for 1 hour. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and blue arrows highlight examples of punctate, fragmented mitochondria. Images taken on the Andor spinning disk at 60x magnification. E) Mitochondrial network complexity scores per condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05.

4.2.5 Gene expression levels of Mff and Drp1 are knocked down in MDM following siRNA treatment

It is clear that both *S. pneumoniae* and *S. aureus* promote mitochondrial fission in MDM at different stages of their interaction with macrophages; *S. pneumoniae* induces fission later during infection, while *S. aureus* induces fission earlier. In order to explore mechanisms of changes in mitochondrial morphology during infection, I took advantage of siRNA targeting of the mitochondrial fission components Mff and Drp1 to knock down their expression in MDM and investigate the mechanisms regulating mitochondrial morphology in our models. Before conducting functional experiments, it was necessary to first validate that the siRNA successfully knocks down Mff and Drp1 expression in MDM. Therefore, MDM were treated with control, Mff or Drp1 siRNA for five days prior to RNA extraction. MDM treated with lipofectamine transfection reagent only were included as a control. cDNA was synthesised, then Mff and Drp1 gene expression levels were determined by RT-qPCR. Relative gene expression levels were calculated relative to the housekeeping genes for β-actin and GAPDH. These experiments were conducted with Dr Brian McHugh.

As shown in Figure 4.8, for both Mff and Drp1 genes, expression levels in MDM following lipofectamine only or control siRNA treatment were very similar, which confirmed that the control siRNA would not alter Mff or Drp1 expression in the subsequent experiments. As expected, Mff or Drp1 siRNA treatment of MDM resulted in a significant reduction in Mff and Drp1 gene expression levels. This confirmed that Mff and Drp1 could be successfully knocked down in MDM by siRNA, therefore this would be a suitable method for investigating the functional

consequences of Mff and Drp1 knockdown in MDM in the context of *S. pneumoniae* or *S. aureus* infection.

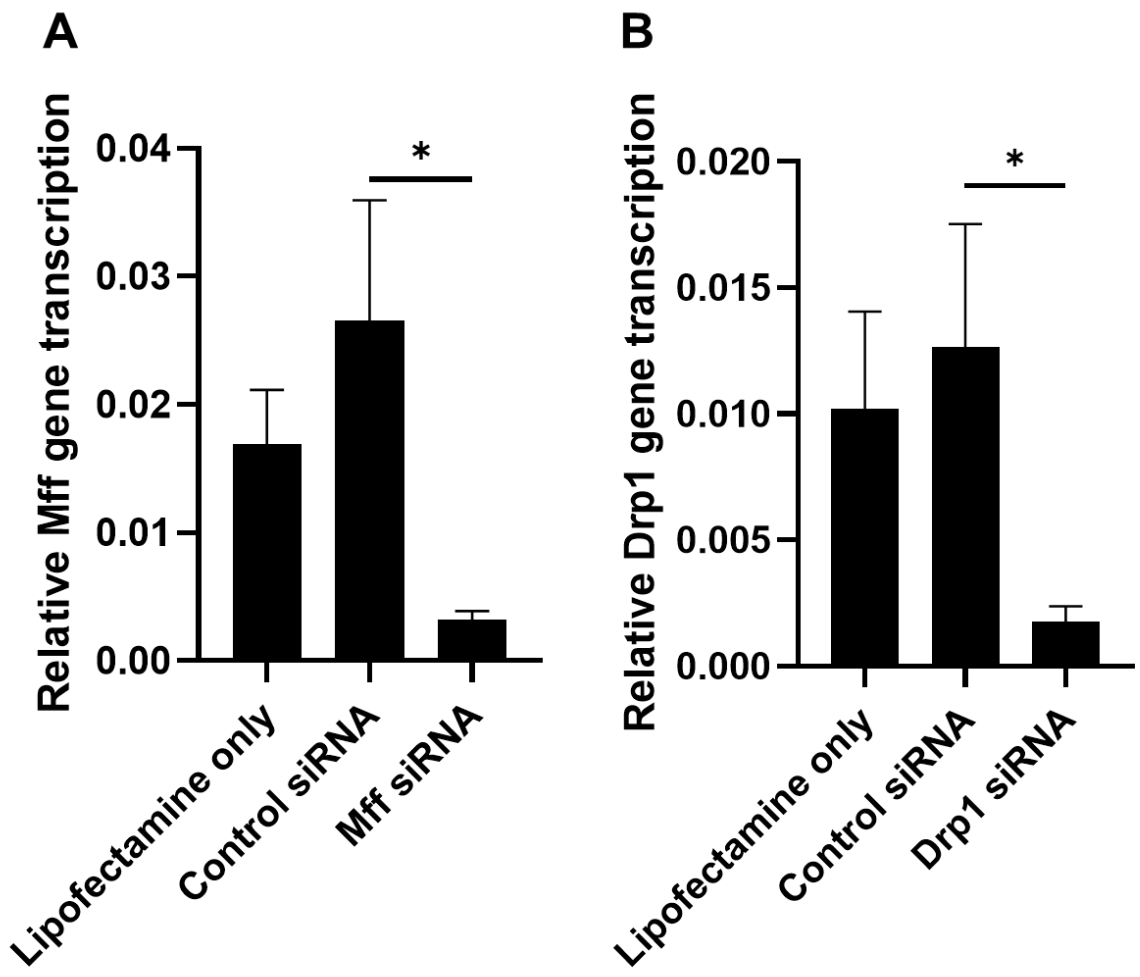


Figure 4.8: Relative gene expression of Mff and Drp1 following siRNA knockdown in MDM. Relative gene expression of A) Mff and B) Drp1 in MDM following lipofectamine only, control siRNA, Drp1 siRNA or Mff siRNA treatment. Error bars represent the SEM. n= 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05.

4.2.6 Mitochondria form elongated, hyperfused mitochondrial networks following knockdown of Mff or Drp1

Upon confirmation of successful Mff and Drp1 knockdown with siRNA, I investigated the influence of Mff and Drp1 knockdown on mitochondria morphology in steady-state conditions. MDM were treated with control, Mff, or Drp1 siRNA for as before, mitochondria were stained and visualised, and mitochondrial network complexity analysed as described above.

Control siRNA-treated MDM exhibited the branched mitochondrial morphology expected in steady-state conditions (Figure 4.9A). Both Mff and Drp1 siRNA knockdown resulted in a hyperfused mitochondrial morphology with highly elongated mitochondrial structures (Figure 4.9B and 4.9C), suggesting that Mff and Drp1 are involved in mitochondrial fission in MDM as expected. The mitochondrial network complexity scores showed that knockdown of Mff led to a modest increase in complexity compared to control, while knockdown of Drp1 resulted in a significant increase in network complexity (Figure 4.9D). These results suggest that Drp1 is the more prominent factor driving mitochondrial fission in MDM.

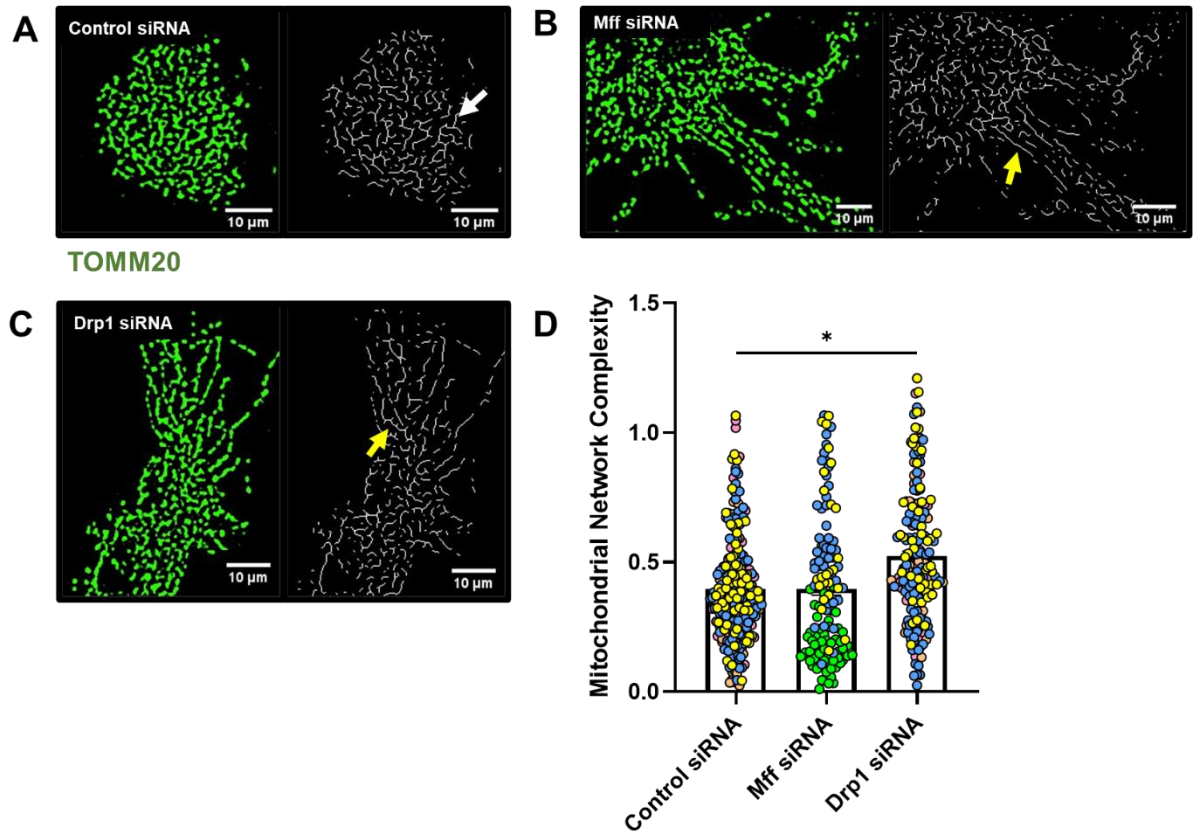


Figure 4.9: Influence of Drp1 or Mff siRNA knockdown on mitochondrial morphology in MDM. A-C) Example images of the mitochondrial morphology in MDM following treatment with control, Mff, or Drp1 siRNA. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria and yellow arrows highlight examples of hyperfused mitochondria. Images taken on the Andor spinning disk at 60x magnification. D) Mitochondrial network complexity scores per condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3-4 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05.

4.2.7 Mff and Drp1 may contribute to *S. pneumoniae*-associated changes in mitochondrial morphology in individual MDM

As I had confirmed that siRNA knockdown of Mff and Drp1 resulted in more hyperfused mitochondrial morphology in steady-state conditions, it was now necessary to establish if these mitochondrial fission components altered mitochondrial morphology in the context of bacterial infection. We began by investigating the effect of *S. pneumoniae* on mitochondria in MDM following Mff or Drp1 knockdown. MDM were treated with control, Mff or Drp1 siRNA as above, then challenged with *S. pneumoniae* for 10 hours. This time point was chosen initially as our previous studies have shown that 10 hours represents the earliest incidences of fission following *S. pneumoniae* challenge and allowed us to exclude any influence of early apoptotic processes, which are typically observed from 14 hours (387). Mitochondria were stained and visualised, and mitochondrial network complexity analysed as described above.

Mitochondria exhibit a complex, branched network of elongated mitochondria under control conditions (Figure 4.10A), and more hyperfused networks following knockdown of Mff or Drp1 (Figure 4.10C and 4.10E) as observed previously. Mitochondrial fragmentation was not observed following 10 hour *S. pneumoniae* challenge in control siRNA-treated MDM (Figure 4.10B), at a global level, likely due to this being the earliest time point associated with the occurrence of fission, however it is possible that changes in mitochondrial morphology may be induced at an individual cell level at this stage. Visually, knockdown of Mff or Drp1 resulted in a more hyperfused mitochondrial morphology following 10 hour *S. pneumoniae* challenge compared to control siRNA and *S. pneumoniae* challenge (Figure 4.10D

and 4.10F). Although the images show these changes in mitochondrial morphology, the population-level mitochondrial network complexity scores do not show significant differences between the conditions (Figure 4.10G). These results suggest that knockdown of Mff or Drp1 may protect mitochondria from S*. pneumoniae*-associated changes in mitochondrial morphology in individual MDM in a population at this time point, therefore Mff and Drp1 may be involved in mediating *S. pneumoniae*-associated changes in mitochondrial morphology in MDM. However, due to the small sample size and variability between MDM in a population, further studies would be beneficial to determine the global effect of Mff and Drp1 in MDM during *S. pneumoniae* challenge.

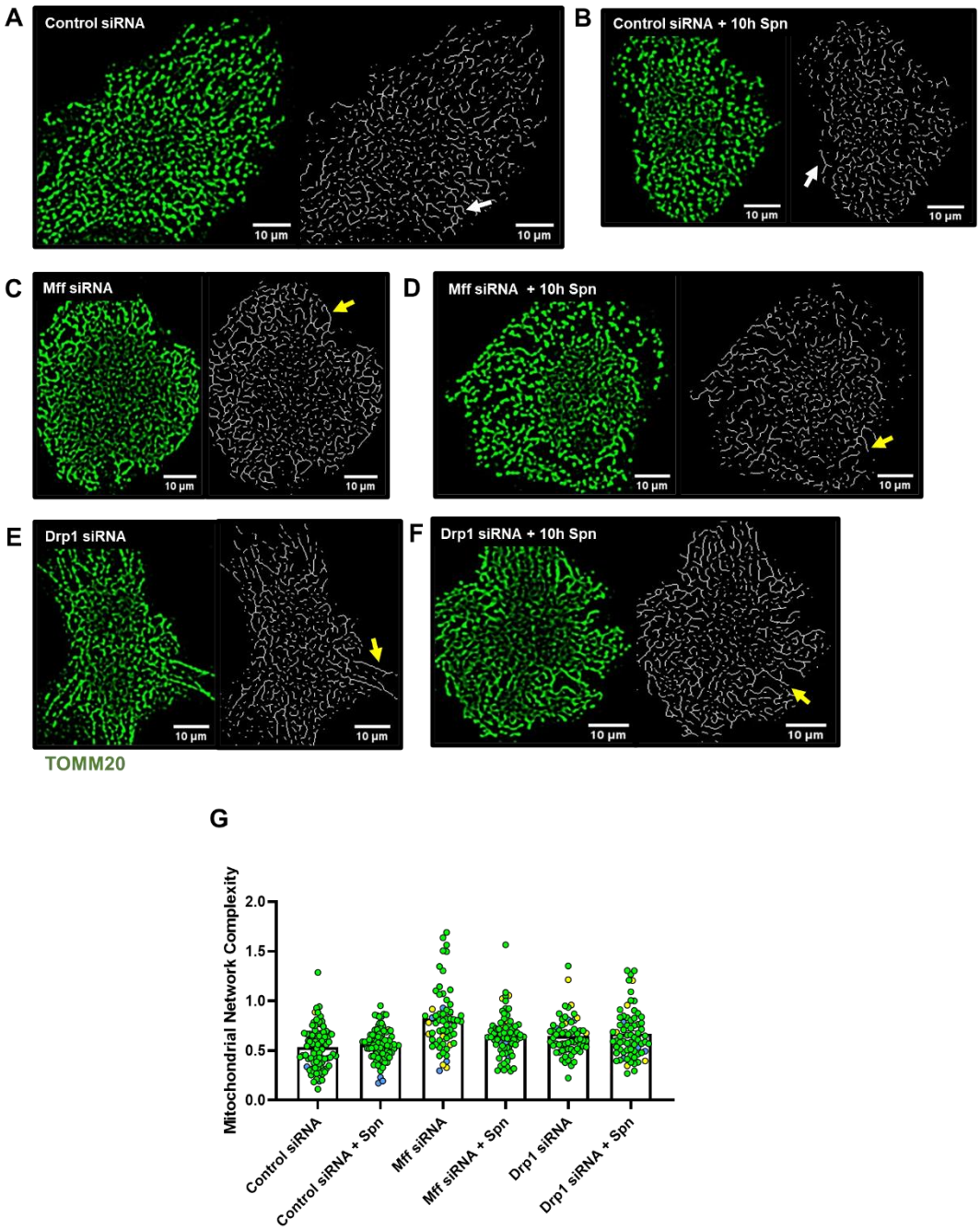


Figure 4.10: Mitochondrial morphology in Spn-challenged MDM following siRNA knockdown of Drp1 or Mff. A-F) Example images of the mitochondrial morphology in MDM following treatment with control, Mff, or Drp1 siRNA and mock infection or *S. pneumoniae* challenge at MOI=10 for 10 hours. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria, and yellow arrows highlight examples of hyperfused mitochondria. Images taken on the Andor spinning disk at 60x magnification. D) Mitochondrial network complexity scores per condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

4.2.8 Mff and Drp1 do not contribute to *S. aureus*-induced mitochondrial fission at a global level

Next**,** the influence of *S. aureus* on mitochondrial morphology following Mff or Drp1 knockdown was investigated. MDM were treated with control, Mff or Drp1 siRNA challenged with *S. aureus* as above. Mitochondria were stained and visualised, and mitochondrial network complexity analysed as described above.

As shown previously, the branched mitochondrial morphology was evident in steady-state conditions following control siRNA treatment (Figure 4.11A), with increases in branching observed following Mff siRNA knockdown (Figure 4.11C) and Drp1 knockdown (Figure 4.11E). This was confirmed by the mitochondrial network complexity scores (Figure 4.11G). 4 hour *S. aureus* challenge of control siRNA-treated MDM resulted in an increase in punctate, fragmented mitochondrial structures (Figure 4.11B) and a reduction in network complexity (Figure 4.11G). Interestingly, 4 hour *S. aureus* challenge following Mff or Drp1 knockdown also resulted in a more fragmented mitochondrial phenotype (Figure 4.11D and 4.11F) and a reduction in network complexity (Figure 4.11G). These results suggest that at a global level, knockdown of Mff or Drp1 does not protect mitochondria from *S. aureus*-associated fission, and that *S. aureus*-associated fission does not require the presence of Mff or Drp1. However, these results must be interpreted carefully due to the variability of individual cell results and the resulting lack of statistical significance.

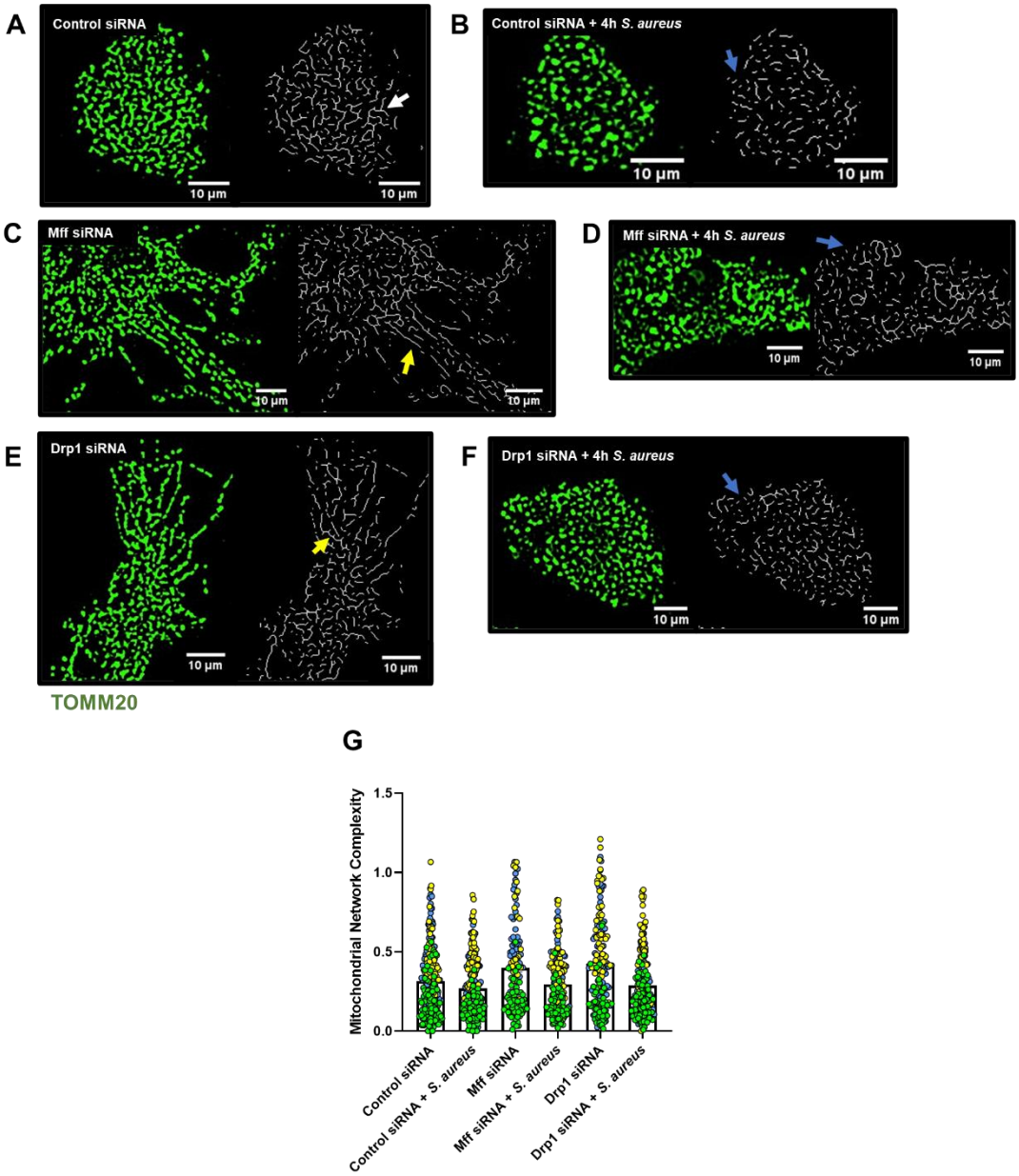


Figure 4.11: Mitochondrial morphology in *S. aureus*-challenged MDM following siRNA knockdown of Mff or Drp1. A-F) Example images of the mitochondrial morphology in MDM following treatment with control, Mff, or Drp1 siRNA, and mock infection or *S. aureus* challenge at MOI=10 for 4 hours. The left images in green represent the processed image and the right images in white represent the skeleton of the processed image. White arrows highlight examples of branched, elongated mitochondria, blue arrows highlight examples of punctate, fragmented mitochondria and yellow arrows highlight examples of hyperfused mitochondria. Images taken on the Andor spinning disk at 60x magnification. D) Mitochondrial network complexity scores per condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3-5 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

4.2.9 *S. aureus* challenge of Drp1 siRNA-treated MDM may result in reduced mROS signal

The lack of impact of Drp1 or Mff knockdown on mitochondrial complexity after *S. aureus* challenge contrasted with the potential role of these factors with *S. pneumoniae.* Next, I wanted to investigate the functional consequences of MFF and Drp1 knockdown and *S. aureus* challenge to mROS production. mROS production increases as a result of enhanced fission and in response to bacterial infection (387), therefore I predicted that Mff and Drp1 knockdown might lead to reduced mROS production due to reduced levels of fission at baseline, but since *S. aureus* challenge of Mff and Drp1 siRNA-treated MDM resulted in similar mitochondrial structure to control siRNA there would not be significant differences in mROS production following Drp1 or Mff knockdown, compared to the control siRNA treated conditions and that all would be enhanced compared to mock-infected control siRNA treatment. MDM were treated with control, Mff, or Drp1 siRNA then challenged with *S. aureus* as before. MitoSOX Red dye was used to detect mROS production. The MitoSOX signal was visualised by confocal microscopy and MitoSOX corrected total cell fluorescence scores were calculated.

It was noteworthy that I did not observe overall induction of mROS after *S. aureus* challenge following control siRNA treatment. In steady-state conditions, knockdown of Mff did not dramatically alter mROS production while knockdown of Drp1 resulted in a modest decrease in mROS signal as shown in Figure 4.12. 4 hour *S. aureus* challenge did not lead to alterations in mROS production in Mff siRNA-treated MDM, but unexpectedly in view of the absence of impact on mitochondrial network complexity resulted in a small reduction in mROS production in Drp1 siRNA-

treated MDM, compared to control or Mff siRNA knockdown. This suggests that at this global level, Drp1 may play a role in modulating mROS production in MDM in response to *S. aureus,* without impacting fission. However, since there was not clear evidence of overall mROS induction after *S. aureus* challenge following control siRNA treatment, and there is variability in the results from individual cells, this must be interpretated cautiously.

