Investigating phagosome dynamics of microbial pathogens

By

Leanne May Smith

UNIVERSITY OF BIRMINGHAM

A thesis submitted to the University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

School of Biosciences

College of Life and Environmental Sciences

University of Birmingham

September 2014

Abstract

Many microbial pathogens are able to evade killing by phagocytes of the innate immune system. This thesis focuses on two pathogens: the fungal pathogen *Cryptococcus neoformans* and the bacterial pathogen *Streptococcus agalactiae*. *C. neoformans* causes severe cryptococcal meningitis in mostly immunocompromised hosts, such as those with HIV infection. In contrast, *S. agalactiae* is the leading cause of neonatal sepsis and meningitis. The interaction between macrophages and these pathogens is likely to be critical in determining dissemination and outcome of disease in both instances.

A collection of *S. agalactiae* clinical isolates, ranging in origin from colonisation cases to severe infection cases, were tested for their ability to persist with a macrophage cell line. Surprisingly, persistence within macrophages was a characteristic shared by all of the isolates tested. Furthermore, by investigating the *Streptococcus*-containing phagosome, it was revealed that streptococci are able to manipulate the acidification of macrophage phagosomes.

Similarly, the maturation of phagosomes containing the fungal pathogen *C. neoformans* was explored. Cryptococci are shown to be able to manipulate the phagosome they reside within. This is driven by modified acquisition of Rab GTPases to the phagosome, as well as altered acidification and cathepsin activity within *Cryptococcus*-containing phagosomes.

Acknowledgements

A PhD is a qualification awarded to an individual. However, no PhD is truly accomplished by an individual alone without the help and support of those around them. I therefore use this section of the thesis to thank some of the people that helped me along my way over the last four years (well… actually 8 years in total, so far!) at the University of Birmingham.

Research itself is often an expensive business so there are a few funding bodies to be thanked for helping to pay for all the fun times. Firstly, the University of Birmingham for the scholarship that kept a roof over my head. Secondly, the funding bodies that paid for the research itself, the Wellcome Trust, Lister Institute, Medical Research Council (MRC) and National Institute for Health Research (NIHR). Finally, the scientific societies that payed for my attendance at various conferences during my PhD studies, providing me with the opportunity to present my work to experts in the field (and also see a little more of the world than I otherwise would!), the Society for General Microbiology (SGM), the Federation of European Microbiological Societies (FEMS) and the Society of Experimental Biology (SEB).

I would also like to thank some people who provided various reagents and microbial strains that made this work possible: Arturo Casadevall, Julian Rutherford, Mark Anthony, Marie-Cécile Lamy and Howard Jenkinson. And, of course, Mark Webber and Bernhard Hube for examining this thesis and making the entire process surprisingly not too scary! Thank you both.

I also owe a great deal of gratitude to Julia Lodge. As my undergraduate tutor it was you who mentored me through my first years at university and taught me many of the skills I

would require for this PhD, such as: at least knowing what end of a pipette is which! Quite crucial really! Thanks for giving me the basics in scientific writing and practical skills. I'm so pleased we ended up moving offices and lab space; hearing your booming laughter from down the corridor always makes the residents of N110 laugh along too!

Next up, my supervisors, Robin May and Anne-Marie Krachler. Both of you have been wonderful and inspiring people to work with and be mentored by. Robin, without a doubt you are the most enthusiastic, optimistic, limb-flailing person anyone could know! These qualities make you one of the best people to come to with any worries, professional or personal. You care a great deal about all of us and we all appreciate that so much. Thanks for making such an amazing environment to scientifically 'grow up' in.

Now for the people in the lab. Firstly: Nicola you were the one to initially show me the ropes in the lab, thank you for you're patience. Simon, you showed me how to use our (slightly tempermental at times) microscopes. Thanks so much for getting me hooked! Kerstin and Elizabeth, thank you both for making those first couple of years that little more hilarious. Becky, although you joined the lab later, it seems as if you've been here the whole time too! Thanks to all of you for being my mini mentors and for being my friends.

The current lab: Robbie, Peter, Andy, Emily, Lea and Dan and the whole HAPI group – thanks for making the 'job' so fantastically comical. Working with you all makes everything so much easier.