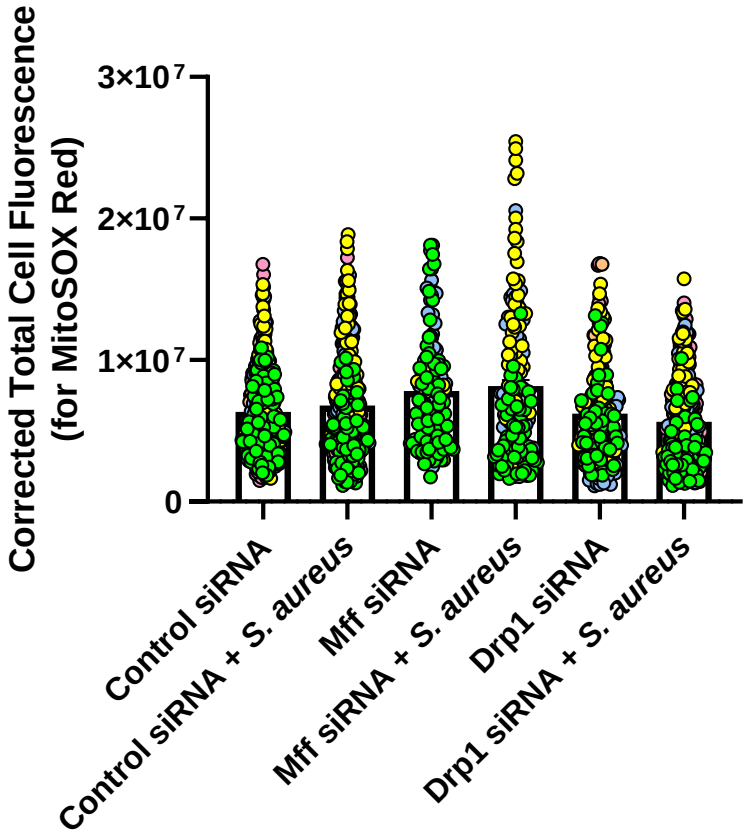


Figure 4.12: MitoSOX Red fluorescence intensity in *S. aureus*-challenged MDM following siRNA knockdown of Mff or Drp1. Corrected total cell fluorescence intensity scores for MitoSOX Red in mock-infected and *S. aureus*-challenged MDM (MOI=10 for 4 hours) following control, Mff, or Drp1 siRNA treatment. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3-5 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

4.2.10 MitoSOX does not brightly stain planktonic *S. aureus* bacteria

The reduction in mROS production in *S. aureus*-challenged MDM in the absence of Drp1 relative to control siRNA, although needing to be interpreted cautiously in the absence of significant induction of control siRNA-treated *S. aureus*-exposed MDM, warranted further investigation. However, it was first necessary to validate that MitoSOX fluorescence was not activated by planktonic bacteria, which would result in a false positive signal associated with bacteria rather than mROS localisation. Heat-killed *S. aureus* bacterial cultures were stained with MitoSOX for 30 minutes, as above for MitoSOX treatment of MDM, followed by DAPI for 12 minutes as above for MDM. 250µl of stained culture was placed into a chamber slide well, and visualised by confocal microscopy.

As shown by the images in Figure 4.13, *S. aureus* bacteria exhibited bright DAPI signal as observed previously. MitoSOX exhibited a low level of baseline signal and a sparse bright signal associated with a small number of individual bacterial cells. This shows that planktonic *S. aureus* does not induce high levels of MitoSOX fluorescence, therefore I can be more confident that the MitoSOX signal associated with *S. aureus* in further experiments is not a false positive derived from non-specific MitoSOX interactions with the bacteria.

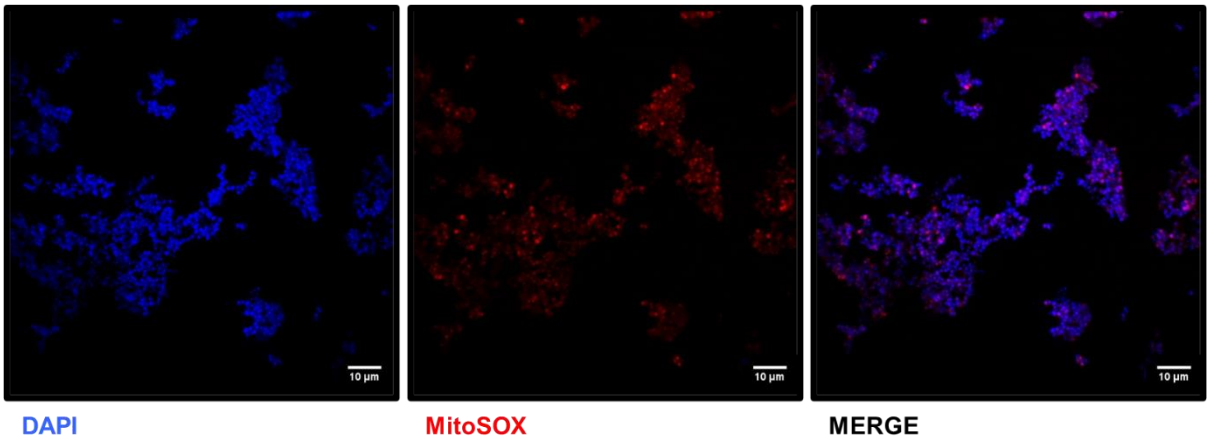


Figure 4.13: Levels of MitoSOX signal in the presence of DAPI-stained planktonic *S. aureus*. Example images of the level of MitoSOX fluorescence (red) present, following MitoSOX staining of planktonic *S. aureus*, also stained with DAPI (blue). Images taken on the Andor spinning disk at 60x magnification.

4.2.11 Drp1 plays a role in mROS localisation to internalised *S. aureus* in individual MDM

Drp1 knockdown did not prevent *S. aureus*-induced fission yet resulted in a modest reduction in mROS production in *S. aureus*-challenged MDM, which was contrary to the expected outcome. Therefore, to probe this further, the localisation of the mROS signal to intracellular bacteria in individual cells was investigated. The localisation of mROS signal to DAPI-stained intracellular *S. aureus* was visualised, and the mROS fluorescent signal associated with each DAPI-stained bacterial region was calculated.

In steady-state conditions, the mROS signal co-localised with the mitochondrial structures (Figure 4.14A). Upon 4 hour *S. aureus* challenge, an intense mROS signal was observed localised to regions of intracellular bacteria (Figure 4.14B), showing that in response to bacterial infection, mROS becomes localised to the intracellular bacteria. However, upon Drp1 knockdown, the mROS signal localised to intracellular *S. aureus* was dramatically reduced (Figure 4.14C). The fluorescence calculations also confirm that a strong mROS signal was associated with intracellular *S. aureus* in steady-state conditions, but in the absence of Drp1, this signal is significantly reduced. Therefore, these results suggest that on an individual cell level, Drp1 is important for the localisation of mROS to intracellular *S. aureus*.

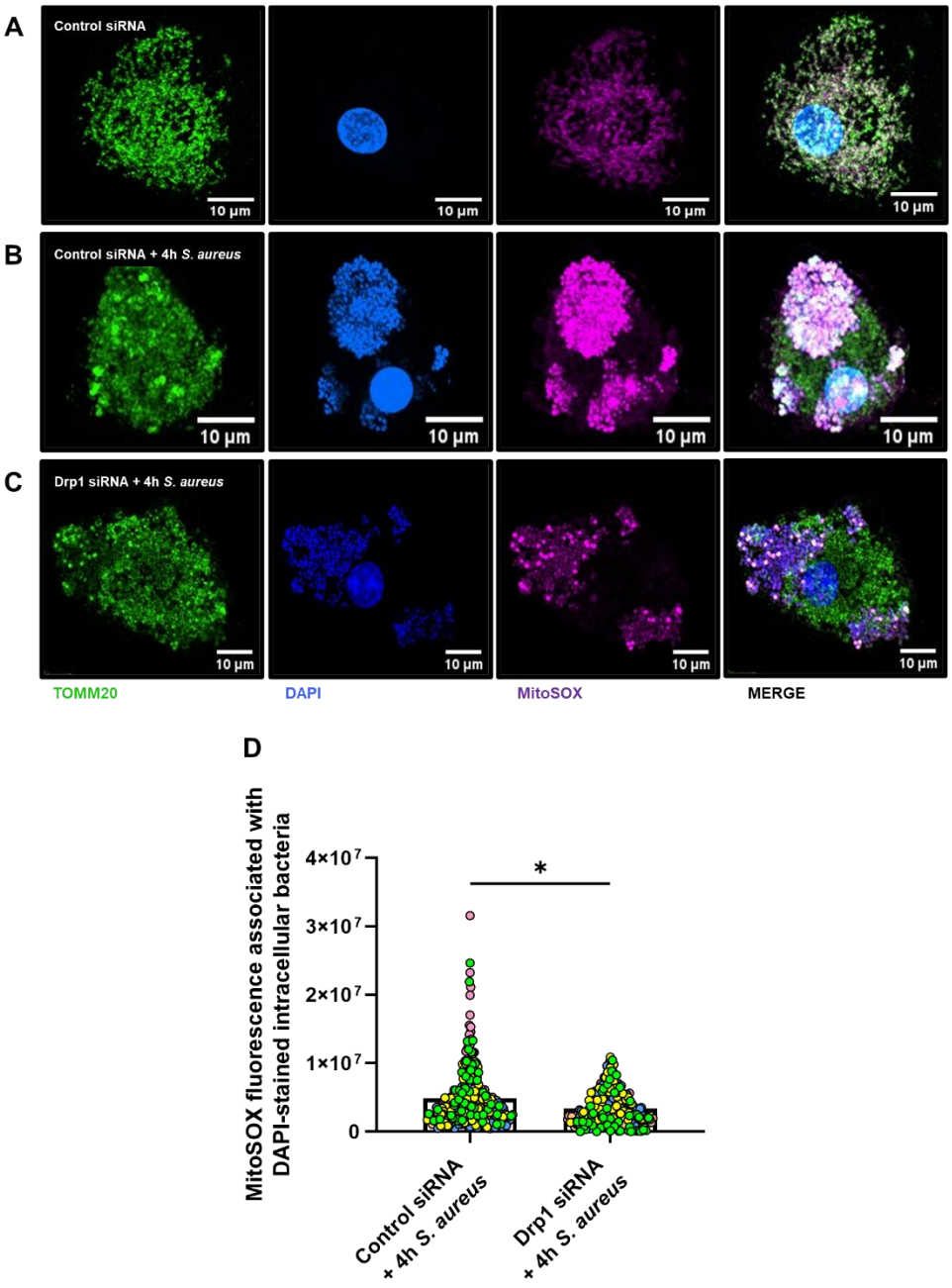


Figure 4.14: Co-localisation of MitoSOX signal with DAPI-stained intracellular *S. aureus* in control siRNA- and Drp1 siRNA-treated MDM. A-C) Example images of the localisation of internalised *S. aureus*, stained with DAPI (blue), and mROS, stained with MitoSOX (magenta), in MDM following treatment with control or Drp1 siRNA. Mitochondria stained with anti-TOMM20 and AlexaFluor 488 antibodies (green). Images taken on the Andor spinning disk at 60x magnification. D) MitoSOX fluorescence associated with DAPI-stained bacterial regions in control siRNA- and Drp1 siRNA-treated MDM. Each point represents a single bacterial region within MDM and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n= 5 independent experiments. Mann-Whitney test carried out. *p<0.05.

4.2.12 Mff modestly reduces, while Drp1 modestly enhances MDM killing of late-stage intracellular *S. pneumoniae*

Since I now had some insight into the roles of Mff and Drp1 in altering mitochondrial dynamics and mROS production in response to bacterial infection, the next step was to investigate the effects of Mff and Drp1 on MDM killing of intracellular *S. pneumoniae* and *S. aureus*. Again, I began by investigating these in our established late-stage *S. pneumoniae* and MDM intracellular killing model. MDM were treated with control, Mff or Drp1 siRNA then challenged with *S. pneumoniae* as above. The supernatant from the MDM culture prior to MDM lysis, and the lysate following MDM lysis, were diluted, and plated to detect extracellular and intracellular bacteria and calculate viable intracellular bacterial counts.

Knockdown of Mff resulted in an increase in viable intracellular bacterial counts from MDM following 14 hour *S. pneumoniae* challenge compared to control siRNA-treated MDM as shown in Figure 4.15. This shows that the Mff may contribute to enhanced MDM killing of intracellular *S. pneumoniae*. In contrast, Drp1 knockdown did not alter viable intracellular *S. pneumoniae* counts compared to control treatment, which suggests that Drp1 does not play a role in MDM killing of late-stage intracellular *S. pneumoniae.*

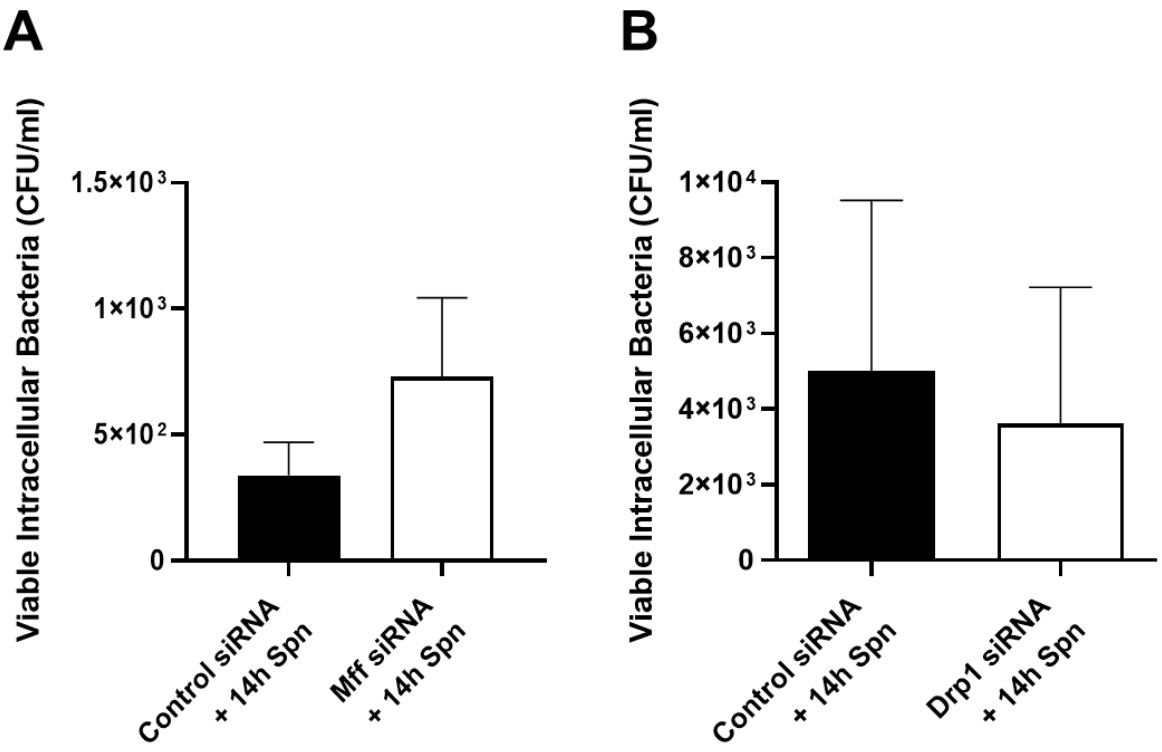


Figure 4.15: Viable intracellular bacterial counts of *S. pneumoniae* from control, Drp1 or Mff siRNA-treated MDM. The mean viable counts obtained from A) control siRNA- or Mff siRNA-treated MDM or B) control siRNA- or Drp1 siRNA-treated MDM following 14 hour *S. pneumoniae* challenge at MOI=10. Assays carried out in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum). Error bars represent the SEM. n = 3 independent experiments. Mann-Whitney tests carried out.

4.2.13 Drp1 may enhance MDM killing of intracellular *S. aureus* at 4 hours but not 8 hours

Next, I investigated the roles of Mff and Drp1 on the MDM killing of *S. aureus*. MDM were treated with control, Mff or Drp1 siRNA, then challenged with *S. aureus* as above. The supernatant from the MDM culture prior to MDM lysis, and the lysate following MDM lysis, were diluted, and plated to detect extracellular and intracellular bacteria and calculate viable intracellular bacterial counts. In this model, in view of the relative resistance to intracellular killing (72), extracellular killing was also measured.

Knockdown of Mff did not alter viable intracellular bacterial counts compared to control siRNA-treated MDM following 4 hour *S. aureus* challenge (Figure 4.16A), indicating that MFF does not play a role in MDM killing of *S. aureus* under these conditions. However, there was a trend towards significantly increased viable intracellular *S. aureus* counts following Drp1 siRNA knockdown (Figure 4.16A), suggesting that Drp1 may contribute to enhanced MDM killing of early-stage intracellular *S. aureus*. Neither Mff nor Drp1 siRNA knockdown altered viable intracellular bacterial counts compared to control siRNA-treated MDM following 8 hour *S. aureus* challenge (Figure 4.16B), suggesting that the potential contribution of Drp1 to MDM killing of *S. aureus* is limited to this early time point and may be overwhelmed at later time points.

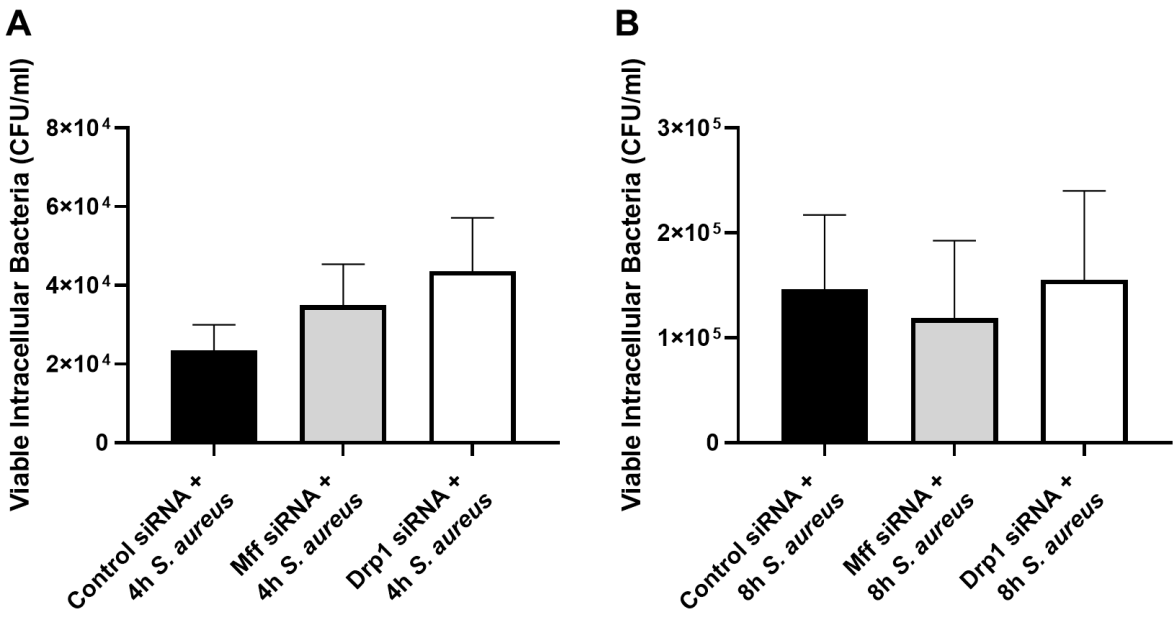


Figure 4.16: Viable intracellular bacterial counts of *S. aureus* from control, Drp1 or Mff siRNA-treated MDM. The mean viable counts obtained from control siRNA-, Mff siRNA-, or Drp1 siRNA-treated MDM following *S. aureus* challenge at MOI=10 for A) 4 hours or B) 8 hours. Assays carried out in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum). Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

4.3 Discussion

In this chapter, I have described how mitochondrial dynamics are altered in human MDM in response to *S. pneumoniae* or *S. aureus* infection, detailed the kinetics of these changes, and how cathelicidin influences mitochondrial dynamics. I have also described roles of the mitochondrial fission components Mff and Drp1 in bacterial-induced mitochondrial fission, mROS localisation to intracellular bacteria, and intracellular bacterial killing. My findings are summarised schematically in Figure 4.17.

We have previously demonstrated that enhanced levels of fission occur during late-stage *S. pneumoniae* infection, upstream of initiation of the apoptosis-associated killing pathway to clear persistent intracellular bacteria (387). As expected, I have confirmed that significant mitochondrial fission occurs following 14 hour *S. pneumoniae* challenge of MDM. However, the influence of *S. aureus* infection on mitochondrial dynamics in MDM is not known. I have shown that mitochondrial fission begins to occur as early as 2 hours following *S. aureus* challenge. This is much earlier than the changes observed with *S. pneumoniae*, therefore it shows that macrophage responses to *S. aureus* infection include a very rapid change in mitochondrial morphology resulting in enhanced fission. In regard to the contrasting intracellular burdens of *S. pneumoniae* and *S. aureus* on MDM, it is also possible that the mitochondrial fission observed at a much earlier time point with *S. aureus* could reflect the higher intracellular burden that *S. aureus* poses to the macrophage compared to *S. pneumoniae* (116). It is likely that the functional consequences of these changes in mitochondrial dynamics would also differ due to the pathogen encountered and the stage of infection. For example, *S. pneumoniae*

represents a lower intracellular burden but when numbers exceed the capacity of canonical killing it can induce apoptosis-associated killing mechanisms during late-stage infection, but *S. aureus* results in higher intracellular burdens earlier, is less efficiently killed by canonical mechanisms and does not induce such mechanisms in MDM (72,543). Therefore, it is likely that enhanced mitochondrial fission in response to *S. aureus* infections, particularly at this early stage of infection, exerts a different function, for example increased mROS production in response to greater stress of canonical killing. mROS enhanced by increased fission is one mechanism that MDM employ to efficiently clear pathogens (309,322), therefore the roles of mROS in MDM during *S. aureus* infection will be explored in the next chapter.

Cathelicidin has been shown to influence mitochondrial dynamics and function in epithelial cell models (418,496,509). For example, studies in the context of skin inflammation have demonstrated that cathelicidin exerts anti-inflammatory effects such as dampening of pro-inflammatory cytokine expression by promoting enhanced mitochondrial biogenesis (509). Furthermore, high concentrations of cathelicidin have been shown to promote apoptosis in airway epithelial cells via translocation of Bax to mitochondria (418,496). However, cathelicidin's effects on mitochondrial morphology in human macrophages during *S. pneumoniae* or *S. aureus* infection remain unclear. I have shown that exogenous cathelicidin exerts contrasting effects on mitochondrial morphology in MDM during *S. pneumoniae* infection; cathelicidin enhances mitochondrial fission following 4 hour bacterial challenge to an extent but protects mitochondria from some of the the increased levels of fission usually observed following 14 hour bacterial challenge. This suggests that cathelicidin may be acting as a regulator of mitochondrial homeostasis in MDM during *S. pneumoniae*

challenge by minimising bacteria-induced changes in mitochondrial morphology, which could be beneficial in the prevention of excessive inflammatory responses related to changes in mitochondrial dynamics.

Furthermore, although the presence of cathelicidin somewhat enhanced fission during early-stage infection, my results detailed in chapter one showed that cathelicidin does not alter MDM killing of intracellular *S. pneumoniae* at this stage. Together, these fission and killing results suggest that the consequences of enhanced cathelicidin-mediated fission are subtle, or that the timing of cathelicidin-mediated fission responses are important for optimal bacterial killing. For example, it is possible that cathelicidin-mediated fission responses are not required for MDM killing of *S. pneumoniae* during early-stage infection; as described previously, macrophages may be capable of killing *S. pneumoniae* at this early time point without additional cathelicidin-induced responses (534). The response might however be an adaption to enable more effective killing later and when the early killing mechanisms are exhausted, as part of a layered approach to microbicidal responses. To investigate the kinetics of this further, it would be interesting to carry out the *S. pneumoniae* and cathelicidin MDM killing assay at the late-stage 14 hour time point and compare the results with the 14 hour *S. pneumoniae* and cathelicidin mitochondrial fission results, to determine if there is an association between cathelicidin's protection of mitochondrial fission and potential cathelicidin-mediated changes in MDM killing at this time point. It would also be to manipulate intracellular numbers to ensure phagolysosomal responses are overwhelmed.

Cathelicidin did not influence changes in mitochondrial morphology following 4 hour *S. aureus* challenge of MDM, showing that cathelicidin is not involved in

modulating mitochondrial dynamics in response to *S. aureus* at this time point. However, my previous data in chapter one suggested that the presence of cathelicidin may enhance MDM killing of high intracellular burdens of *S. aureus* at this early stage, therefore together, these results suggest that the mechanisms by which cathelicidin may enhance MDM killing in this model do not involve an increase in mitochondrial fission. Again, it is possible that the study of cathelicidin's effects on mitochondrial dynamics and *S. aureus* killing is limited by focussing on a single time point, therefore further study into the effects of cathelicidin following different time points of *S. aureus* challenge would provide a deeper understanding of cathelicidin's influence on mitochondrial-associated macrophages responses during infection.

Mitochondrial fission occurs in response to bacterial infection and enhances macrophage pro-inflammatory responses to efficiently clear pathogens (309,322,387). Mff and Drp1 have been extensively characterised as primary components of the mitochondrial fission machinery, however the mechanistic details and roles of these components in MDM mitochondrial dynamics and antimicrobial responses during *S. pneumoniae* and *S. aureus* infections are unknown. I have demonstrated that Drp1 is the more prominent factor in mediating mitochondrial fission, as shown by significant mitochondrial hyperfusion in the absence of Drp1, while significance was not observed in the absence of Mff. This result was not very surprising as Drp1 possesses the functionality to oligomerise and facilitate fission via Drp1 constriction, while Mff acts as just one receptor involved in recruiting Drp1 to mitochondrial membranes. Therefore, even in the absence of Mff, Drp1 could still facilitate fission via interactions with another receptor such as Fis1 (544). This emphasises the critical importance of Drp1 in the regulation of mitochondrial

dynamics in MDM. However, to build a more comprehensive understanding of the mechanism of Drp1-mediated fission in MDM, it would be interesting to also investigate the role of Fis1 via siRNA knockdown. This would provide more insight into which receptor is most important for Drp1 recruitment and fission activity in MDM.

In the context of bacterial exposure, I have shown that Mff and Drp1 may play a role in mediating changes in mitochondrial morphology following 10 hour *S. pneumoniae* challenge of MDM, however it should be noted that high levels of fission are not typically expected at this early 10 hour time point when increased fission is only starting to be observed, and the results were not statistically significant. Furthermore, the network complexity results, which represent the population-wide results, suggested that there was no significant change in mitochondrial morphology under these conditions, while the individual cell images showed clear changes in mitochondrial morphology. Therefore, Mff and Drp1 may play a role in regulating *S. pneumoniae*-induced mitochondrial fission in individual cells, but further study into the population effects would be beneficial. In addition, exploration of later time points when enhanced fission typically occurs would be required to confirm these observations. Interestingly, my data shows that the absence of Mff and Drp1 did not prevent mitochondrial fission induced following 4 hour *S. aureus* challenge, therefore these factors do not contribute to early-stage *S. aureus*-induced fission at a global level. However, when mROS production was investigated, there was a suggestion that Drp1 might be involved in modulating mROS production in response to *S. aureus* at a global level, as mROS production was modestly reduced in the absence of Drp1, relative to control or Mff knockdown. It is noteworthy there was not clear

evidence of induction of mROS in association with the loss of mitochondrial complexity after *S. aureus* challenge, so it is possible that the loss of complexity did not reflect fission leading to enhanced mROS but rather an alternative process such as release of mitochondrial-derived vesicles, as has recently been described following *S. aureus* exposure (407). This could lead to failure to induce mROS globally but rather a shift in localisation. This warranted further investigation as it was a very surprising result. The mitochondrial fission and mROS fluorescence results corresponded to the global cell population effects, however this meant that potentially more subtle, individual cell effects would be masked. Therefore, it was possible that the small reduction in mROS production in *S. aureus*-challenged MDM in the absence of Drp1 reflected more localised effects in individual cells. In accordance with this, I have shown that Drp1 plays an important role in the localisation of mROS to intracellular *S. aureus*, as shown by significantly reduced mROS signal associated with intracellular bacteria in the absence of Drp1. This suggests that the roles of Drp1 in MDM during infection extend beyond facilitating mitochondrial fission, to also regulating the correct localisation of mROS to intracellular *S. aureus*.

Finally, I have also determined a possible role of Mff in enhancing MDM killing of intracellular later-stage *S. pneumoniae* and Drp1 on early-stage *S. aureus*, with evidence of enhanced *S. aureus* killing trending toward significance. While results suggest there may be differences on process by micro-organisms overall, they suggest that altering mitochondrial fragmentation and potentially also localisation may impact direct intracellular killing. In regard to early stage *S. aureus*, the combination of reduced mROS localisation and MDM killing in Drp1-siRNA treated

MDM suggests that promoting mROS localisation to intracellular bacteria is a mechanism by which Drp1 may enhance MDM killing of *S. aureus*, as mROS is known to possess microbicidal capacity against *S. aureus* (407). This therefore emphasises the importance of regulating mitochondrial dynamics, mROS production and localisation in modulating macrophage antimicrobial responses and enhancing bacterial killing. mROS production in siRNA-treated MDM following *S. pneumoniae* challenge was not investigated here, therefore this would be beneficial in order to identify if macrophages employ a similar Drp1-associated mechanism to enhance killing of *S. pneumoniae*. It is, however, an ongoing focus in the research group.

To summarise, this chapter details the contrasting influences of *S. pneumoniae* and *S. aureus* infection on changes in mitochondrial dynamics, the role of cathelicidin in these contexts, and the roles of the key mitochondrial fission components Mff and Drp1 in modulating mitochondrial morphology, mitochondrial functions, and MDM killing during *S. pneumoniae* and *S. aureus* infections. I have shown that mitochondrial fission occurs in response to both *S. pneumoniae* and *S. aureus* challenge of MDM, but this response occurs much earlier in response to *S. aureus* than *S. pneumoniae*, demonstrating that macrophages adapt their responses to pathogens depending on the organism and context. Cathelicidin exerted contrasting effects on mitochondrial morphology at different stages of *S. pneumoniae* infection but had no effect on *S. aureus*-induced mitochondrial fission, which also suggests that the consequences of cathelicidin and mitochondrial interactions in MDM in response to infection are dynamic and context-dependent. They may also be influenced by the extracellular impacts on both micro-organisms, which I have shown differ by micro-organism, and could alter intracellular burden. Mechanistically,

Mff and Drp1 play a role in mediating *S. pneumoniae*-induced fission, but not *S. aureus*-induced fission, at a global level. Furthermore, I have shown that on a more localised, individual cell level, there is a role for Drp1 in promoting mROS localisation to intracellular *S. aureus*, as a potential mechanism for enhancing MDM killing of *S. aureus*. Both Mff and Drp1 may therefore play roles in promoting MDM killing of these bacteria, but with contributions differing by context.

The main limitations of these studies are similar to those outlined in chapter three. Many of the group sizes are fairly small and would benefit from increased numbers, and it would be very interesting to investigate changes in mitochondrial morphology during infection in a more physiologically relevant model such as tissue-resident alveolar macrophages. It would also be beneficial to expand on these findings and build a more detailed picture of the kinetics of mitochondrial dynamics during infection by exploring more time points of infection, or by conducting live cell confocal microscopy studies to track changes in mitochondrial dynamics in real time. Nevertheless, I have shown that macrophages modulate mitochondrial dynamics during bacterial infection, and mitochondrial fission components, in particular Drp1, play important roles in mROS localisation and MDM killing of bacteria, in particular *S. aureus*. This is a very interesting field of study and continued research in this area would be extremely beneficial for further understanding the roles of mitochondrial dynamics in macrophage antimicrobial responses during infection.

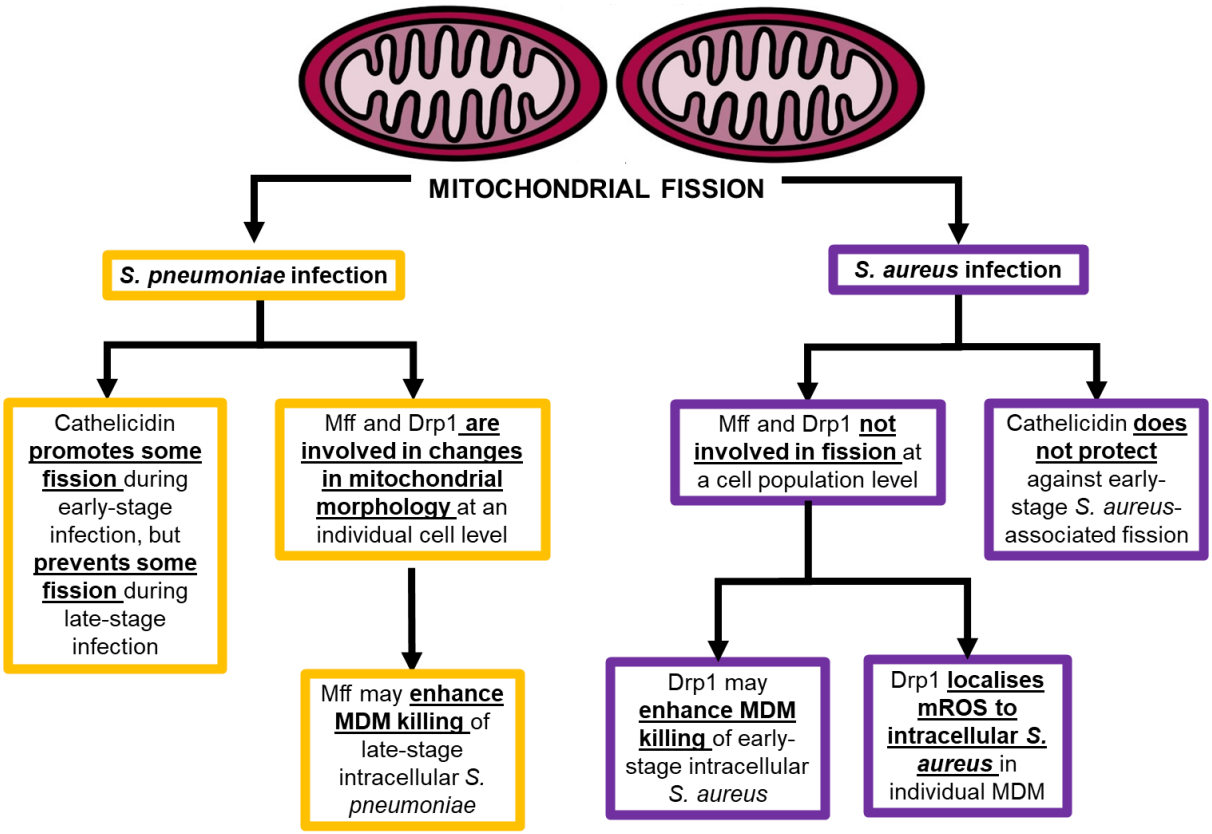


Figure 4.17: Schematic representation of the alterations in mitochondrial dynamics in human MDM in response to *S. pneumoniae* or *S. aureus* infection, the influence of cathelicidin on mitochondrial morphology, and the roles of Mff and Drp1 in bacterial-induced fission, mROS localisation and intracellular bacterial killing.

Chapter 5

Mitochondrial ROS production and mitochondrial functions in macrophage antimicrobial responses to *Streptococcus pneumoniae* and *Staphylococcus aureus*

5.1 Introduction

Mitochondrial ROS (mROS) production occurs during oxidative phosphorylation and at low levels, contributes to maintenance of cellular homeostasis (319,320). However, mROS production can be enhanced by a number of factors including bacterial infection and in the case of immune cells such as macrophages, this increase in mROS production is important for enhancing pro-inflammatory responses and pathogen clearance (309,322). Studies using the electron transport chain Complex I inhibitor rotenone have shown that mROS production can occur by different mechanisms in different contexts, the most prevalent of which are traditional forward electron flow from Complex I to Complex IV of the electron transport chain, and RET, where electrons flow in reverse to generate high levels of mROS at Complex I (329). Rotenone was demonstrated to have contrasting effects on mROS production depending on the mechanism; it inhibits mROS production if RET is occurring (329) but enhances mROS production if forward electron flow is occurring (545). RET has been described using an LPS-activated BMDM model of mROS production (329), but the Dockrell group has previously shown that rotenone treatment of healthy and COPD alveolar macrophages enhances mROS-dependant *S. pneumoniae* killing, suggesting mROS production can also occur via forward electron flow during bacterial infection (403).

However, the mechanisms of mROS production in primary human MDM in response to *S. pneumoniae* or *S. aureus* are not clear. Therefore, I applied rotenone treatment to my MDM with different time points of bacterial challenge to investigate potential mechanisms of mROS production. Furthermore, although mROS production increases in response to bacterial infection and is important for macrophage microbicidal responses, and I have shown that early-stage *S. aureus* induces enhanced mitochondrial fission (see Chapter 4), the influence of early-stage *S. aureus* challenge on mROS production in primary human MDM is not well established. Therefore, I also investigated this via mROS staining and microscopy.

mROS production is critical in later macrophage responses to infection, but the primary early source of ROS in macrophages is the NADPH oxidase system (204,281). NADPH oxidase can generate an oxidative burst following recruitment to phagosomes to kill intracellular pathogens, as well as modulate other macrophage pro-inflammatory responses (239,283–285). However, due to limitations on timing relative to other microbicidals, localisation and potency of macrophage ROS, they may be insufficient to clear pathogens alone, therefore may combine with other microbicidal strategies to efficiently clear pathogens (291,292). For example, superoxide been shown to interact with NO in phagosomes to produce the more potent and highly microbicidal ROS peroxynitrite (281). It is well established that NADPH oxidase-derived ROS is critical for clearance of certain bacteria, including *S. aureus*, as chronic granulomatous disease (CGD) patients, who have defective NADPH oxidase machinery and cannot generate ROS, are more susceptible to bacterial and fungal diseases with *S. aureus* being one of the most prevalent pathogens (546). However, details on the contribution of mROS to intracellular

killing of early-stage *S. aureus* in primary human MDM, particularly alongside NADPH oxidase-derived ROS, remain unclear. Therefore, by taking advantage of compounds that modulate ROS production from these sources, I investigated the influence of mROS and NADPH oxidase-derived ROS on *S. aureus* killing in my MDM model.

Changes in ROS production are evidently important for promoting efficient antimicrobial host defence responses in macrophages. However, as emphasised throughout this thesis, a multi-layered macrophage immune response with combinations of responses is required for optimal macrophage antimicrobial function and pathogen clearance. Cathelicidin has been shown to enhance ROS production in THP-1 cells, and a synergy between cathelicidin and ROS was required for enhanced intracellular bacterial killing (505). Furthermore, cathelicidin has also been shown to promote ROS production, bacterial killing and enhanced antimicrobial responses in neutrophils (547,548). However, interactions between cathelicidin and mROS production on bacterial killing in primary human MDM have not been established. Therefore, I investigated if exogenous cathelicidin treatment altered mROS production in MDM in the presence and absence of *S. aureus*, and if cathelicidin influenced MDM killing of intracellular *S. aureus* following modulation of both NADPH oxidase-derived ROS and mROS production.

To provide another example, sustained intracellular bacterial persistence and reactive species can induce LMP and the activation of the cathepsin family of proteases (110). In particular, cathepsins B and D have been shown to enhance mROS production in isolated purified mitochondria (549). The Dockrell group has previously shown that cathepsin D is required to initiate macrophage apoptosis and

promote clearance of intracellular *S. pneumoniae*, as demonstrated by reduced bacterial clearance and increased apoptosis resistance in murine BMDM lacking cathepsin D (272). However, the roles of cathepsin B on mROS production and *S. pneumoniae* clearance in macrophages are yet to be characterised and since it does not impact apoptosis in MDM challenged with *S. pneumoniae*, I used it to explore relationships with mROS upstream of apoptosis (272). Therefore, I sought to determine the influence of cathepsin B on the regulation of mROS production and intracellular bacterial killing in BMDM during the later stages of *S. pneumoniae* challenge.

Enhanced mROS production is one of the primary mitochondrial adaptations to infection, and a key mechanism for this in pro-inflammatory macrophages is a metabolic shift from oxidative phosphorylation to glycolysis for cellular energy generation (198,323). This allows for rapid ATP generation via glycolysis and a switch in mitochondrial function from energy generation to enhanced mROS production during infection (325–327). Another functional consequence of mitochondrial adaptation in pro-inflammatory macrophages in response to bacterial challenge is a loss of $\Delta\psi_m$ (366). This occurs in response to factors including bacterial exposure or mitochondrial dysfunction and is associated with apoptosis; the Dockrell group has shown previously that a loss of $\Delta\psi_m$ occurs in macrophages during the later stages of *S. pneumoniae* challenge and in association with apoptosis-associated killing (110,306), However, it is possible that subtle changes in $\Delta\psi_m$ could occur earlier during bacterial challenge, for example as a result of hyperpolarisation, and these could be associated with altered mROS production (550,551), but this is yet to be established. Therefore, I confirmed the loss of $\Delta\psi_m$

during late-stage *S. pneumoniae* challenge and investigated the influence of earlier *S. pneumoniae* challenge on $\Delta\psi_m$ by flow cytometry. Furthermore, although *S. aureus* does not induce apoptosis-associated killing (72), the details of early-stage *S. aureus* challenge on $\Delta\psi_m$ have not been confirmed; therefore, I also investigated this via flow cytometry.

5.2 Results

5.2.1 mROS production in MDM can be modulated by MitoPQ and MitoQ treatment

Firstly, I aimed to determine if I could modulate mROS production with chemical compounds in my MDM model. I used the mitochondrial redox cycler MitoPQ (321) and the mROS scavenger MitoQ (511), to induce and inhibit mROS, respectively. MDM were mock treated, MitoPQ treated for 1 hour, or MitoQ treated for 1 hour followed by another hour of MitoPQ treatment. MDM were stained with MitoSOX and fixed, visualised by confocal microscopy, then MitoSOX fluorescence intensity scores were calculated.

As shown in Figure 5.1A, mock-treated MDM display a low level of baseline MitoSOX fluorescence which becomes dramatically brighter following MitoPQ treatment, showing that MitoPQ can induce mROS production in my MDM. The addition of MitoQ reduces the fluorescence intensity induced by MitoPQ, showing that MitoQ is capable of inhibiting some of the MitoPQ-induced mROS production. These observations are confirmed by the fluorescence intensity scores (Figure 5.1B). MitoPQ-treated MDM exhibited significantly higher fluorescence than mock-treated MDM, and this MitoPQ-induced fluorescence was reduced by the addition of

MitoQ. In fact, the contribution on mitochondrial staining was greater than the score suggests since there was still some nuclear staining, as expected, because of the known capacity of MitoSOX to intercalate with nuclear DNA (552). This shows that chemical compounds such as MitoPQ and MitoQ are able to modulate mROS production in my MDM, which would be useful in further studies investigating the influence of ROS production on other macrophage antimicrobial responses, such as intracellular bacterial killing.

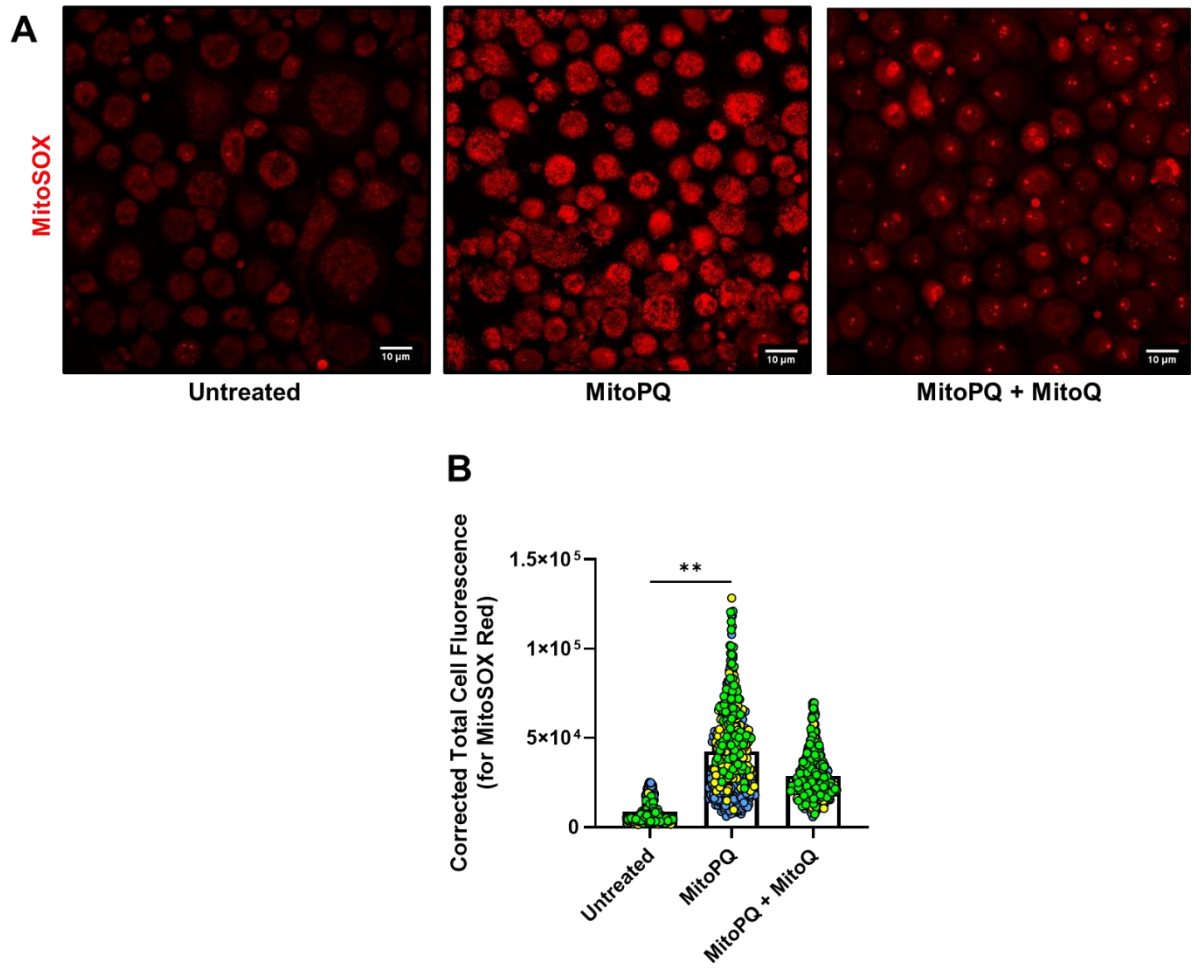


Figure 5.1: Influence of MitoPQ and MitoQ treatment on mROS production in MDM. A) Example images of MitoSOX Red fluorescence in untreated MDM, MitoPQ-treated MDM after 1 hour stimulation, and MDM treated with MitoQ for 1 hour followed by MitoPQ for 1 hour. Images taken on the Leica SP8 at 40x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red from MDM in each treatment condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. **p<0.01.

5.2.2 Rotenone treatment does not influence late-stage *S. pneumoniae*-induced mROS production

In order to investigate the mechanisms of mROS production in MDM during bacterial infection, I took advantage of rotenone, and the contrasting effects it has been reported to have on mROS production depending on mechanism. I began by investigating the effect of rotenone on mROS production during late-stage *S. pneumoniae* challenge. MDM were treated with rotenone prior to 24 hour LPS stimulation or 14 hour *S. pneumoniae* challenge. It has previously been shown that rotenone inhibits mROS production in LPS-stimulated macrophages (329), therefore LPS was used as a control and comparator to bacteria in these experiments. MDM were MitoSOX stained, visualised, and analysed as before.

As shown by both the example images and fluorescence intensity scores in Figure 5.2, rotenone treatment induced mROS production in mock-infected MDM, which was the expected outcome for rotenone treatment of MDM in steady-state conditions. 24 hour LPS stimulation significantly enhanced mROS production, while rotenone reduced LPS-induced mROS production as expected. 14 hour *S. pneumoniae* challenge also significantly enhanced mROS production, however rotenone treatment did not alter this. These results suggest that during the later stages of *S. pneumoniae* challenge of MDM, mROS production is not occurring via RET. However, it was important to consider that 14 hours is a late time point for *S. pneumoniae* challenge and could be too late to see changes if rotenone effects occur at an earlier stage. Therefore, it was necessary to investigate an earlier time point of *S. pneumoniae* challenge to probe this further.

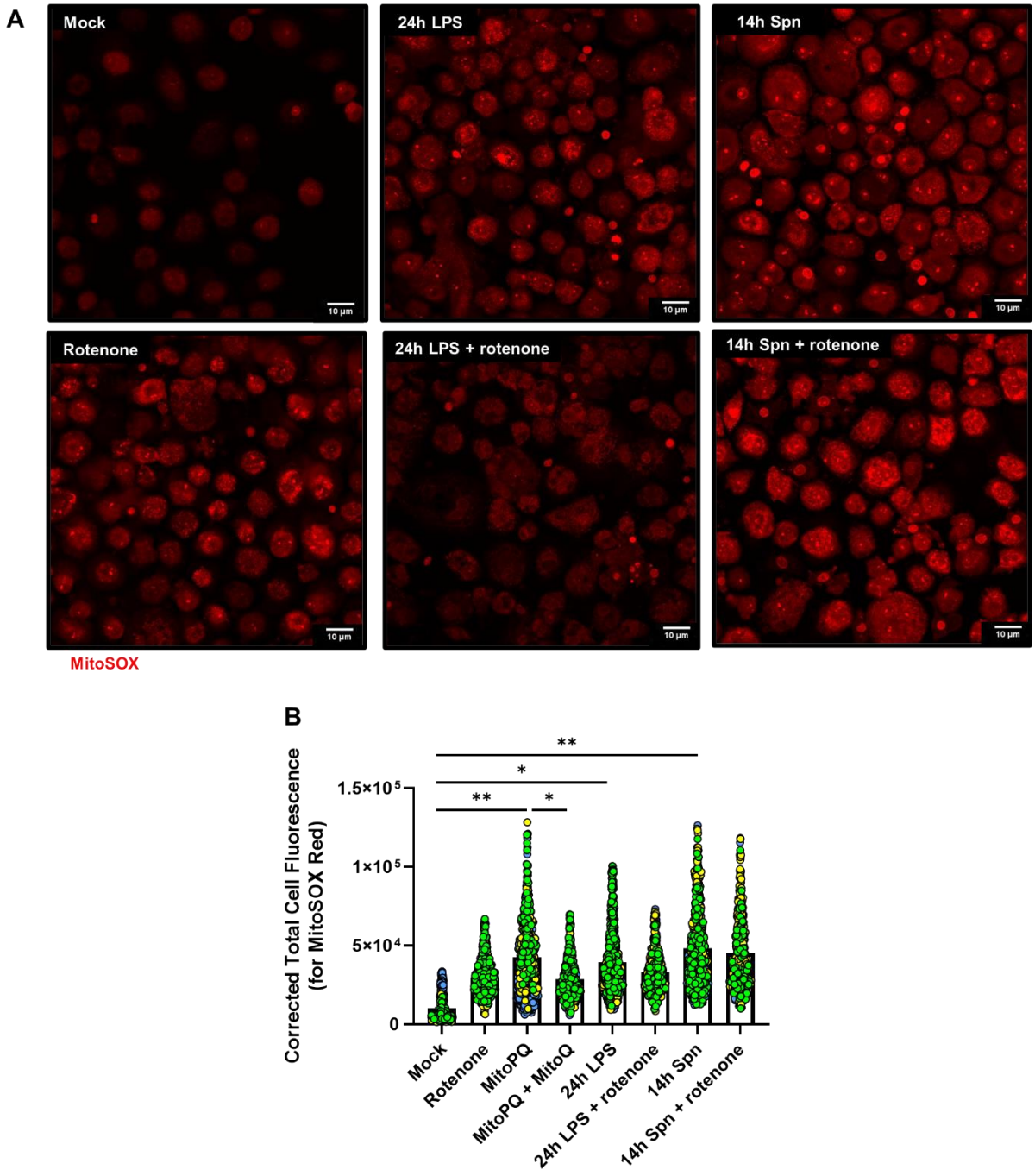


Figure 5.2: Influence of rotenone on mROS production in MDM following late-stage LPS treatment or *S. pneumoniae* challenge. A) Example images of MitoSOX Red fluorescence in MDM following mock treatment, 24 hour LPS treatment, or *S. pneumoniae* challenge (MOI=10 for 14 hours), with or without rotenone treatment. Images taken on the Leica SP8 at 40x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red from MDM in each treatment condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, **p<0.01.

5.2.3 Rotenone treatment reduces earlier-stage *S. pneumoniae*-induced mROS production

Next, I wanted to investigate the effect of rotenone earlier during *S. pneumoniae* challenge to determine if rotenone's influence on mROS production was similar at earlier time points to that I observed after 14 hours *S. pneumoniae* challenge. MDM were treated with rotenone, then stimulated with LPS or challenged with *S. pneumoniae* for 8 hours. MitoSOX staining, visualisation and analysis were then conducted. To enable a more similar comparison of results, LPS stimulation was also applied for 8 hours in these experiments.

As shown by the example images (Figure 5.3A) and fluorescence intensity scores (Figure 5.3B), rotenone treatment alone enhanced mROS production as observed previously. LPS stimulation did not alter mROS production compared to mock-infected MDM at this time point, but rotenone treatment significantly enhanced mROS production when combined with LPS treatment. However, these results need to be interpreted with caution since there was minimal induction of mROS by LPS at this time, so the predominant effect was induction of mROS by rotenone as in the unstimulated MDM. 8 hour *S. pneumoniae* challenge significantly enhanced mROS production and rotenone treatment resulted in inhibition of *S. pneumoniae*-induced mROS production, suggesting that RET may be the mechanism for mROS production at this time. This also suggests that the mROS production mechanism early after bacterial challenge may involve RET but changes over the course of bacterial challenge and is predominantly via forward electron flow at later stages.

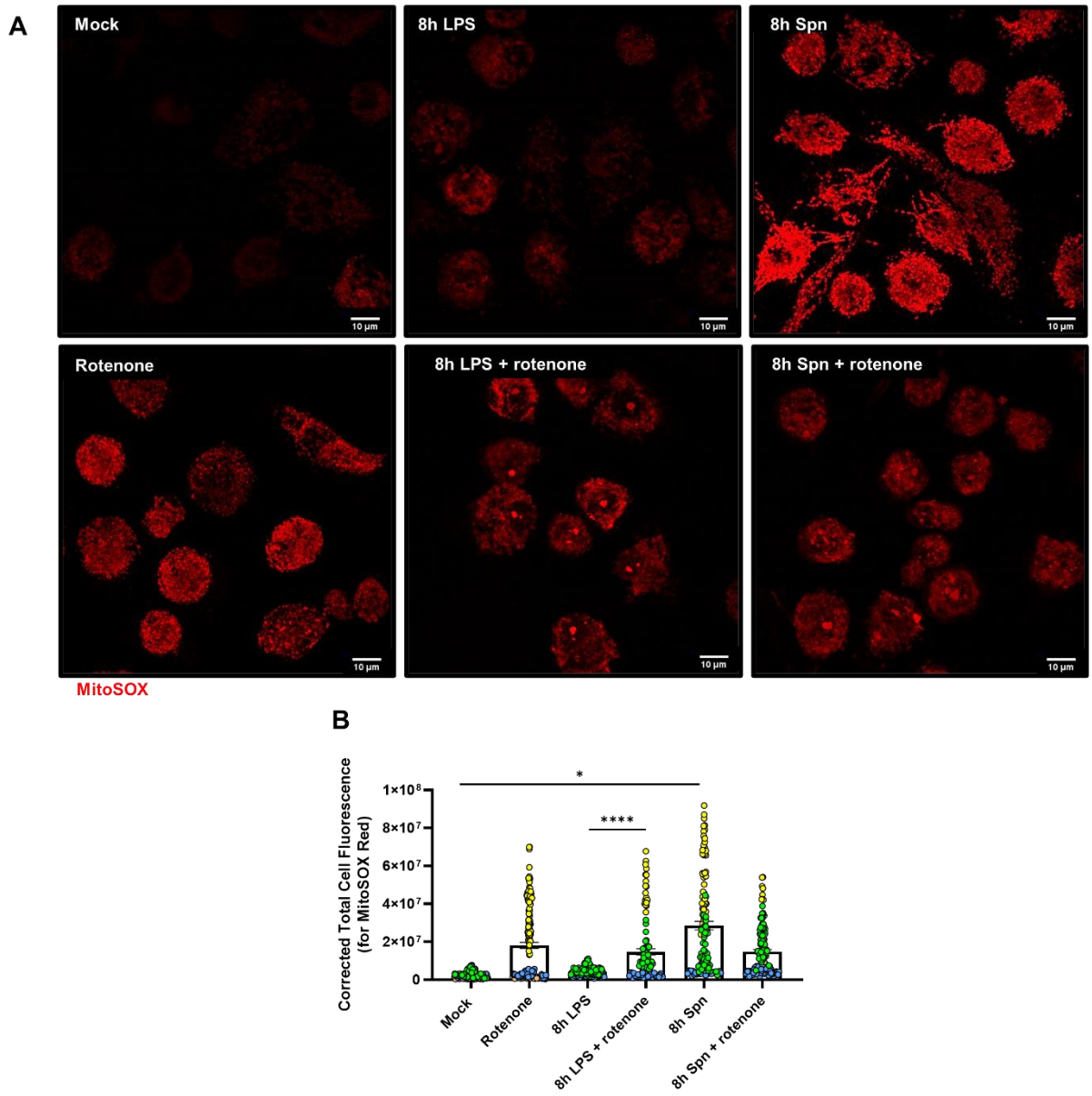


Figure 5.3: Influence of rotenone on mROS production in MDM following early-stage LPS treatment or *S. pneumoniae* challenge. A) Example images of MitoSOX Red fluorescence in MDM following mock treatment, 8 hour LPS treatment, or *S. pneumoniae* challenge (MOI=10 for 8 hours), with or without rotenone treatment. Images taken on the Andor spinning disk at 60x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red from MDM in each treatment condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, ****p<0.0001.

5.2.4 Rotenone treatment does not influence early-stage *S. aureus*-induced mROS production

After establishing the effect of rotenone on mROS production during *S. pneumoniae* challenge, I investigated its effects during *S. aureus* challenge. As I have shown evidence of early-stage *S. aureus* challenge inducing other mitochondrial adaptations, such as enhanced mitochondrial fission, 4 and 8 hour *S. aureus* challenge time points were chosen. As for the *S. pneumoniae* experiments, MDM were treated with rotenone and stimulated with LPS for 8 hours or challenged with *S. aureus* for 4 or 8 hours. MDM were then MitoSOX, stained, visualised, and analysed as before.

As shown by the example images (Figure 5.4A) and fluorescence intensity scores (Figure 5.4B), rotenone treatment alone enhanced mROS production as before. 8h LPS stimulation did not alter mROS production as before, but the addition of rotenone again increased mROS production. Both 4 and 8 hour *S. aureus* challenges resulted in enhanced mROS production, suggesting that in addition to enhanced mitochondrial fission at these early time points, mROS production is also enhanced in response to *S. aureus* challenge. However, rotenone treatment did not alter mROS production induced by *S. aureus* at either time point, suggesting that RET is not the mechanism for mROS production at this time. Further studies to investigate the effect of rotenone at earlier *S. aureus* challenge time points would be beneficial to gain more insight into the mechanism behind early-stage *S. aureus*-induced mROS production, and determine if the mechanism involves RET.

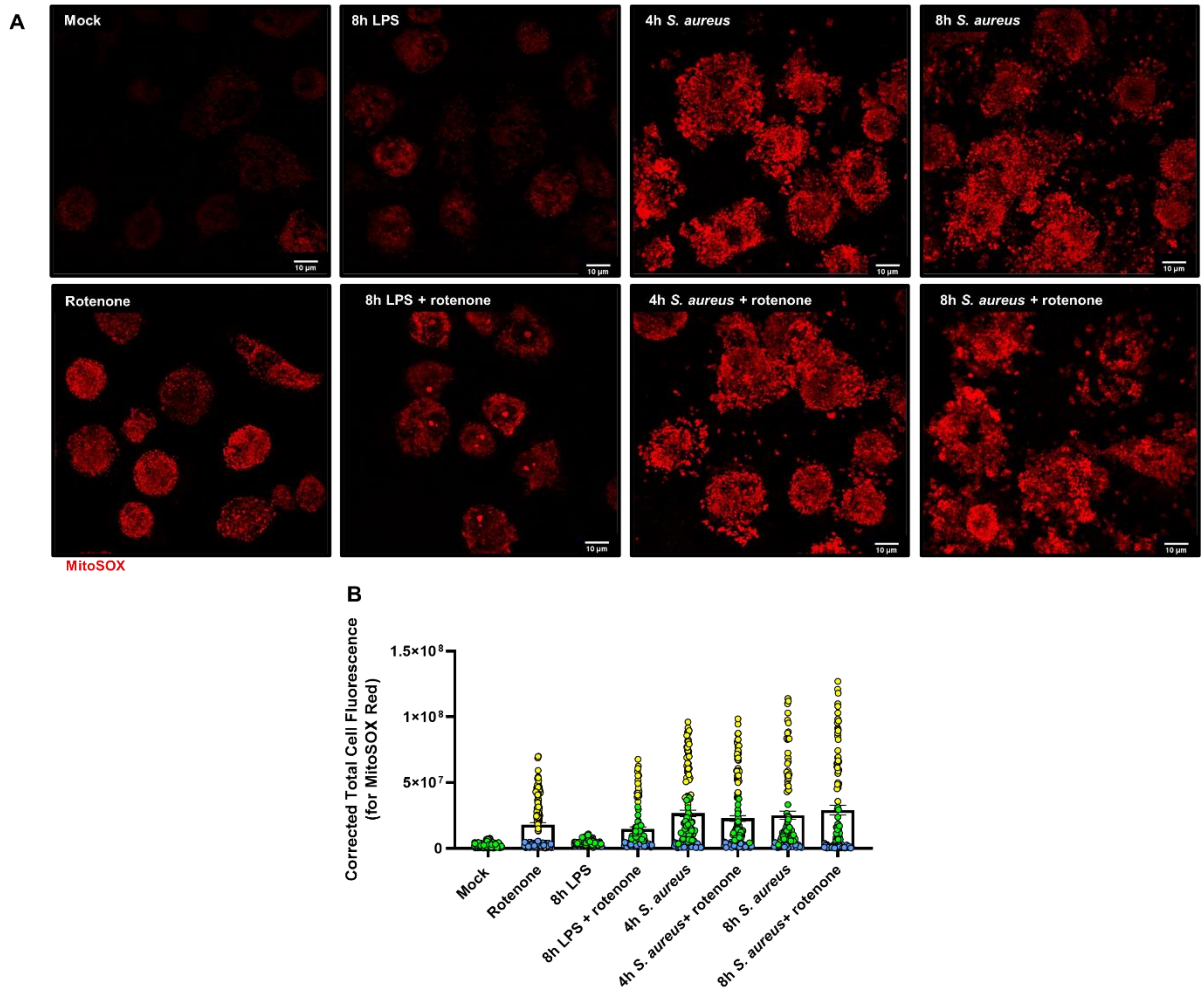


Figure 5.4: Influence of rotenone on mROS production in MDM following early-stage LPS treatment or *S. pneumoniae* challenge. A) Example images of MitoSOX Red fluorescence in MDM following mock treatment, 8 hour LPS treatment, or *S. aureus* challenge (MOI=10 for 4 or 8 hours), with or without rotenone treatment. Images taken on the Andor spinning disk at 60x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red in MDM in each condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

5.2.5 Early-stage *S. aureus* may enhance mROS production in MDM, and this is not influenced by mROS modulation

After establishing mROS production's relationship to RET in MDM in response to bacteria, I was next interested to investigate how early-stage *S. aureus* challenge influences mROS production in MDM. The focus was on *S. aureus* as *S. aureus* imposes a higher bacterial burden than *S. pneumoniae* and my previous results showed that early-stage *S. aureus* enhances mitochondrial fission at early time points. I determined whether mROS production following *S. aureus* challenge could be further modulated by compounds known to manipulate mROS. Modulation of mROS was carried out using MitoPQ to induce mROS production and MitoQ to inhibit mROS production; these will be referred to as mROS modulator compounds. Again, *S, aureus* was the focus rather than *S. pneumoniae* as the group has already demonstrated that *S. pneumoniae*-induced mROS production can be modulated via inhibition by MitoTEMPO (387,553) (see also Figure 9A of this chapter). In addition, as mentioned previously, other studies have also demonstrated that LPS-induced mROS signals can be influenced by compound modulation (329). However, much less is known about the influence of *S. aureus.* MDM were treated with one of these compounds for an hour prior to 4 hour mock infection or *S. aureus* challenge. MDM were stained with MitoSOX, visualised, and analysed as before.

As shown by the example images (Figure 5.5A) and fluorescence intensity scores (Figure 5.5B), inhibition of mROS production by MitoQ resulted in a reduction in MitoSOX fluorescence intensity compared to mock-treated MDM, suggesting that MitoQ treatment inhibited mROS production as expected. However, while MitoPQ treatment was expected to induce mROS production, significant induction was not

observed under these conditions despite MitoPQ induction of mROS in MDM being observed previously (Figure 5.1). It is possible that in this case, the timing of visualising mROS production was not optimal to observe the MitoPQ induction effect. These MDM were visualised following 4 hour mock infection or *S. aureus* challenge, in contrast to visualisation after 1 hour as shown in Figure 5.1, therefore if MitoPQ has a more rapid but transient effect, this 4 hour time point may be too late to visualise a significant effect. Nevertheless, in response to *S. aureus* challenge, an increase in MitoSOX fluorescence intensity was observed for all conditions compared to the mock-infected counterparts, with significance observed in MitoQ-treated MDM, and this *S. aureus*-induced increase in fluorescence was similar across all conditions. This casts doubts on the cause of the MitoSOX fluorescence since for both LPS and *S. pneumoniae*, the MitoSOX fluorescence is reduced by mROS inhibition. Further experiments would be required to confirm whether this result was due to failure to optimise the MitoQ inhibition or reflected a cause of fluorescence other than specific mROS generation.

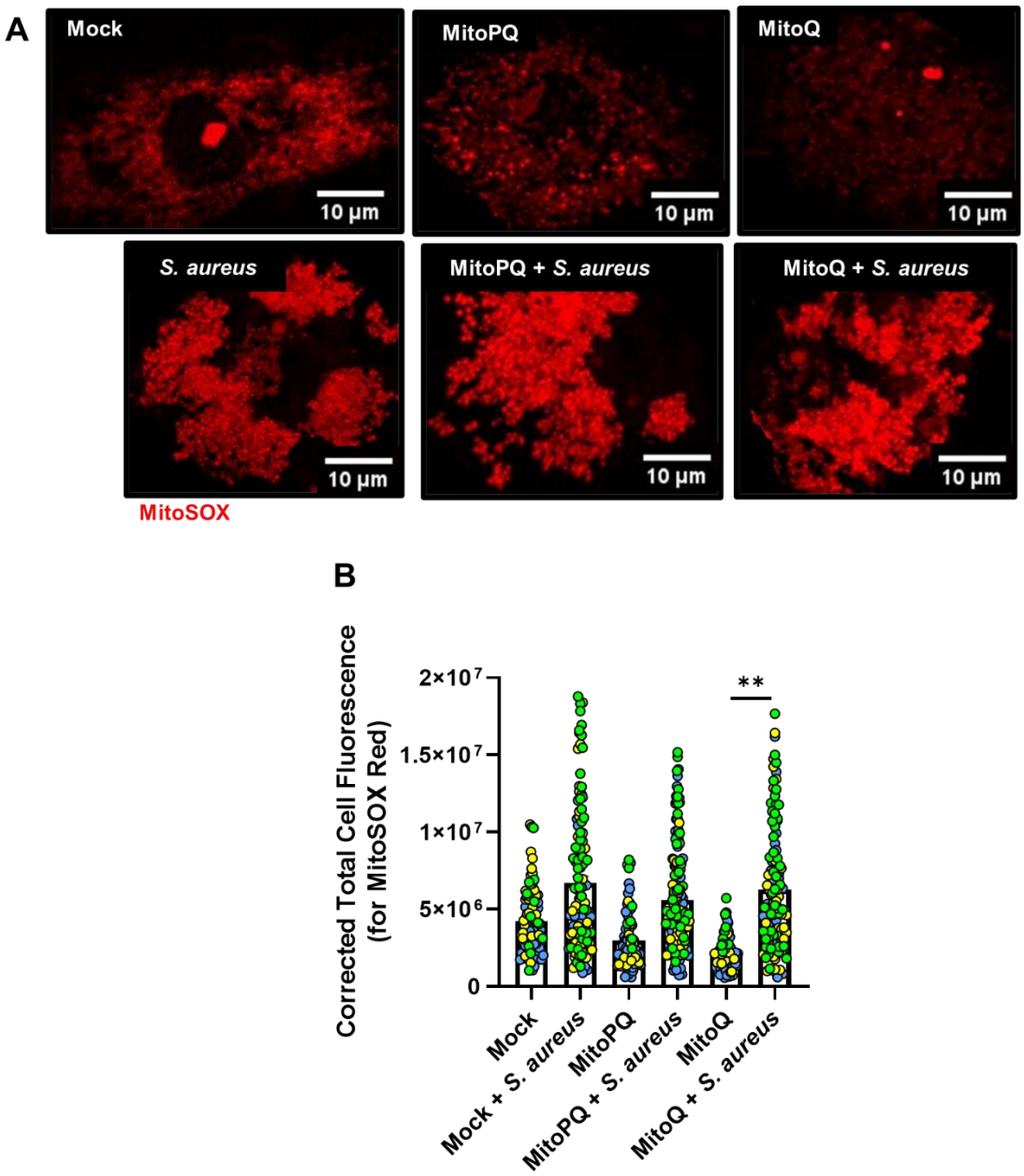


Figure 5.5: MitoSOX fluorescence in MDM treated with MitoPQ or MitoQ, with or without early-stage *S. aureus* challenge. A) Example images of MitoSOX Red fluorescence in mock-infected and *S. aureus*-challenged MDM (MOI=10 for 4 hours) following treatment with MitoPQ or MitoQ for 1 hour. Images taken on the Andor spinning disk at 60x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red in MDM in each condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. **p<0.01.

5.2.6 Cathelicidin enhances MitoSOX fluorescence in the absence of *S. aureus*, but may reduce MitoSOX fluorescence in the presence of *S. aureus*

Following on from the influence of *S. aureus* challenge on MitoSOX fluorescence, acknowledging that I could not be completely sure the signal after *S. aureus* was due to mROS production *per se*, my next step was to investigate the role of cathelicidin on both steady state and *S. aureus* challenge-associated signal to determine if cathelicidin altered this. The focus was still on *S. aureus* as my previous results indicated that cathelicidin may contribute to MDM killing of early-stage intracellular *S. aureus*, but not *S. pneumoniae*, therefore cathelicidin-mediated changes in mROS production was one possible mechanism for this *S. aureus* killing effect to explore further. MDM were mock infected or challenged with *S. aureus* for 4 hours as before, then treated with exogenous cathelicidin for 1 hour. MDM were stained with MitoSOX, visualised, and analysed as before.

As demonstrated by the example images (Figure 5.6A) and fluorescence intensity scores (Figure 5.6B), in steady state conditions, cathelicidin treatment resulted in an increase in MitoSOX fluorescence. This suggests that in the absence of bacteria, cathelicidin can enhance MitoSOX fluorescence in MDM. Following bacterial challenge, cathelicidin treatment resulted in a reduction in MitoSOX fluorescence in MDM following *S. aureus* challenge which suggests that in the presence of *S. aureus*, cathelicidin may act to reduce *S. aureus*-induced mROS production. The lack of significant changes in MitoSOX fluorescence in *S. aureus*-challenged MDM with cathelicidin compared to mock-infected MDM with cathelicidin also suggests that cathelicidin may serve as a brake on increasing mROS in

response to *S. aureus* in order to regulate mROS production and prevent excessive inflammatory responses induced by high levels of mROS. Interpretation is however, tempered by the fact that I have not specifically inhibited the *S. aureus* associated MitoSOX fluorescence with mROS inhibitors, and by the small sample size and individual cell variability.

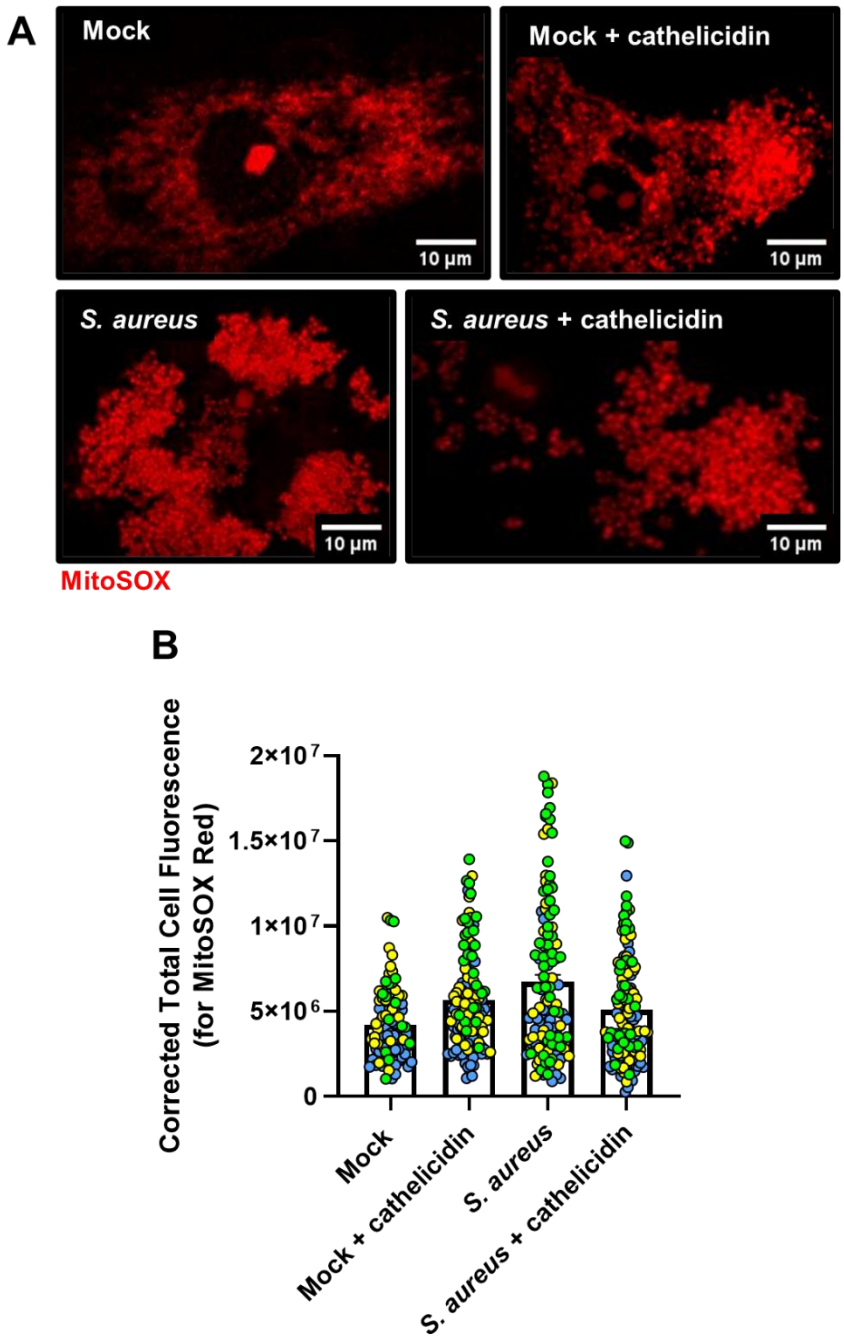


Figure 5.6: Cathelicidin's influence on MitoSOX fluorescence production in MDM in the presence and absence of *S. aureus*. A) Example images of MitoSOX Red fluorescence in MDM following mock infection or *S. aureus* challenge (MOI=10 for 4 hours), followed by exogenous cathelicidin treatment. Images taken on the Andor spinning disk at 60x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red in MDM in each condition. Each point represents a single MDM, and each colour represents one donor. White bars represent the mean mitochondrial network complexity score. Error bars represent the SEM. n = 3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out.

5.2.7 NADPH oxidase-derived ROS and mROS contribute to MDM killing of early-stage intracellular *S. aureus*

To explore the microbicidal consequences of any potential changes in mROS production, I investigated the influence of mROS production on MDM killing of intracellular *S. aureus*. In addition, I was also interested to investigate the influence of NADPH oxidase-derived ROS production in my model of MDM killing of *S. aureus*, as this source of ROS is an important early macrophage response to infection and has been shown to play an important role in the clearance of *S. aureus* (546). MDM were treated with MitoQ for 1 hour, with or without subsequent MitoPQ treatment for an additional hour as before or treated with the NADPH oxidase inhibitor DPI for 1 hour, prior to 4 hour *S. aureus* challenge. For simplicity, these three compounds will be collectively referred to as ROS modulating compounds in later results. Following antimicrobial treatment to kill remaining extracellular bacteria, the supernatant from the MDM culture prior to MDM lysis, and the lysate following MDM lysis, were diluted, and plated to calculate viable intracellular bacterial counts as before.

As shown in Figure 5.7, DPI treatment of MDM resulted in significantly higher viable *S. aureus* counts compared to bacteria-only MDM, suggesting that NADPH oxidase-derived ROS contributes significantly to macrophage killing of intracellular *S. aureus*, as expected. MitoPQ treatment did not result in a reduction in viable bacterial counts as expected; it is possible that pre-treatment of MDM for 1 hour prior to 4 hour bacterial challenge resulted in early production of mROS at a point where it was not required for initial killing of *S. aureus*. Therefore, further investigation into the optimal time for MitoPQ treatment to contribute to bacterial killing is required to develop this experimental model. However, inhibition of mROS production by MitoQ

treatment, with and without MitoPQ, also resulted in significantly higher viable *S. aureus* counts, albeit to a lesser extent than those observed with DPI treatment. This therefore suggests that although NADPH oxidase-derived ROS is the primary source of ROS contributing to macrophage killing of early-stage intracellular *S. aureus*, mROS also contributes to *S. aureus* killing at this point.

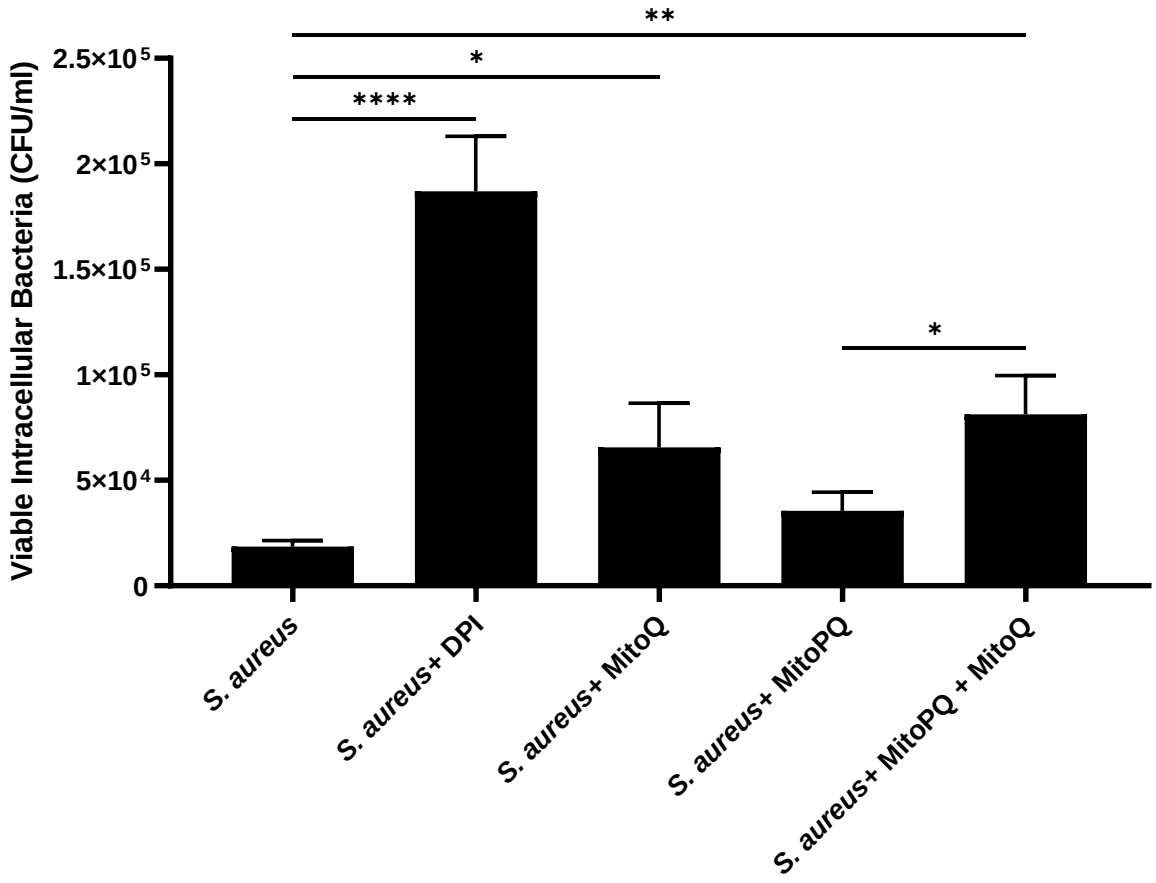


Figure 5.7: Influence of mROS and NADPH oxidase-derived ROS compound treatment on MDM killing of early-stage intracellular *S. aureus*. The mean viable counts obtained from MDM following mock treatment or ROS compound treatment, then mock-infected or challenged with *S. aureus* (MOI=10 for 4 hours). Assays carried out in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum). Error bars represent the SEM. n= 6 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, **p<0.01, ****p<0.0001.

5.2.8 Cathelicidin contributes to MDM killing of early-stage *S. aureus* when NADPH oxidase-derived ROS production is impaired

As the influence of NADPH oxidase-derived ROS and mROS on MDM killing of early-stage *S. aureus* had been studied, I next investigated the role of cathelicidin in this system to determine if cathelicidin altered mROS- or ROS-mediated bacterial killing and if there was any evidence of possible interactions between mROS, ROS or cathelicidin. MDM were treated with ROS modulating compounds, mock infected or challenged with *S. aureus* for 4 hours, then treated with exogenous cathelicidin as described before. Following antimicrobial treatment to kill remaining extracellular bacteria, the supernatant from the MDM culture prior to MDM lysis, and the lysate following MDM lysis, were diluted, and plated to calculate viable intracellular bacterial counts as before.

As demonstrated in Figure 5.8, exogenous cathelicidin treatment did not alter MDM killing of *S. aureus* when MDM were challenged with *S. aureus* only, or when MDM were also treated with MitoQ or MitoPQ. However, in MDM treated with both MitoPQ and MitoQ, there was a non-significant reduction in viable bacterial counts in the presence of cathelicidin. This suggests that cathelicidin may contribute to macrophage killing of *S. aureus* to a greater extent when mROS production is altered, but interpretation of this must be cautious as this effect was not observed with mROS induction or inhibition alone. Furthermore, the presence of cathelicidin in MDM with DPI treatment clearly resulted in a significant reduction of viable bacterial counts compared to MDM without cathelicidin treatment. This suggests that cathelicidin contributes to MDM killing of *S. aureus* when NADPH oxidase-derived ROS production, an important early killing mechanisms employed by macrophages,

is impaired. This demonstrates an example of a multi-layered, combinatory antimicrobial response in macrophages; early ROS production contributes significantly to bacterial killing but when this is suboptimal, cathelicidin can exert a greater killing effect to assist the clearance of early-stage *S. aureus.*

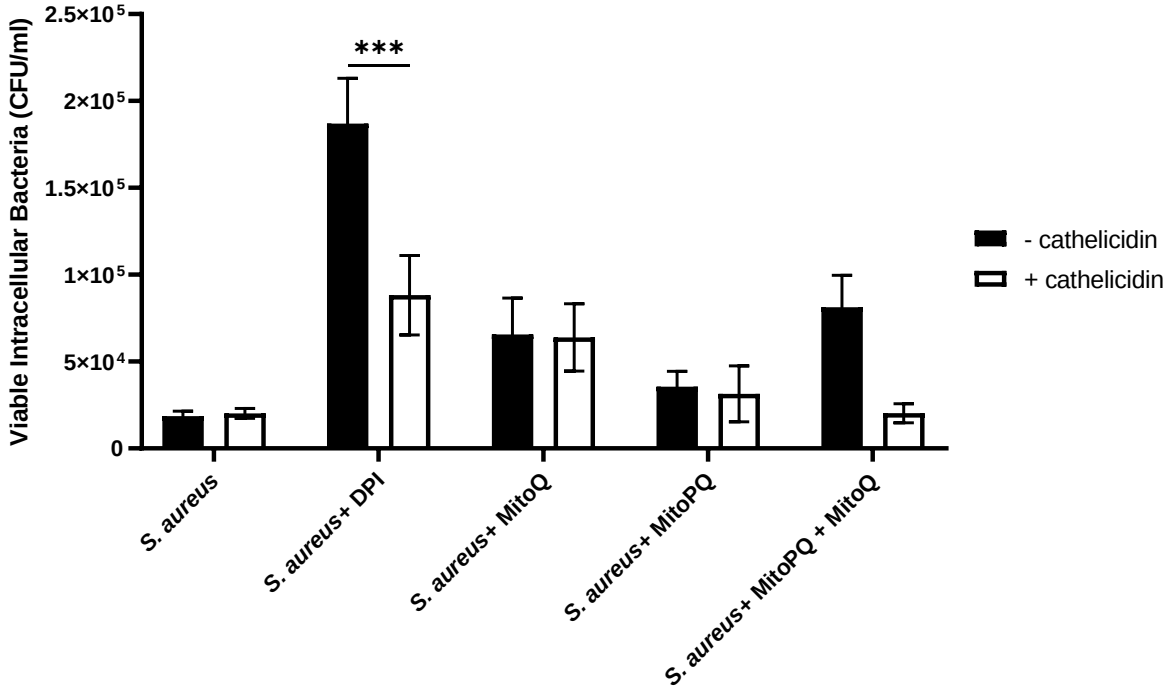


Figure 5.8: Influence of cathelicidin on ROS-mediated killing of early-stage intracellular *S. aureus*. The mean viable counts obtained from MDM following mock treatment or ROS compound treatment, then mock-infected or challenged with *S. aureus* (MOI=10 for 4 hours), then mock or cathelicidin treated for one hour. Assays carried out in serum-free media (RPMI 1640 + 2 millimolar L-glutamine). Error bars represent the SEM. n= 6 independent experiments. Two-way ANOVA with Sidak's multiple comparisons test carried out. *p<0.05, ***p<0.001, ****p<0.0001. Overall ANOVA result: ****ROS compound treatment, ***presence of cathelicidin, **interaction between ROS compound treatment and cathelicidin.

5.2.9 Cathepsin B contributes to mROS production and intracellular killing by murine BMDM during late-stage *S. pneumoniae* challenge

Cathelicidin is one example of an antimicrobial factor that can interact with other macrophage responses to enhance pathogen clearance, but lysosomal proteases represent a second example that have been shown to play roles in enhancing macrophage microbicidal responses, particularly against *S. pneumoniae*. As mentioned previously, the lysosomal proteases cathepsin D and B have been shown to enhance mROS production in isolated mitochondrial models (549), and cathepsin D is required to initiate macrophage apoptosis and promote clearance of intracellular *S. pneumoniae* (272). Therefore, to investigate the role of cathepsin B more in depth on mROS production and intracellular killing of late-stage *S. pneumoniae*, murine BMDM, cells were treated with the cathepsin B inhibitor CA074-me for 1 hour prior to *S. pneumoniae* challenge. Following 14 hour *S. pneumoniae* challenge, BMDM were MitoSOX stained, visualised, and analysed as before. To confirm that the signal detected was mROS, the mitochondrial-specific mROS inhibitor MitoTEMPO was included as a control. Intracellular bacterial killing assays was conducted for 12 and 16 hour *S. pneumoniae* challenge time points, following the killing assay protocol detailed previously. 12 hours represents a late-stage challenge time point prior to apoptosis, while 16 hours represents a time point associated with apoptosis as a late-stage bacterial killing mechanism. The following experiments were conducted with Dockrell group members Jennifer Marshall and Dr Clark Russell, and BMDM were used to complement data in our recent manuscript which focused on BMDM as the experimental cell type. The manuscript is currently available on the Bioarchive pre-print server (BioRxiv, doi: https://doi.org/10.1101/722603) (387).

mROS production was dramatically increased following 14 hour *S. pneumoniae* challenge of BMDM and was reduced with the addition of MitoTEMPO treatment as expected (Figure 5.9A). Inhibition of cathepsin B also resulted in a marked reduction in *S. pneumoniae*-induced mROS signal (Figure 9A). The MitoSOX fluorescence intensity scores calculated per condition are in accordance with the visual findings (Figure 5.9B); *S. pneumoniae* challenge significantly enhanced MitoSOX fluorescence intensity compared to mock infection, while both CA074-me and MitoTEMPO treatment significantly reduced *S. pneumoniae*-induced MitoSOX fluorescence intensity. This suggests that cathepsin B contributes to enhanced mROS production in macrophages in response to late-stage *S. pneumoniae* challenge. Inhibition of cathepsin B also resulted in an increase in viable intracellular bacterial following 12 and 16 hour *S. pneumoniae* challenge, with significance observed for 12 hours (Figure 5.9C). This suggests that cathepsin B also contributes to intracellular killing of late-stage *S. pneumoniae* in BMDM. Together, these results demonstrate that cathepsin B regulates mROS production and killing of late-stage *S. pneumoniae* in macrophages and further illustrates how different macrophage responses, in this case activation of cathepsin B and mROS production, contribute to enhanced macrophage killing of persistent intracellular bacteria.

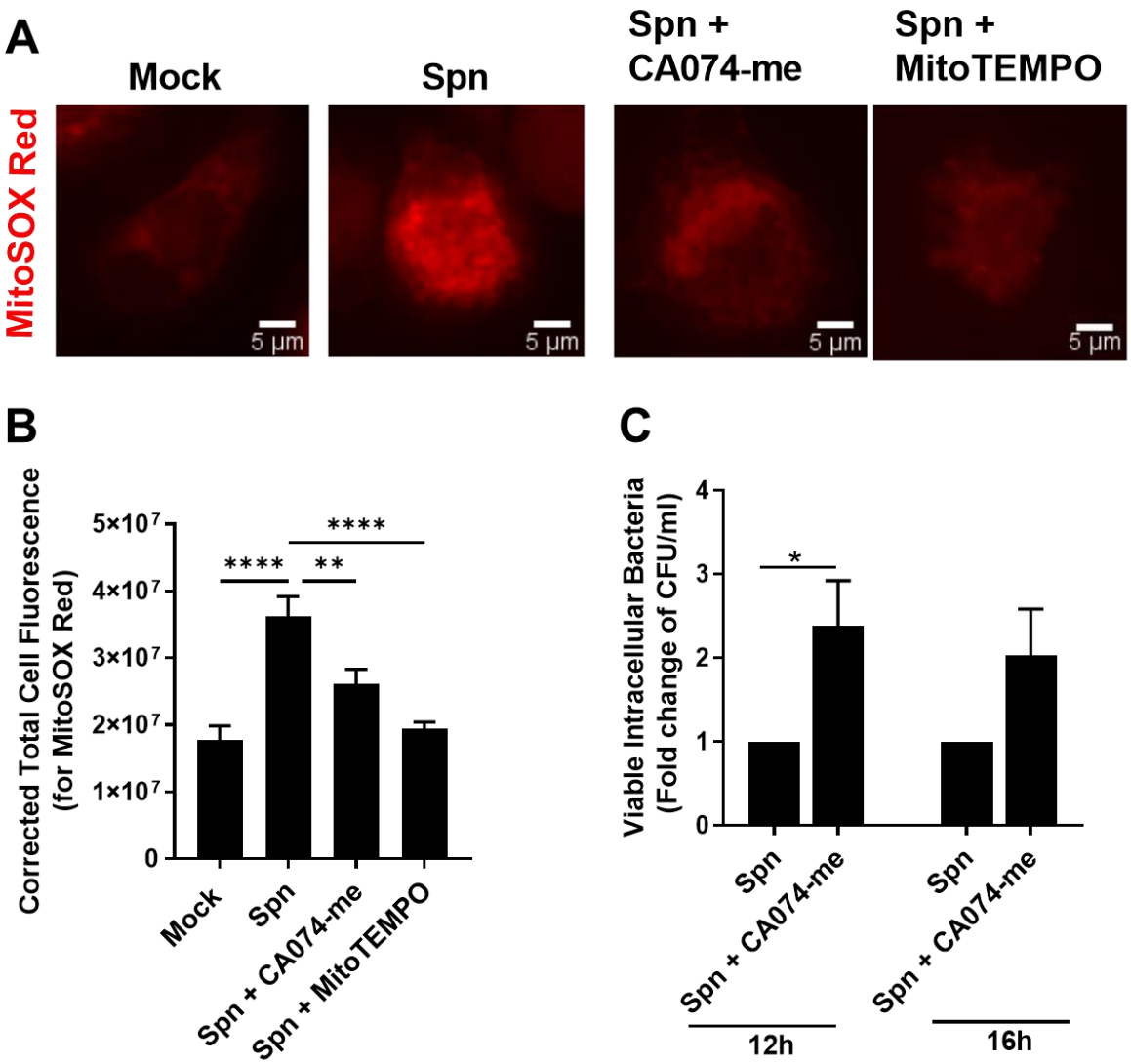


Figure 5.9: Influence of cathepsin B inhibition on mROS production and intracellular bacterial killing in murine BMDM following *S. pneumoniae* challenge. A) Example images of MitoSOX Red fluorescence in BMDM following *S. pneumoniae* challenge (MOI 10 for 14 hours), with and without prior CA074-me or MitoTEMPO treatment. mROS images taken on the Leica SP8 at 63x magnification and the Andor spinning disk at 60x magnification. B) Corrected total cell fluorescence intensity scores for MitoSOX Red from BMDM in each treatment condition. C) Fold change of viable intracellular bacterial counts from BMDM challenged with *S. pneumoniae* at MOI 10 for 12 or 16 hours, with and without prior CA074-me treatment. Assays carried out in complete media (RPMI 1640 + 2 millimolar L-glutamine + 10% low endotoxin foetal bovine serum). Error bars represent the SEM. n= 4 independent experiments for mROS analysis and 3 independent experiments for bacterial killing. One-way ANOVA with Sidak's multiple comparisons test and student paired *t* test carried out. *p<0.05, **p<0.01, ****p<0.0001.

5.2.10 Loss of inner mitochondrial transmembrane potential ($\Delta\psi_m$) occurs during late-stage *S. pneumoniae* challenge, and is unaltered by cathelicidin, detected using JC-1

Enhanced mROS production is one of the primary consequences of mitochondrial adaptations to infection, and can be facilitated by a metabolic shift from oxidative phosphorylation to glycolysis for cellular energy generation in pro-inflammatory macrophages (198,323). Mitochondrial adaptations to infection result in other functional changes, such as a loss of $\Delta\psi_m$. This is associated with apoptosis; the Dockrell group has previously shown that a dramatic loss of $\Delta\psi_m$ occurs in response to late-stage *S. pneumoniae* challenge (110,306), as macrophages implement apoptosis-associated killing as a mechanism to clear persistent intracellular bacteria. Therefore, I was interested to confirm this in my MDM model, and also investigate the role of cathelicidin on *S. pneumoniae*-mediated changes in $\Delta\psi_m$. MDM were challenged with *S. pneumoniae* for 4 or 14 hours, with or without 1 hour of subsequent exogenous cathelicidin treatment. MDM were then stained with JC-1 dye to detect changes in $\Delta\psi_m$ and analysed by flow cytometry.

Under steady-state conditions, MDM exhibit a high level of JC-1 red aggregate fluorescence, indicative of a high $\Delta\psi_m$ and healthy, non-depolarised mitochondria. This was unaltered following 4 hour *S. pneumoniae* challenge as shown by the lack of shift in the scatter plot and the histogram (Figure 5.10A and 5.10B), showing that loss of inner mitochondrial transmembrane potential is not triggered in response to early stages of *S. pneumoniae* challenge. However, a marked shift in JC-1 red fluorescence following 14 hour *S. pneumoniae* challenge was observed, indicative of loss of $\Delta\psi_m$ (Figure 5.10A and 5.10B). This shows that

late-stage *S. pneumoniae* challenge triggers loss of $\Delta\psi_m$, as expected. These results are validated by the mean fluorescence intensity values for JC-1 red (Figure 5.10C); 4 hour *S. pneumoniae* challenge did not alter fluorescence intensity but was significantly reduced following 14 hour *S. pneumoniae* challenge. In regard to the influence of cathelicidin on *S. pneumoniae*-induced changes in $\Delta\psi_m$, exogenous cathelicidin treatment did not significantly alter changes in $\Delta\psi_m$ under any condition, suggesting that cathelicidin does not alter $\Delta\psi_m$ under these conditions.

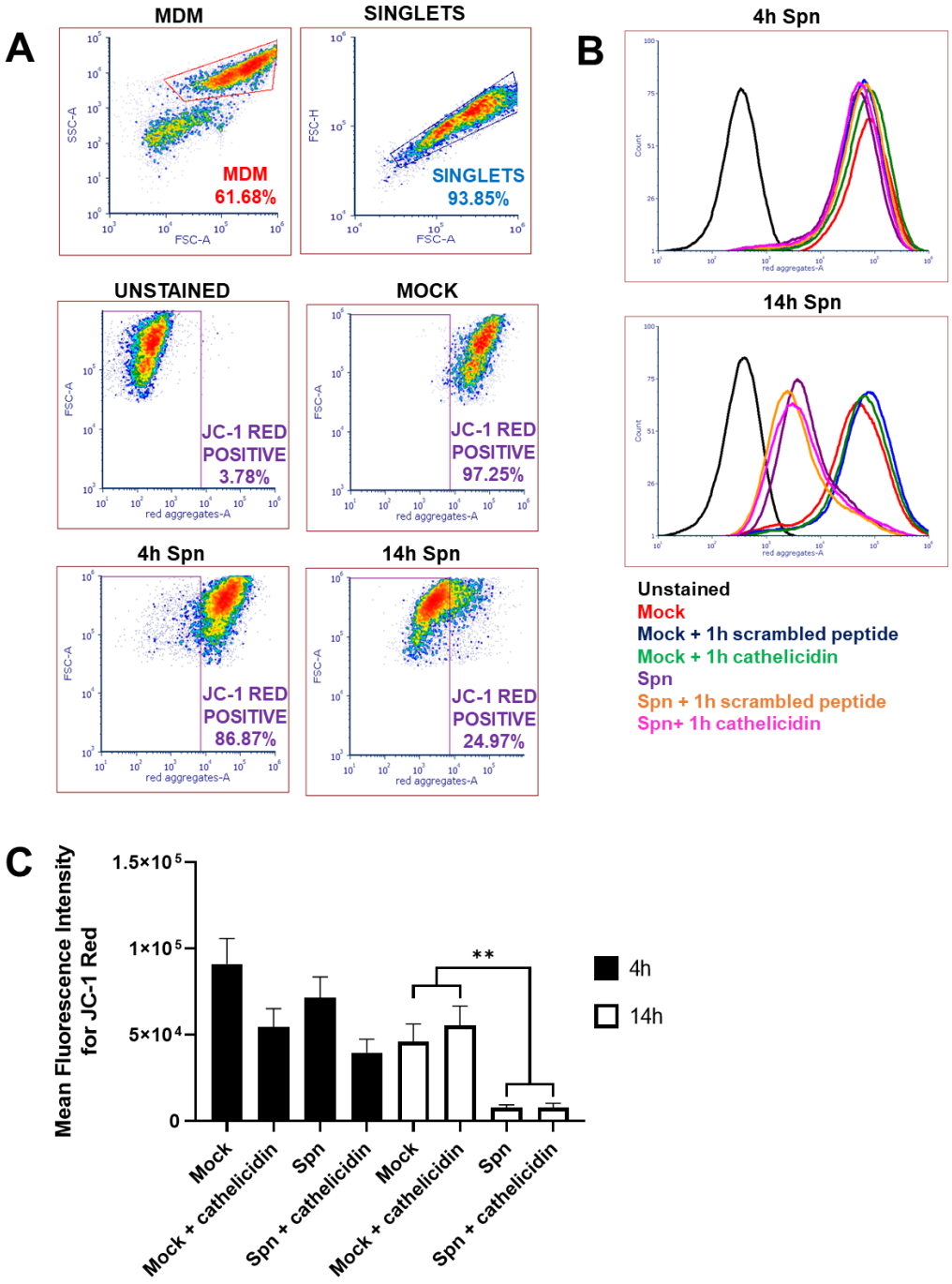


Figure 5.10: Flow cytometry analysis of changes in inner mitochondrial transmembrane potential ($\Delta\psi_m$) in MDM during early- and late-stage *S. pneumoniae* challenge, detected using JC-1. A) Gating strategy to select for MDM, singlets, then JC-1-positive cells. B) Example histograms showing changes in JC-1 red aggregate fluorescence levels in MDM following 4 hour or 14 hour *S. pneumoniae* challenge. C) Quantification of JC-1 red aggregate mean fluorescence intensity values from MDM following 4 or 14 hour *S. pneumoniae* challenge. Error bars represent the SEM. n= 3 independent experiments. Two-way ANOVA with Tukey's multiple comparisons test carried out. **p<0.01. Overall ANOVA result: for 4 hour *S. pneumoniae*- *presence of cathelicidin. For 14 hour *S. pneumoniae*- ***presence of *S. pneumoniae.*

5.2.11 Early-stage *S. pneumoniae* or *S. aureus* challenge do not induce changes in inner mitochondrial transmembrane potential ($\Delta\psi_m$), detected using TMRM

JC-1 is well known to be useful in the detection of major shifts in $\Delta\psi_m$, but it has also been suggested that it may not be sensitive enough to detect very subtle changes in $\Delta\psi_m$ (517). TMRM, another mitochondrial-specific dye, has been reported to be more sensitive in detecting subtle changes in $\Delta\psi_m$ (517). Therefore, to investigate potential subtle changes in $\Delta\psi_m$ during early stages of bacterial challenge in more detail, MDM were challenged with *S. pneumoniae* for 4 hours, or *S. aureus* for 1, 4, or 8 hours, stained with TMRM and analysed by flow cytometry as described above for JC-1.

Figure 5.11 shows that FCCP treatment, used as a positive control to induce loss of $\Delta\psi_m$ (513,514), resulted in the expected significant loss of $\Delta\psi_m$ as shown previously. 4 hour *S. pneumoniae* challenge did not significantly alter TMRM fluorescence, suggesting no change in $\Delta\psi_m$ (Figure 5.11B), which agrees with the JC-1 result. A high TMRM fluorescent signal was detected at all time points following *S. aureus* challenge (Figure 5.11C). This suggests that early-stage *S. aureus* challenge does not trigger a loss of $\Delta\psi_m$, which is in line with results from previous studies showing that *S. aureus* does not induce apoptosis in macrophages (72), therefore a dramatic loss of $\Delta\psi_m$ would not be expected.

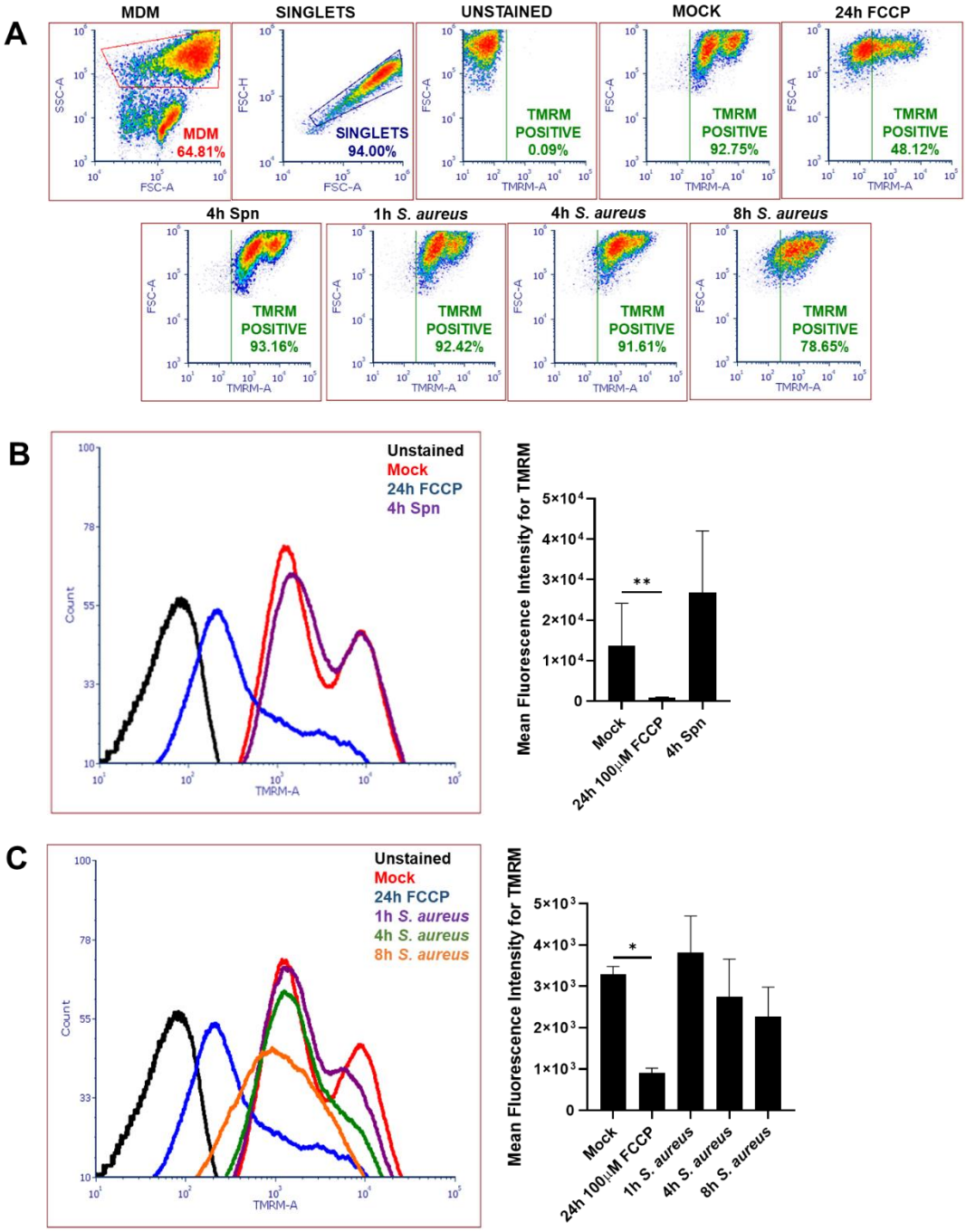


Figure 5.11: Flow cytometry analysis of changes in inner mitochondrial transmembrane potential ($\Delta\psi_m$) in MDM during early-stage *S. pneumoniae* and *S. aureus* challenge, using TMRM. A) Gating strategy to select for MDM, singlets, then TMRM-positive cells. B) Example histogram showing TMRM fluorescence levels, and quantification of TMRM mean fluorescence intensity values, in MDM following 4 hour *S. pneumoniae* challenge. C) Example histogram showing TMRM fluorescence levels, and quantification of TMRM mean fluorescence intensity values, in MDM following 1, 4, or 8 hour *S. aureus* challenge. Error bars represent the SEM. n=3 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons test carried out. *p<0.05, **p<0.01.

5.3 Discussion

In this chapter, I have described some mechanistic details of mROS production in human MDM in response to different stages of *S. pneumoniae* and *S. aureus* challenge, and how chemical modulation and cathelicidin influence mROS production and mROS- or NADPH oxidase-derived ROS-mediated intracellular *S. aureus* killing in MDM. I have also identified the lysosomal protease cathepsin B as a regulatory factor of mROS production and intracellular killing of late-stage *S. pneumoniae* in BMDM. Finally, I have also described the influence of different stages of *S. pneumoniae* and *S. aureus* challenge on the loss of $\Delta\psi_m$ in MDM. Summaries of my findings in relation to mROS production, bacterial killing, and $\Delta\psi_m$ are represented schematically in Figures 5.12, 5.13 and 5.14, respectively.

mROS production has been shown to occur by different mechanisms in different contexts. The most well characterised include production at a variety of electron transport chain sites during traditional forward electron flow, for example sites I_F located in Complex I, II_F located in Complex II, or III_{Qo} located in Complex III; and RET, which has been shown to generate superoxide via site I_Q in Complex I. In recent years, studies into the functional roles of RET-derived mROS in macrophages have shown that RET-derived mROS is particularly associated with metabolic reprogramming, where increased succinate oxidation has been shown to be a primary driver for enhanced I_Q-derived mROS production (329,554), and increased pro-inflammatory cytokine production to promote pro-inflammatory macrophage functions in response to LPS (400), but little is known about the impact of live bacteria. Rotenone has been widely regarded as Complex I inhibitor associated with inducing enhanced mROS production. However, it has now been shown that this is

only the case when mROS is produced via forward electron flow and rotenone inhibits mROS production if RET is the mechanism; this therefore allows rotenone to be used to probe mechanisms of mROS production *in vitro.* In an LPS-activated BMDM model of mROS production, this method was employed to suggest that LPS-induced mROS production occurred via RET (329). The Dockrell group has also previously shown that rotenone treatment of healthy and COPD alveolar macrophages enhances mROS-dependent *S. pneumoniae* killing (403). However, the mechanisms of mROS production in primary human MDM in response to *S. pneumoniae* or *S. aureus* have not been investigated. I have shown that the potential mechanism of mROS production in MDM in response to *S. pneumoniae* changes over time; my findings suggests that mROS produced early in response to bacterial challenge may occur via RET, while mROS produced during late-stage bacterial challenge occurs via forward electron flow. As RET-derived mROS production is associated with promoting pro-inflammatory macrophage function (400), it is possible that MDM could produce mROS via RET in response to early-stage *S. pneumoniae* challenge as a method of driving other pro-inflammatory responses to promote bacterial clearance. It would be interesting to investigate this further by probing the effects of rotenone treatment on MDM challenged with *S. pneumoniae* at other time points to gain further insight into the kinetics of RET and mROS production over time. It would also be beneficial to measure other functional consequences of rotenone treatment and different *S. pneumoniae* challenge time points, such as pro-inflammatory cytokine production or MDM killing of early-stage *S. pneumoniae* to determine if there is correlation between RET-derived mROS production, pro-inflammatory cytokine production and killing of early-stage *S. pneumoniae*. Similarly, the lack of significant rotenone effect on mROS production following 4 or 8 hour *S.*

aureus challenge suggests that *S. aureus*-induced mROS production occurs via forward electron flow and RET is not involved. However, further studies investigating other *S. aureus* challenge time points would be beneficial to determine if RET occurs at earlier time points.

The conclusions relating to *S. aureus* must also be interpreted more cautiously since I was not able to show the MitoSOX fluorescence following *S. aureus* challenge was inhibitable with the mROS inhibitor MitoQ. While this may have reflected challenges in the use of MitoQ in the experiment, such as timings, or imprecision in the measure of fluorescence with confounding by nuclear or bacterial-associated signals, further work is needed to confirm that the MitoSOX signal with *S. aureus* does indeed involve mROS. This should use other measures of mROS production. Examples of such measures include visualisation and analysis of H_2O_2, produced via detoxification of mitochondrial superoxide, by using a H_2O_2-specific dye such as MitoPY1 (555); or use of a different superoxide dye that does not intercalate with DNA, such as MitoNeoD (552). Additionally, alternative analysis of MitoSOX fluorescence could be carried out, for example focusing on the mitochondrial signal specifically with co-staining of mitochondria, and more optimisation of MitoQ and use of alternative inhibitors, such as MitoTEMPO, would also be beneficial. Furthermore, some of the results in this chapter are limited by small sample sizes and individual cell variability, which means that although clear differences were observed between conditions in several experiments, they were not statistically significant. However, it is highly likely that further experiments to increase sample sizes and reduce variability would achieve statistical significance in many cases.

I have also shown that modulation of mROS production via MitoPQ does not alter the intensity of early-stage *S. aureus*-induced mROS production as detected by MitoSOX fluorescence. This suggests that *S. aureus* challenge enhances mROS production in MDM to an extent where further enhancement is undetectable; this may reflect the high intracellular burden *S. aureus* imposes on the macrophage and the intensity of responses activated by the macrophage to manage this challenge. However, it is important to consider another possibility, that MitoPQ and MitoQ may not exert a global effect under these conditions and potential effects may only occur in a subset of MDM. Therefore, as described previously for Drp1 in the context of mROS production in Chapter 4, measurement of mROS fluorescence in the total cell population or in the whole cell may mask any compound effects on individual cells or even changes in localisation of production within a cell. To investigate this further, it may be beneficial to analyse the number of MDM in each condition that exhibit changes in mROS fluorescence to gain further understanding of the effects of mROS modulation in individual MDM of a population. It would also be important to examine the impact of modulators on mROS specifically localised to mitochondria adjacent to bacteria, to gain further insight into the specific importance of localisation of mROS in macrophage antibacterial responses in addition to levels of mROS production.

Both NADPH oxidase-derived ROS and mROS, early and late ROS sources respectively, are known to be enhanced in macrophages in response to bacterial challenge, and are important for optimal pathogen clearance and activation of other macrophage antimicrobial responses (309,322). However, macrophages may combine ROS with other antimicrobial factors to overcome spatial, temporal, and potency limitations of macrophage ROS (291,292), which further emphasises the

importance of a multi-layered macrophage immune response for optimal pathogen clearance.

In regard to MDM intracellular killing of *S. aureus*, DPI inhibition of NADPH oxidase showed that ROS is the primary ROS source that contributes to MDM killing of early-stage intracellular *S. aureus* in my model. This result agrees with previous studies that have illustrated the importance of NADPH oxidase-derived ROS in the clearance of *S. aureus* by macrophages and the increased susceptibility of CGD patients, who cannot generate NADPH oxidase-derived ROS, to *S. aureus* infections (546). However, my results showed that mROS also contributes to *S. aureus* killing at this stage, therefore suggesting that mROS has a microbicidal effect on early-stage intracellular *S. aureus* alongside NADPH oxidase-derived ROS, albeit the extent of involvement may be less for mROS. This also indicates that the two different ROS sources contribute to *S. aureus* killing during bacterial challenge, potentially acting at different periods. The greater impact of inhibition of NADPH oxidase-derived would be consistent with its role early after ingestion and the known observation that the majority of killing in phagocytes occurs in the initial stages of phagosomal maturation (556). The modest impact of mROS inhibition might reflect the lower numbers of bacteria that are available after initial killing, and which would be available for killing by mROS. More detailed studies into the kinetics and localisation of mROS and NADPH oxidase-derived ROS during *S. aureus* challenge, for example via confocal microscopy or live cell imaging, would provide further insight into the relative contributions and roles of mROS and NADPH oxidase-derived ROS in *S. aureus* killing.

Cathelicidin is an example of an antimicrobial factor that has been shown to enhance ROS production and interact with ROS to enhance bacterial killing, in THP-1 cells and neutrophils (505,547,548). However, cathelicidin's influence on mROS production, and interactions between cathelicidin and different sources of ROS on bacterial killing, in primary human MDM have not been established. I have shown that cathelicidin can enhance mROS production from MDM in the absence of *S. aureus*. However, in the presence of *S. aureus*, cathelicidin treatment resulted in a reduction in mROS production in MDM challenged with *S. aureus*. This suggests that cathelicidin may be serving as a brake on *S. aureus*-induced enhancement of mROS in order to regulate mROS production and prevent excessive induction of inflammatory responses in response to high levels of mROS. Interpretation of this finding however has to be cautious in view of the above concerns with interpretation of MitoSOX fluorescence following *S. aureus* challenge. Furthermore, I have also shown that cathelicidin provides a greater contribution to MDM killing of intracellular *S. aureus* when NADPH oxidase-derived ROS production in impaired but had no effect on *S. aureus* killing following modulation of mROS production. This suggests that macrophages employ a system where cathelicidin exerts a greater bacterial killing effect when a primary early macrophage antimicrobial response, in this case NADPH oxidase-derived ROS production, is suboptimal, therefore demonstrating another example of a multi-layered, combinatory macrophage response to infection. In relation to mROS, it may have a distinct role acting as a brake and limiting excessive oxidative stress.

In the context of macrophage killing of *S. pneumoniae*, I have demonstrated that the lysosomal protease cathepsin B contributes to enhanced mROS production

and enhanced intracellular bacterial killing in BMDM during the later stages of *S. pneumoniae* challenge (387), therefore functioning as a mechanism regulating *S. pneumoniae*-induced macrophage microbicidal responses. Cathepsin B can be activated following lysosomal membrane permeabilisation, which occurs in response to late-stage intracellular *S. pneumoniae* persistence and functions as a sensor of intracellular bacterial viability (110,557). Previous work has shown that lysosomal membrane permeabilisation is associated with enhanced mitochondrial fission (558), and another lysosomal protease, cathepsin D, is required for the initiation of apoptosis and clearance of persistent intracellular *S. pneumoniae* (272). Therefore, these cathepsin B findings show that another lysosomal protease contributes to the regulation of mitochondrial function and macrophage microbicidal responses, also linking lysosomal permeabilisation to mitochondrial fission. Therefore, cathepsin B demonstrates another example of how multiple macrophage antimicrobial responses co-operate to enhance bacterial killing by macrophages.

An increase in mROS production is a key functional consequence of mitochondrial adaptations in macrophages in response to bacterial challenge. Another change in mitochondria that can be associated with exposure to bacteria is alteration of $\Delta\psi_m$ in mitochondria. A marked loss of $\Delta\psi_m$ has been shown to occur in response to late-stage *S. pneumoniae* challenge as an initial step in the activation of apoptosis-associated killing in macrophages, but does not occur in response to earlier stages of *S. pneumoniae* challenge (110,306). Moreover, induction of mROS isn't essential for apoptosis induction, suggesting mROS and loss of $\Delta\psi_m$ are likely dissociated (292). Therefore, I was also interested to examine if cathelicidin had any impact on $\Delta\psi_m$. I confirmed that a significant loss of $\Delta\psi_m$ occurs in response to late-

stage (14 hour), but not early-stage (4 hour), *S. pneumoniae* challenge via flow cytometry analysis with JC-1 dye. The addition of cathelicidin treatment did not alter $\Delta\psi_m$ in any condition at either time point, therefore cathelicidin does not influence $\Delta\psi_m$ under these conditions. However, although JC-1 is useful for detecting large shifts in $\Delta\psi_m$, such as those observed in apoptosis, another dye, TMRM, has been shown to be more sensitive for detecting more subtle changes in $\Delta\psi_m$ that may be observed at earlier time points (517). This not only allowed investigation of subtle loss of $\Delta\psi_m$ but also hyperpolarisation which could aid enhanced mROS generation (559,560). The TMRM analysis confirmed that 4 hour *S. pneumoniae* challenge does not alter $\Delta\psi_m$, as observed with JC-1. In addition, the influence of *S. aureus*, which does not induce apoptosis-associated killing (72), on $\Delta\psi_m$ in MDM is unclear. By investigating potential early *S. aureus*-induced changes in $\Delta\psi_m$ using TMRM, I have shown that alteration of $\Delta\psi_m$ does not occur in response to *S. aureus* in my MDM. Therefore, although I have observed changes in mitochondrial dynamics and mROS production in response to early-stage *S. aureus* challenge of MDM, I did not identify any alteration in $\Delta\psi_m$ following *S. aureus* challenge. However, it should be noted that in some of the flow plots for TMRM positive conditions (Figure 5.11A), such as Mock, 4h Spn, and 1h *S. aureus*, there appears to be two subsets of MDM. As the TMRM fluorescence intensity correlates with the level of TMRM present by mitochondria, it is likely that the larger subset represents MDM with intermediate TMRM uptake while the smaller subset represents MDM with a high level of TMRM uptake. In addition, this high TMRM subset appears to diminish following 4h or 8h *S. aureus* challenge, therefore it is possible that this represents a subtle loss of $\Delta\psi_m$ in these conditions that would not be detected by measuring the TMRM positive population as a whole. Further studies to characterise these TMRM subsets and determine any subtle

effects on the loss of $\Delta\psi_m$ would be beneficial to further understand the effect of *S. aureus* on mitochondrial function in human MDM.

In summary, this chapter describes some details of the mechanisms and functions of mROS production, ROS production, and changes in $\Delta\psi_m$ in macrophages in response to *S. pneumoniae* and *S. aureus* challenge. I have shown that mROS production is potentially enhanced by early-stage *S. aureus* challenge and confirmed induction by late-stage *S. pneumoniae* challenge. Mechanistically, my findings suggest that mROS production occurs via RET early in response to *S. pneumoniae* but occurs via forward electron flow in response to late-stage *S. pneumoniae* and early-stage *S. aureus*. In regard to bacterial killing, NADPH oxidase-derived ROS is the primary ROS source mediating early-stage *S. aureus* killing, but mROS also contributes to killing at this time, demonstrating that different ROS sources contribute to enhanced bacterial killing in MDM in the early stages of killing. Cathelicidin enhances mROS production in steady-state MDM but potentially reduces *S. aureus*-induced mROS production, therefore acting as a brake to regulate mROS production which may prevent excessive inflammation. Interestingly, I have shown that cathelicidin contributes to *S. aureus* killing when NADPH oxidase-derived ROS production is impaired, suggesting that cathelicidin has a greater bacterial killing effect in MDM when a primary early response, in this case NADPH oxidase-derived ROS production, is suboptimal. For *S. pneumoniae*, I have described how cathepsin B functions as a regulator of mROS production and enhanced macrophage killing of persistent intracellular *S. pneumoniae*, providing further evidence of interactions between different macrophage microbicidal responses to enhance bacterial killing. Finally, I have confirmed that loss of $\Delta\psi_m$,

another mitochondrial functional change in response to bacterial infection which is associated with induction of apoptosis, occurs in response to late-stage but not early-stage *S. pneumoniae*. Furthermore, *S. aureus*, which does not induce apoptosis-associated killing, did not trigger alteration of $\Delta\psi_m$. Although *S. aureus* does induce mitochondrial adaptations to infection such as increased fission and potentially mROS production, it does not appear to induce hyperpolarisation of the inner mitochondrial membrane to enhance mROS induction.

My findings provide valuable insight into mechanistic details and functional consequences of mROS production. They also provide details on mitochondrial adaptations to bacterial challenge, as well as interactions between these responses and the other antimicrobial factors, such as cathelicidin or cathepsin B, enhancing macrophage antimicrobial responses and bacterial killing. However, it is evident that the role of mitochondrial adaptations in macrophages during bacterial infection, and the interactions between multiple macrophage antimicrobial responses, are diverse and complex. Therefore, this field warrants further investigation to understand these processes further, as it is clear that they are critical for optimal macrophage microbicidal functionality.

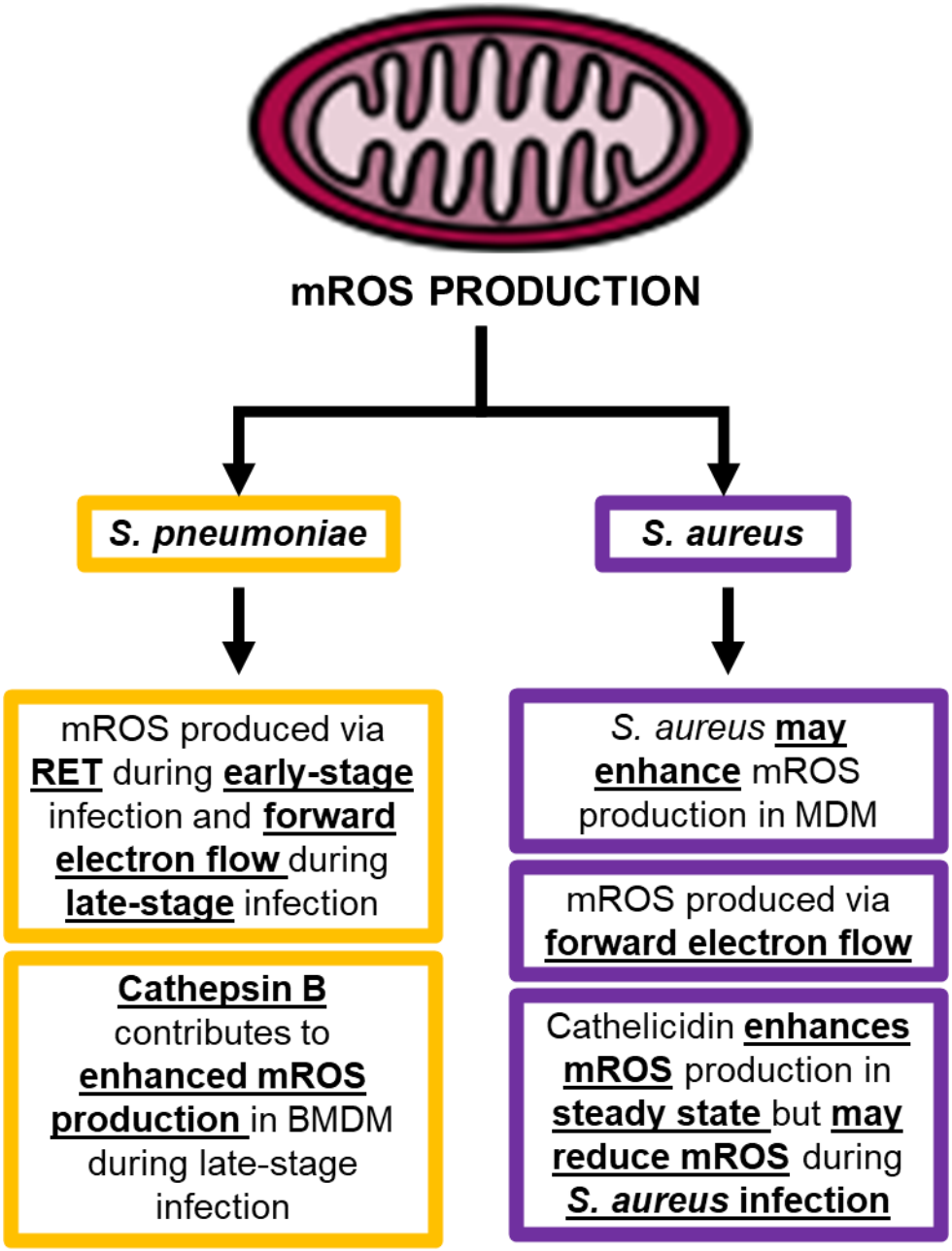


Figure 5.12: Schematic representation of the influence of *S. pneumoniae* and *S. aureus* on mROS production in MDM, the mechanisms behind this mROS production, and the contribution of the additional antimicrobial factors, cathepsin B and cathelicidin, on mROS production.

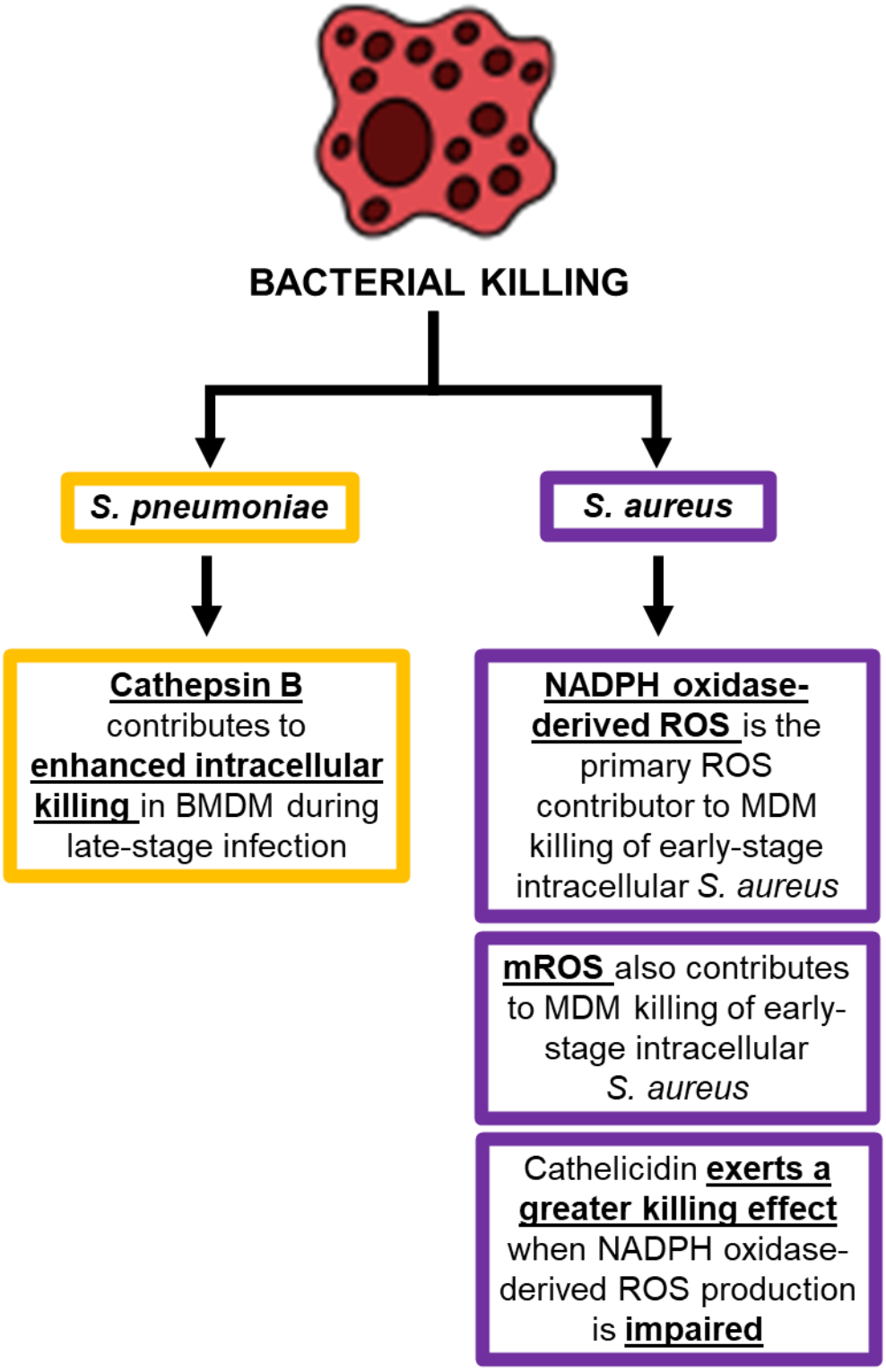


Figure 5.13: Schematic representation of the influence of cathepsin B on *S. pneumoniae* killing in MDM, and of NADPH oxidase-derived ROS, mROS and cathelicidin on *S. aureus* killing by MDM.

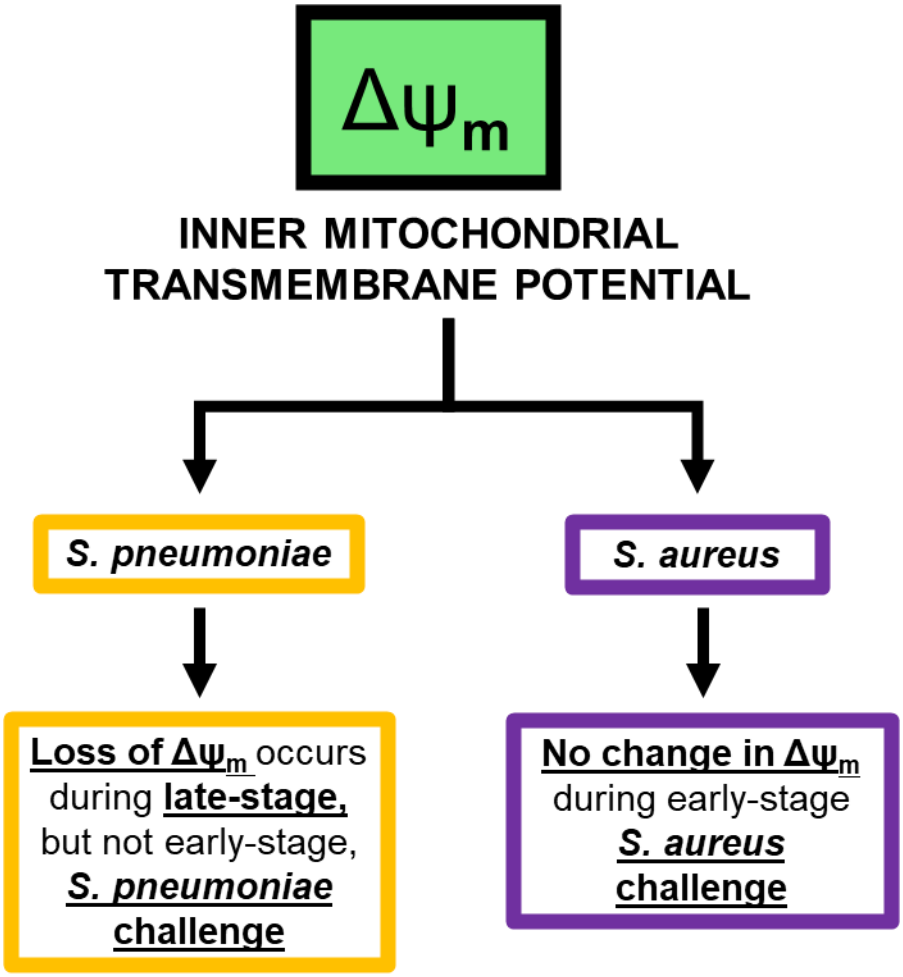


Figure 5.14: Schematic representation of the influence of *S. pneumoniae* and *S. aureus* on inner mitochondrial transmembrane potential ($\Delta\psi_m$) in MDM.

Chapter 6

Discussion

6.1 Overview of project and findings

This thesis aimed to investigate novel macrophage microbicidal responses against the gram-positive bacteria *Streptococcus pneumoniae* and *Staphylococcus aureus*, two of the most prevalent pathogens posing a significant threat to global health due to antimicrobial resistance (12). Although macrophages are adept at pathogen clearance, limitations on individual responses alone suggest that macrophages require multiple different microbicidal mechanisms with different spatial and temporal niches to combine in a multi-layered immune response to optimally clear pathogens (17,292). Therefore, with a particular focus on mitochondrial dynamics, mROS production, and the cationic host defence peptide cathelicidin, I aimed to investigate the roles and potential interactions of these responses in human macrophages in response to *S. pneumoniae* and *S. aureus* challenge.

I have demonstrated that alterations in mitochondrial dynamics and mROS production play roles in macrophage antimicrobial responses to *S. pneumoniae* and *S. aureus*, with different effects depending on the bacteria encountered which may be reflective of the contrasting intracellular burdens. Furthermore, vitamin D- and phenylbutyrate-induced cathelicidin expression in human macrophages can be modulated by bacterial challenge or exposure to pro-inflammatory cytokines. In addition to direct bactericidal functionality against *S. pneumoniae* and, to a more modest extent, *S. aureus*, cathelicidin may also synergise with other macrophage

responses to enhance macrophage bacterial killing, particularly of *S. aureus*. Furthermore, cathelicidin may serve as a brake on mitochondrial-associated antimicrobial responses during bacterial challenge, therefore acting to regulate mitochondrial homeostasis and prevent host tissue damage via excessive induction of oxidative stress.

6.2 Multi-layered macrophage responses to the contrasting challenges of *S. pneumoniae* and *S. aureus*

As emphasised throughout this thesis, macrophages require combinations of microbicidal responses with different spatial and temporal niches, described as the multi-layered immune response to facilitate optimal killing of *S. pneumoniae* and *S. aureus*, as these pathogens are adapted to resist killing by individual mechanisms. Furthermore, these pathogens present contrasting challenges to the macrophage. *S. pneumoniae* is more difficult to ingest, while *S. aureus* is readily ingested (72,108). However, as a result, the lower intracellular burden of *S. pneumoniae* is cleared more easily by macrophage intracellular killing mechanisms than *S. aureus*, which poses a much higher intracellular burden and could therefore overwhelm individual killing mechanisms more. Furthermore, persistent *S. pneumoniae* challenge induces a delayed apoptosis-associated killing mechanism in macrophages to facilitate clearance (292,296). *S. aureus* does not induce macrophage apoptosis, which can result in a persistent higher bacterial burden (72). Therefore, the contrasting challenges of these pathogens stress macrophage microbicidal responses in different ways. this contrast enabled investigation of macrophage adaptations in response to these different pathogens, and how macrophage responses facilitate their clearance.

The Dockrell group has extensively studied macrophage responses to *S. pneumoniae* and have described the kinetics and roles of a number of macrophage microbicidal mechanisms across different stages of *S. pneumoniae* challenge, which are represented schematically in Figure 6.1. The *S. pneumoniae*-associated findings presented in this thesis expand this model and provide further mechanistic details on *S. pneumoniae*-induced mROS production and describe a potential role for cathelicidin. This is also represented schematically in Figure 6.1.

In contrast, relatively little has been described for the mechanisms and interactions of macrophage responses against *S. aureus*. However, it is clear from my data and from previous studies that many macrophage responses are induced earlier in response to *S. aureus* (407), compared to *S. pneumoniae*. Therefore, many of the *S. aureus*-associated experiments described in this thesis focused on an early challenge time point (4-8 hours) but investigated the interactions of multiple responses during early-stage *S. aureus* challenge. This multi-layered response is represented schematically in Figure 6.2.

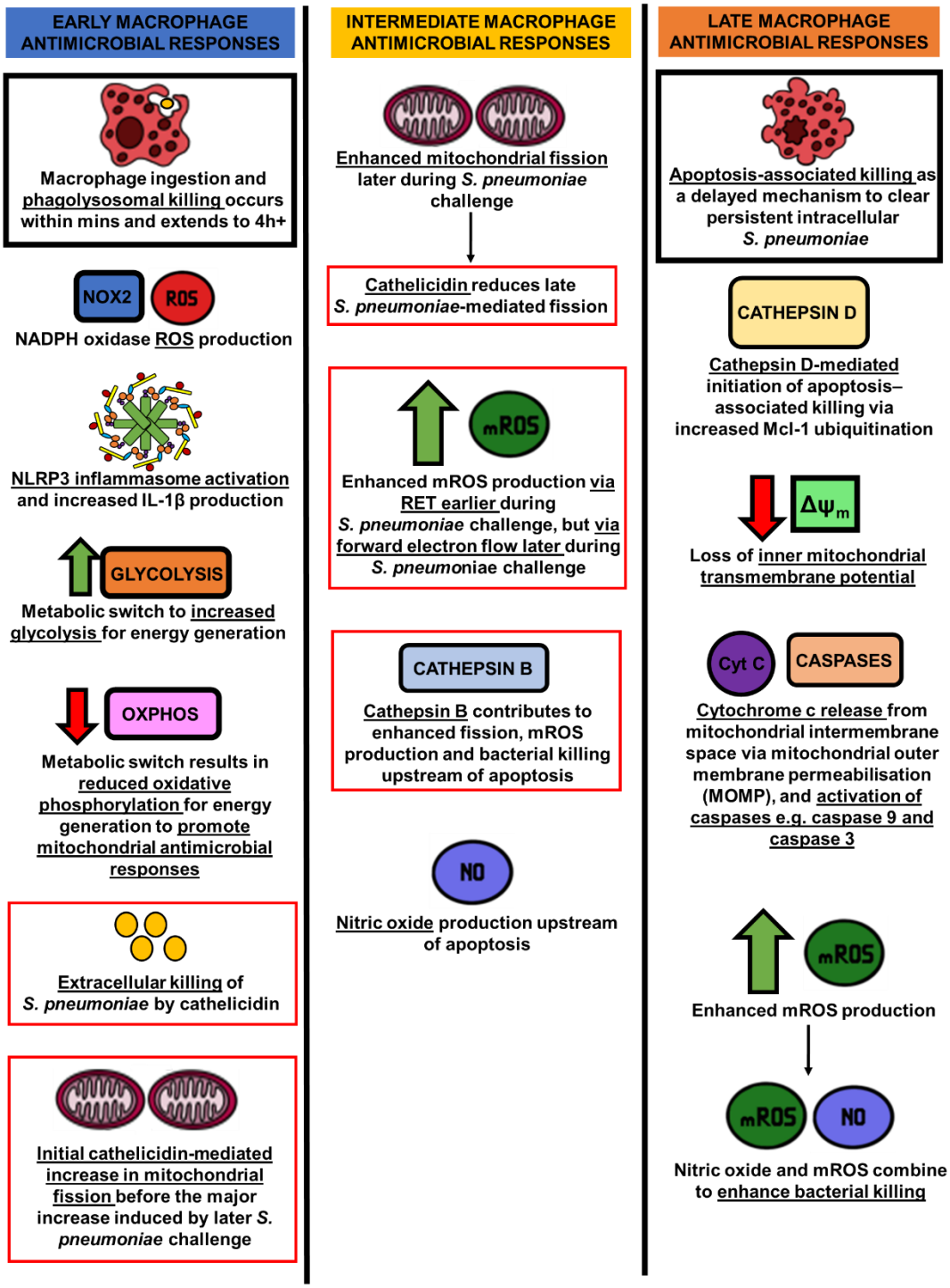


Figure 6.1: Schematic representation of the macrophage multi-layered immune response in response to *S. pneumoniae* over time. Killing mechanisms (black borders) and other antimicrobial responses employed by macrophages at early, intermediate, and late stages of *S. pneumoniae* challenge. The red borders represent the responses that this thesis has contributed to this system, while the other responses have been described previously by the Dockrell group and others.

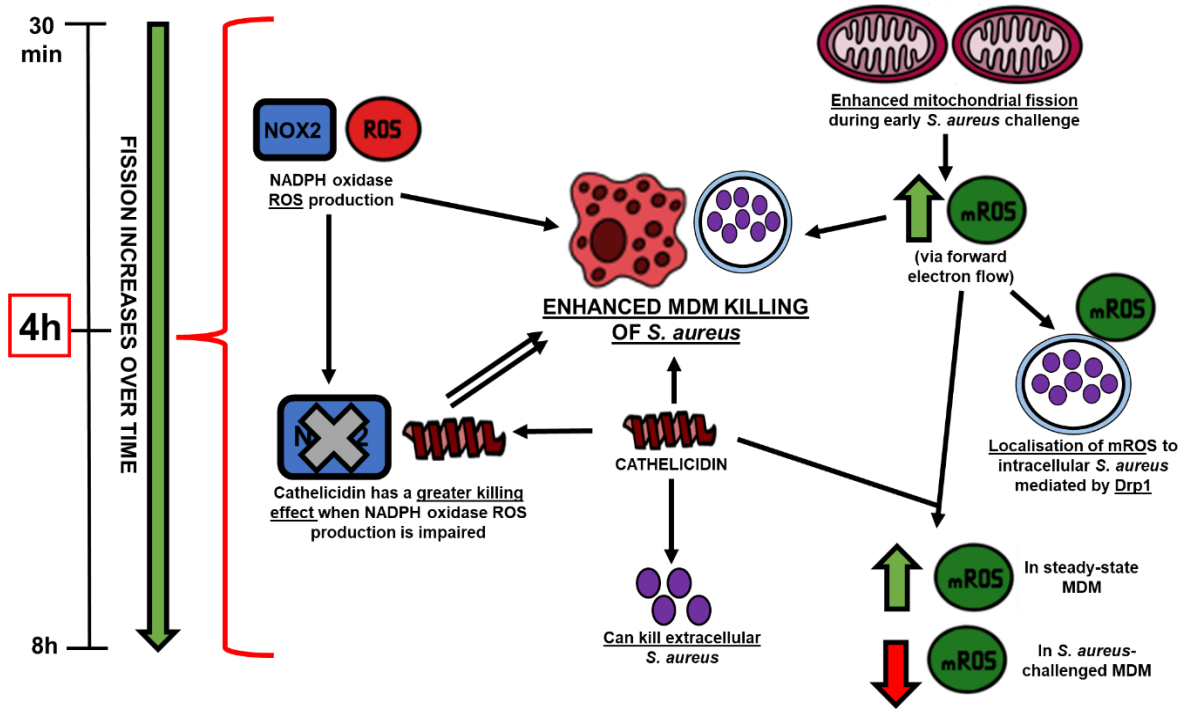


Figure 6.2: Schematic representation of the macrophage multi-layered immune response in response to *S. aureus* following 4 hour bacterial challenge, as described in this thesis.

6.3 Roles of mitochondrial dynamics and mROS production in macrophages in responses to *S. pneumoniae* or *S. aureus*

Mitochondrial dynamics and mROS production contribute to macrophage responses against both *S. pneumoniae* and *S. aureus*, but the roles and kinetics of these responses differ between the two pathogens. It has been shown previously that enhanced fission and mROS production are evident during the late stages of *S. pneumoniae* challenge (387,403), which has also been confirmed by my data. However, my data show that enhanced fission occurred as early as 2 hours in response to *S. aureus* and continued up to 8 hours post-challenge, and mROS production was potentially enhanced by 4 hours of *S. aureus* challenge (taking into account the caveats described previously regarding MitoSOX staining), which were much earlier than those observed with *S. pneumoniae*. This temporal difference between *S. aureus* and *S. pneumoniae* is likely reflective of the higher intracellular burden posed by *S. aureus* and suggests that macrophages adapt the timing of induction of microbicidal mechanisms in response to the different challenges posed by different pathogens.

In regard to mechanisms of mROS production, my data suggest that reverse electron transfer (RET) contributes to *S. pneumoniae*-induced mROS production during mid-stage challenge (8 hours), but not during late-stage *S. pneumoniae* challenge or *S. aureus* challenge. RET-derived mROS production is associated with promoting macrophage antimicrobial functions, such as increased production of pro-inflammatory cytokines (400). This further supports the notion of a multi-layered macrophage immune response where different responses work co-ordinately to enhance macrophage microbicidal functionality across all stages of bacterial

challenge. In addition, high levels of fission were not expected 8 hours post-*S. pneumoniae* challenge, therefore the process of RET could be employed at this stage to enhance mROS production before the onset of increased fission. In contrast, the lack of RET contribution during late-stage *S. pneumoniae* challenge or *S. aureus* challenge suggests that mROS production likely occurs by traditional forward electron flow. However, it is known that extensive fission leads to dysregulation of the electron transport chain, enhanced leakage of electrons and formation of superoxide (395,396). There are multiple potential sites for this at different complexes (554). For example, site I_F located in Complex I produces superoxide via electron leakage from reduced flavin (554). Site II_F in Complex II uses the oxidation of the TCA cycle succinate to fumarate to drive the reduction of ubiquinone to ubiquinol, which subsequently allows for superoxide generation (554). Site III_{Qo} located in Complex III generates superoxide via interactions between semiquinone and oxygen (554). Therefore, it is also possible that due to the enhanced level of fission induced by late-stage *S. pneumoniae* and early-stage *S. aureus*, increased mROS production occurs via this increased leakage of electrons and superoxide generation.

The mechanistic contributions of the mitochondrial fission regulators Drp1 and Mff to macrophage responses also differed between *S. pneumoniae* and *S. aureus*. My data suggest that Drp1 and Mff contribute to mediating fission at an individual cell level following late-stage *S. pneumoniae* challenge or early-stage *S. aureus* challenge, suggesting that under these conditions, fission may be mediated by mechanisms that don't require Drp1 or Mff, or the contributions of Drp1 and Mff were heterogeneous within the cell population. However, upon further investigation of

these factors on a more localised, individual cell level in the context of *S. aureus* challenge, it became clear that Drp1 is potentially important for mediating the localisation of mROS to intracellular *S. aureus*. My data also showed there was a trend toward Drp1 contributing to macrophage killing, so in conjunction with the mROS localisation data, this suggests that Drp1-mediated localisation of mROS may contribute to enhanced macrophage killing of intracellular *S. aureus*. It should be noted that my data demonstrating a role for Drp1 in mROS localisation does contrast with the 2018 Cell study describing localisation of mROS to intracellular MRSA via the Parkin-dependent formation of MDVs, as this study showed that this mechanism was Drp1-independent (407). However, that published study was conducted in murine BMDM and the RAW264.7 macrophage cell line, not primary human MDM. Another study has also demonstrated that MDV formation is Drp1-independent, but this was conducted in HeLa and COS7 cell line which are again distinct from primary human MDM (409). Therefore, it is possible that in my human MDM model, MDVs could be formed by a Drp1-dependent mechanism or that Drp1-mediated localisation of mROS involves another mechanism that may compliment the role of Drp-1 independent production of MDVs. This would explain why I failed to find a role for canonical Drp-1 mediated fission in the mitochondrial adaption with *S. aureus* yet still found some roles for this process in localisation of mROS to *S. aureus* and resultant killing of the bacteria.

As highlighted above and throughout this thesis, combinations of different factors and responses are required for optimal macrophage microbicidal functionality. The above result demonstrates a mechanism by which different mitochondrial factors, in this case the fission regulator Drp1 and mROS, can interact

to promote macrophage microbicidal responses against *S. aureus*. In regard to macrophage antimicrobial factors interacting with mROS to aid in the clearance of *S. pneumoniae*, I have also shown that the lysosomal protease cathepsin B contributes to macrophage microbicidal functionality at the later stages of bacterial challenge by enhancing mROS production and intracellular killing. This supports and expands upon previous studies from the Dockrell group which described a role for the lysosomal protease cathepsin D in macrophage microbicidal responses against *S. pneumoniae* mediated via apoptosis induction (272). In this work, cathepsin B was found to not be associated with induction of apoptosis so it appears to have a distinct microbicidal mechanism. Therefore, these cathepsin B results not only delineate a role for a second lysosomal protease in the regulation of macrophage microbicidal functions, but also start to outline a different macrophage microbicidal response, in this case a lysosomal protease acting to directly enhance mROS production, to enhance *S. pneumoniae* killing.

Finally, with a particular focus on macrophage killing *S. aureus*, my data confirmed that NADPH oxidase-derived ROS is important for bacterial killing and is the primary ROS source contributing to killing at the early stages of challenge, which is well-established in the literature (204,269,281,283). However, my results have expanded upon this to show that mROS also contributes to early *S. aureus* killing. This demonstrates that two distinct sources of ROS contribute to macrophage killing of *S. aureus* at this early stage. It is known that NADPH oxidase-derived ROS is produced rapidly following pathogen ingestion (204,281), so although the kinetics and mechanistic details of the functions of these two ROS sources are still unclear, it is possible that NADPH oxidase-derived ROS mediates bacterial killing first to

initially reduce the intracellular burden. The pivotal role of NADPH oxidase in generating ROS into bacteria-containing phagosomes and mediating a superoxide burst is well known (283), but NADPH oxidase also contributes to killing of gram-positive bacteria in phagosomes by acting as a substrate for caspase-1 and its subsequent regulation of phagosome acidification (561). Studies have shown that in response to internalisation of *S. aureus* by macrophages, NADPH oxidase-derived ROS production can neutralise the acidic phagosomal pH generated by v-ATPase activity (561). Therefore, caspase-1 produced following activation of the NLRP3 inflammasome enhances phagosomal acidification by hydrolysing NADPH oxidase components (561). While this could enhance phagosomal acidification, it might put greater requirement on other sources of ROS such as mitochondria. Following NADPH oxidase-mediated killing, mROS may then contribute to subsequent killing of the remaining bacteria based on my results. However, the aforementioned study suggested that a source of ROS distinct from NADPH oxidase is the activator of the NLRP3 inflammasome (561). Other studies have shown that mROS can induce inflammasome activation (270,413), therefore it is possible that the contribution of mROS to early killing of *S. aureus* I observed could be related to its activation of the inflammasome, which then subsequently results in enhanced caspase-1-mediated phagosome acidification via NADPH oxidase hydrolysis.

6.4 Roles of cathelicidin in macrophages in responses to *S. pneumoniae* or *S. aureus*

Cathelicidin is widely known for its microbicidal and immunomodulatory properties and is an important component of host innate immunity to pathogens (418,460,461). In this thesis, some of the roles of cathelicidin in macrophage

responses to *S. pneumoniae* and *S. aureus* were explored, and there appears to be both similarities and differences between the responses to these bacteria.

I have demonstrated that cathelicidin rapidly kills extracellular *S. pneumoniae*, and also enhances mitochondrial fission in the presence of early-stage *S. pneumoniae*. However, cathelicidin did not contribute to macrophage early killing of *S. pneumoniae.* One possible hypothesis is that cathelicidin could contribute by killing some of the bacteria before ingestion by macrophages, therefore reducing the extracellular bacterial population, and subsequently reducing the bacterial burden such that macrophages can then kill the intracellular bacteria without requiring an additional contribution from intracellular cathelicidin. In regard to enhanced fission occurring in the absence of greater macrophage killing, it is possible that the consequences of enhanced cathelicidin-mediated fission are too subtle to have detected under these specific experimental conditions, or that cathelicidin-mediated fission responses are not required for bacterial killing at this time point. The response might however be an adaption to enable more effective killing at later time points and/or when the early killing mechanism are exhausted, as part of a layered approach to microbicidal responses

In contrast to *S. pneumoniae*, cathelicidin not only killed extracellular *S. aureus* directly, albeit to a more modest extent, but it also contributed to enhanced macrophage killing of *S. aureus*. Again, this could support the notion that additional antimicrobial responses are required in macrophages to overcome the greater burden of *S. aureus* than is required for responses against *S. pneumoniae* at the intracellular load levels in these studies. When investigating potential interactions between mROS and cathelicidin on bacterial killing, cathelicidin had a greater killing

effect when NADPH oxidase-derived ROS production was impaired. This is compatible with a macrophage mechanism where additional microbicidal responses have a greater contribution to bacterial killing only when other responses are sub-optimal. In this example, as previously mentioned, NADPH oxidase-derived ROS production is one of the key early macrophage responses to *S. aureus* challenge and is required for optimal clearance of this pathogen (269). A recent study has further illustrated the importance of NADPH oxidase in *S. aureus* killing, as NOX2 deficiency in BMDM resulted in enhanced intracellular survival of *S. aureus*, as well as improved viability of infected BMDM which led to the systemic spread of *S. aureus* in NOX2-deficient mice (562). However, it is possible that when NADPH oxidase-derived ROS production is impaired, macrophages enhance the activity of additional responses to play a greater role in bacterial killing to compensate for the reduced efficacy of a primary response. This could also suggest that the optimal timing or localisation of cathelicidin and its killing effects overlaps with the timing and localisation of NADPH oxidase-derived ROS, further emphasising the importance of timing and localisation of individual responses for the regulation of a multi-layered combinatory macrophage immune response.

Finally, my data also suggest that cathelicidin contributes to the regulation of mitochondrial dynamics and mROS production in macrophages in response to bacterial challenge, possibly as a brake to minimise bacterial-induced mitochondrial alterations as well as maintain mitochondrial integrity and homeostasis. Cathelicidin is known a diverse range of immunomodulatory properties which can exert pro-or anti-inflammatory effects in different contexts. In monocytes and macrophages, anti-inflammatory properties include reducing pro-inflammatory cytokine expression, for

example by neutralising LPS and LTA and preventing endotoxin-mediated TLR signalling (417,563), and upregulating anti-inflammatory cytokine expression (564).This evidence of cathelicidin's dampening of inflammatory responses, and my results, imply that cathelicidin may function to prevent excessive activation of mitochondrial-associated pro-inflammatory macrophage responses during bacterial infection which could help to minimise inflammation-induced damage to the host. In addition, my data suggests that cathelicidin may also contribute to enhanced MDM killing of intracellular *S. aureus* when mROS production is impaired, therefore cathelicidin's killing effects may spatially and temporally overlap with both a typically early ROS source (NADPH oxidase) and a late source (mROS). However, this mROS observation must be taken cautiously as it was only observed with both MitoPQ and MitoQ treatment and not each treatment individually, therefore it is still unclear which mROS alteration influences cathelicidin's effects.

6.5 Potential therapeutic applications

This thesis has provided a greater understanding of some of the macrophage microbicidal responses induced in response to *S. pneumoniae* or *S. aureus* challenge. A greater understanding of host responses to infection is crucial to inform the development of host-targeted therapeutic strategies to prevent antimicrobial resistance, and it is possible that some of the results presented in this thesis could help to inform future developments. Some particular examples are discussed below.

The use of cathelicidin, and modulation of its expression, has already been under investigation in different contexts as a novel antimicrobial strategy. In the context of *Mycobacterium tuberculosis* (Mtb), vitamin D- and PBA-induced

cathelicidin production has been shown to be important for the macrophage clearance of Mtb infection (453,565). Clinical trials to date have demonstrated that vitamin D supplementation, independently and in combination with phenylbutyrate intake, enhances vitamin D-mediated immune responses and increased bacterial clearance in tuberculosis patients (525–527). Therefore, as it is evident that therapeutic induction of *CAMP* by vitamin D and phenylbutyrate has a positive impact on clearing Mtb, it is possible that this would also be beneficial for enhancing macrophage immune responses against other bacterial infections. In the context of *S. pneumoniae* and *S. aureus* infections, vitamin D levels have been shown to influence susceptibility to disease. Vitamin D deficiency has been associated with a significantly increased susceptibility to developing community-acquired pneumonia (566), while vitamin D treatment on human neutrophils *in vitro* has been shown to promote neutrophil-mediated killing of *S. pneumoniae* (567). Studies into paediatric skin and soft tissue infections caused by *S. aureus* have demonstrated that children deficient in vitamin D are more likely to develop recurrent infections (568). Therefore, these studies suggest that sufficient vitamin D levels are important for the optimal clearance of *S. pneumoniae* and *S. aureus,* and further support the benefits of incorporating vitamin D supplementation into the treatment of *S. pneumoniae* and *S. aureus* infections.

In addition, my results on the impairment of vitamin D- and PBA-mediated *CAMP* expression by TNF-α and IFN-γ, which concur with studies in epithelial cells showing that pro-inflammatory cytokines impair vitamin D-mediated cathelicidin expression (436), highlight pro-inflammatory cytokine production as another potential mechanism that could be modulated to enhance *CAMP* expression in macrophages.

An association between vitamin D levels and cytokine expression has been described in a number of contexts; not only can cytokines influence vitamin D-mediated host defence responses, but vitamin D levels can influence the expression of both pro- and anti-inflammatory cytokines. High vitamin D levels have been associated with regulating inflammatory responses by promoting the expression of anti-inflammatory cytokines while reducing the expression of pro-inflammatory cytokines (569). Vitamin D deficiency has been linked to increased pro-inflammatory cytokine expression and more severe inflammation in a number of chronic conditions, such as atherosclerosis, asthma and inflammatory bowel disease (569,570). Therefore, dampening the production of pro-inflammatory cytokines or preventing the development of a sustained pro-inflammatory environment, by increased vitamin D supplementation or by other methods, could contribute to enhanced *CAMP* expression by limiting pro-inflammatory cytokine levels and also aid in the management of chronic inflammatory conditions. However, it is important to consider that this could also have pathological consequences if inhibition of the cytokines prevents them from activating other macrophage microbicidal responses.

Exogenous cathelicidin also shows a high degree of promise as a therapeutic treatment of bacterial infections due to its diverse microbicidal and immunomodulatory properties (418,460,461). There has been particular focus on the use of cathelicidin as a treatment for skin infections, as it is known to be produced in abundance by epithelial cells in response to infection (141,142,425). However, my data suggest that cathelicidin could be used to enhance macrophage microbicidal mechanisms and aid in bacterial killing, particularly of *S. aureus*. The evidence of greater cathelicidin microbicidal activity in the absence of NADPH oxidase-derived

ROS also suggests that cathelicidin could be particularly beneficial in the treatment of bacterial infections in a context of other macrophage responses being impaired. For example, cathelicidin could be a useful therapeutic factor in the treatment of bacterial infections in CGD patients, where NADPH oxidase is defective and susceptibility to *S. aureus* infections in particular is high (269). This should be investigated in future studies. An association between NADPH oxidase and vitamin D-mediated cathelicidin expression has been described in macrophages in the context of Mtb infection, where the absence of NOX2 function inhibited vitamin D-mediated upregulation of cathelicidin expression and subsequent cathelicidin-mediated antimycobacterial responses (571). If this were true in other settings cathelicidin, which could act as a safety net in microbicidal responses when NADPH oxidase is inhibited might actually not be being effectively generated under these circumstances and might require exogenous supplementation. However, treatment of NOX2-inhibited macrophages with exogenous cathelicidin was sufficient to rescue vitamin D-induced antimycobacterial activity in this example (571). Therefore, this suggests that deficiencies in NADPH oxidase could also impact endogenous cathelicidin expression and associated antimicrobial responses in macrophages, but also supports the therapeutic potential of cathelicidin as exogenous restoration of cathelicidin levels could mitigate some of these effects and improve macrophage antimicrobial functionality.

As mentioned previously, cathelicidin's diverse immunomodulatory properties can influence both pro- and anti-inflammatory responses in a context-dependent manner, and cathelicidin can employ both bactericidal and anti-inflammatory mechanisms during infection. A recent study in a murine bacterial sepsis model has

shown that cathelicidin promoted neutrophil bactericidal activity via release of NETs and ectosomes containing neutrophil granule components, while also preventing macrophage pyroptosis and release of pro-inflammatory cytokines to manage host inflammatory damage, in a murine sepsis model (572). Therefore, based on cathelicidin's diverse immunomodulatory properties, and my evidence that cathelicidin can enhance MDM killing of *S. aureus* while also serving as a brake on pathogen-mediated mitochondrial adaptations and mROS production, cathelicidin could be a useful therapeutic candidate for promoting bacterial clearance while also balancing potentially damaging excessive inflammatory responses in the host.

Finally for cathelicidin, a number of studies have demonstrated the synergy between cathelicidin and antimicrobial drugs on bacterial killing, where this synergy can often sensitise bacteria to lower MICs of the drugs or restore the effectiveness of drugs that bacteria have developed resistance against (573). For example, the synergy between cathelicidin and gentamicin has been shown to enhance the speed of bacterial membrane permeability over cathelicidin alone (573). Cathelicidin-derived short peptides have demonstrated synergy with vancomycin and enhanced the killing of *Pseudomonas aeruginosa,* a pathogen known to have low susceptibility to vancomycin treatment (574). In the case of *S. aureus*, cathelicidin synergy with teicoplanin was shown to overcome *S. aureus* resistance to cathelicidin and improve efficacy of cathelicidin in *S. aureus* killing (575). These studies further illustrate the diverse therapeutic potential of cathelicidin, and how antimicrobials that are currently considered ineffective or weak due to the development of resistance to be used as effective therapeutics once again.

Mitochondrial manipulation is also a promising avenue for therapeutic development due to the importance of mitochondria in mediating macrophage responses to infection. Several different approaches have been suggested, including enhancing mitochondrial biogenesis, altering mitochondrial signalling pathways, altering mitochondrial dynamics, and modulating mROS production (576). Research into the influence of current drugs, which are known to modulate mitochondrial function, in the treatment of various conditions has begun. For example, the mitochondrial electron transport chain complex I inhibitor, and therefore RET inhibitor, metformin is widely regarded as the frontline treatment for Type II diabetes (577), but is now being studied for its potential as a therapeutic in a number of other conditions. Metformin has been shown to be beneficial in the treatment of, and reducing the risk of, bacterial pneumonia in diabetes patients (578), has demonstrated anti-cancer potential (579), and can reduce pro-inflammatory cytokine production while increasing anti-inflammatory cytokine production in LPS-stimulated murine BMDM (580). This suggests that enhancing mROS may not be the optimal strategy as in many cases it may need to be reduced. Therefore, therapeutically altering mitochondrial processes would require careful control as excessive alterations could result in increased inflammation, and modulation would likely have to be individualised with both enhancement when sub-optimal and inhibition when excessive. My data highlight additional processes that could be investigated for therapeutic potential. In regard to *S. pneumoniae*, enhancing cathepsin B expression could promote mROS production and increased bacterial clearance by macrophages. For *S. aureus*, combining selective induction of NADPH oxidase-derived ROS and mROS in macrophages could aid in enhancing bacterial clearance, as well as modulating Drp1-mediated localisation of mROS to ensure its localisation

with intracellular bacteria. As with any other responses, it is likely that an optimal response would be to enhance early production but to ensure this is limited and turned off early so as to prevent prolonged and ineffectual responses.

6.6 Future directions

6.6.1 Detailed analysis of the kinetics, localisation and interactions of mitochondria, ROS production, and cathelicidin in macrophages in real time

My work has demonstrated that the mitochondrial dynamics and mROS production, NADPH oxidase-derived ROS production, and cathelicidin all contribute to macrophage microbicidal responses against bacterial challenge in different ways. However, the details of how these responses function and interact remain to be elucidated. Live imaging of macrophages in real-time over the course of bacterial challenge would be invaluable for investigating this. Combinations of these factors in live macrophages challenged with *S. pneumoniae* or *S. aureus* could be fluorescently stained and imaged in real time to determine the timing of expression and localisation of these factors and highlight any co-localisation or interactions between different factors. As well as visual representation, the real-time live images or videos could be analysed in a number of ways, including but not limited to fluorescence intensity of each factor over time, co-localisation analysis, or analysis of individual cells exhibiting a certain signal (for example, number of fluorescent ROS-positive cells in the population), to gain a much clearer understanding of exactly how these factors contribute to macrophage microbicidal responses. In addition, super resolution microscopy would be a useful technique in these studies, as the higher

resolution would provide much more precise visuals on the location of different antimicrobial factors in macrophages compared to confocal microscopy.

6.6.2 Roles of mitochondrial adaptations, ROS production and cathelicidin in tissue-resident macrophage populations, *in vivo* models, and patient groups

As mentioned previously, almost all of the studies conducted for this thesis used primary human monocyte-derived macrophages as the model. Although this is an appropriate and relevant model for analysis of human macrophage responses to bacteria, it provides less insight into how highly specialised tissue-resident macrophage populations which have distinct origins from MDM, such as alveolar macrophages (207), have adapted their antimicrobial responses to their niche and the pathogens encountered within. Therefore, it would be extremely interesting to investigate some of the macrophage responses detailed in this thesis, such as intracellular killing, mitochondrial dynamics, mROS production and *CAMP* expression, in different tissue-resident populations. Specialist macrophage populations can respond differently to bacterial challenges; for example, alveolar macrophages are well adapted for pneumococcal clearance in the lung (207,292,534), but a subset of splenic macrophages have been shown to exhibit defective pneumococcal killing and serve as an intracellular niche for the development of septicaemia (581). Therefore, future studies would highlight macrophage populations with defects in bacterial killing, provide more insight into how macrophages adapt their responses to their different niches and challenges, and potentially inform the development of host macrophage-based therapeutic strategies that are particularly adept at enhancing the responses of a specific macrophage population. Following these studies, it would be necessary to

investigate key findings *in vivo* in animal models such as mice or zebrafish. However, particularly in the case of mice, *in vivo* studies involving Drp1 knockouts have proved challenging, as complete Drp1 knockouts are embryonic lethal (344). To overcome this, methods such as tamoxifen-inducible Cre/loxP gene expression knockout systems have been developed (582,583). These conditional knockout systems allow for spatial and temporal control of, for example, Drp1 gene expression in different cells and tissues (583), or permit studies into systemic Drp1-knockout effects in post-natal and adult mice (582), as a means to overcome this embryonic lethality. Finally, it would be very interesting to study key findings in macrophages from patient groups that are particularly susceptible to *S. pneumoniae* or *S. aureus* disease, for example very young and very old patients or patients with other co-morbidities, in order to determine patient-relevant outcomes of these studies.

6.6.3 Impact of new or repurposed drugs on the modulation of mitochondrial adaptations, ROS production and cathelicidin expression in human macrophages

One of the overarching goals in this field of study is the discovery and development of new therapeutic strategies to prevent antimicrobial resistance, which includes research into new antimicrobial drugs, and repurposing of existing drugs, and their impact on modulating host defence responses. Using the example above, metformin has been shown to improve disease outcome in pneumonia patients (578) and in other conditions (579,580). Therefore, it would be beneficial to conduct mechanistic studies into how drugs such as metformin impact mitochondrial-associated responses and cathelicidin expression in human macrophages, both in steady-state conditions and in the context of *S. pneumoniae* or *S. aureus* challenge.

For example, does mitochondrial ETC complex I inhibition by metformin prevent excessive mROS production in these contexts, or does metformin treatment mediate spatial and temporal regulation of mROS for optimal pathogen clearance, by preventing early mROS production via RET and promoting mROS production from other mitochondrial sites? Answers to questions such as these would be very beneficial in informing how new or currently available drugs could be used in a novel way to treat bacterial infections. Furthermore, as synergy between cathelicidin and antimicrobials in relation to enhanced bacterial killing is known, it would be very interesting to determine if cathelicidin could combine with other drugs in a similar manner to enhance macrophage antimicrobial responses further, as evidence of another potential host-based therapeutic strategy.

6.7 Conclusion

In summary, this thesis has demonstrated that mitochondrial dynamics, mROS production and the cationic host defence peptide cathelicidin play important roles in macrophage microbicidal responses against *S. pneumoniae* and *S. aureus*, but the effects of these responses differ depending on the pathogen encountered. *S. pneumoniae* and *S. aureus* pose contrasting challenges to the macrophage, including representing low and high intracellular burdens, respectively. I have shown that *S. aureus* typically induces mitochondrial-associated macrophage responses much earlier than *S. pneumoniae,* likely as a result of the greater intracellular burden. Furthermore, cathelicidin plays particular roles in enhancing macrophage killing of *S. aureus*, as well as regulating mitochondrial dynamics and function to minimise pathogen-associated alterations. These findings demonstrate that macrophages require combinations of different factors to function in a multi-layered

immune response to optimally clear pathogens. In addition, *CAMP* expression can be modulated by a number of factors in macrophages, highlighting this as a potential avenue, alongside the use of exogenous cathelicidin and modulation of mROS production and localisation, for investigation as a potential host-based therapeutic approach to help combat antimicrobial resistance. Further studies into the mechanisms regulating the microbicidal responses discussed and more in-depth analysis of the timing and localisation of macrophage mitochondrial adaptations and cathelicidin in response to bacterial challenge, and the responses of different specialist tissue-resident macrophage populations to bacterial challenge, would provide a greater understanding of the complex interweaving of macrophage microbicidal responses as well as identify other possible avenues for host-based therapeutic development.

Chapter 7

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