Finally, to my Family – thanks for doing your best to understand what was going on! And, of course, Becki – thank you for supporting me and putting up with all the panic moments that no one else saw.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AIDS	acquired immune deficiency syndrome
AMPs	anti-microbial peptides
Arp	actin-related protein
ATP	adenosine-5'-triphosphate
BBB	blood brain barrier
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCPs	*Cryptococcus*-containing phagosomes
CD	cluster of differentiation
CFU	colony forming unit
CNS	central nervous system
CR	complement receptor
CSF	cerebrospinal fluid
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EOD	early-onset disease
FBS	fetal bovine serum
Fc	fragment crystallisable
FcαR	Fc alpha receptor
FcεR	Fc epsilon receptor
FcγR	Fc gamma receptor
GAS	group A *Streptococcus*
GBS	group B *Streptococcus*
GDI	guanosine dissociation inhibitor
GEF	guanine exchange factor
GM-CSF	granulocyte macrophage-colony stimulating factor
GTP	guanosine-5'-triphosphate
GXM	glucuronoxylomannan

GXMGal	glucuronoxylomannangalactan
HIV	human immunodeficiency virus
HMDM	human monocyte derived macrophage
IAP	intrapartum antibiotic prophalaxis
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
LAMP	lysosomal associated membrane protein
LB	Luria-Bertani
LMS	Leanne May Smith
LOD	late-onset disease
LPS	lipopolysaccharide
LTA	lipoteichoic acid
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MHC	major histocompatibility complex
MLST	multilocus sequence typing
MNGC	multi-nucleated giant cell
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa-B
NK	not known
NK cell	natural killer cell
NM	no multilocus sequence type data
NO	nitric oxide
NT	non-typable
N-WASP	neuronal Wiskott-Aldrich syndrome protein
OD	optical density
PAMPs	pathogen associated molecular patterns
PBS	phosphate buffered saline

PFA	paraformaldehyde
PI	post infection
PI3P	phosphatidylinositol 3-phosphate
PI3K	phosphoinositide 3-kinase
PMA	phorbol myristic acetate
ppGpp	guanosine tetraphosphate
PRRs	pattern recognition receptors
PS	phosphatidylserine
PV	parasitophorous vacuole
RILP	Rab-interacting lysosomal protein
RNS	reactive nitrogen species
ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SAJ	Simon Andrew Johnston
SCPs	*Streptococcus*-containing phagosomes
SNARE	soluble NSF attachment protein receptor
T3SS	type three secretion system
T4SS	type four secretion system
T-cell	Thymus cell
Th	T-helper
THY	Todd-Hewitt yeast
TLR	Toll-like receptor
TNFα	tumour necrosis factor alpha
TRITC	Tetramethylrhodamine isothiocyanate
TraDIS	transposon-directed insertion site sequencing
vATPase	vacuolar-type adenosine triphosphatase
WASH	WASP and SCAR homologue
YPD	yeast peptone dextrose

Chapter I. Introduction

Much of this introduction has been previously published as a review article.

Smith, L.M. and May, R.C. (2013) Mechanisms of microbial escape from phagocyte killing. *Biochem Soc Trans* **41**: 475-490.

Phagocytosis and phagosome maturation are crucial processes in biology. Phagocytosis and the subsequent digestion of phagocytosed particles is used by both single-celled and multi-celled eukaryotic organisms. The phagocytosis of apoptotic cells and intruding microorganisms has been extensively studied in the scientific community. This introduction focuses on a central cell of innate immunity, the macrophage, and this cell's interactions with microbial pathogens. Our current knowledge of phagosome maturation is summarised and the myriad of mechanisms microbes have established to escape phagocytic killing will be discussed.

Phagocytosis is arguably one of the most important processes in biology. From single celled amoebae using phagocytosis for nutrition, to phagocytic cells of the vertebrate immune system using phagocytosis to destroy microbes and present antigens, phagocytosis plays an important role in many aspects of biology. We will consider the diverse strategies that microbial pathogens use to escape killing by phagocytes. These strategies fall into three broad categories: diversion and modification of the phagosome maturation process, general resistance and persistence within the phagolysosome, or physical escape from either the phagosome or the entire phagocyte.

Phagosome Maturation

Phagocytosis is typically defined as the uptake of particles larger than 0.5 μm, which includes most bacteria, fungi, protozoa and other eukaryotic cells. There is, however, a limit to the size of an object that can be phagocytosed (Chen *et al.*, 1997). Phagocytosis is initiated via receptor engagement, either via microbial ligands or host ligands deposited on the pathogen surface. Pattern recognition receptors (PRRs) recognise pathogen associated molecular patterns (PAMPs) on the surface of microbes and these include Mannose receptor, Dectin-1, CD14, scavenger receptor A, CD36 and MARCO (Jaumouillé and Grinstein, 2011). Opsonic receptors recognize host molecules deposited on the surface of microbes and include FcγR variants, FcαR, FcεR, CR1, CR3, CR4 and the integrin $\alpha_5\beta_1$ (Flannagan *et al.*, 2012; Jaumouillé and Grinstein, 2011). For most microbes, entry into phagocytes probably involves a combination of these receptors recognising different aspects of the microbial surface. However, the receptor that dominates in this initiation of phagocytosis is likely to play a major role in determining the precise route of phagosome maturation and indeed microbial fate.

Once internalised, the contents of the phagosome must be digested. For antigen presenting phagocytes such as macrophages and dendritic cells, antigens derived from the degraded microbe are then secondarily presented on the surface of the phagocyte. To achieve this degradation, phagosomes undergo a series of maturation stages, receiving new material from early endosomes, late endosomes and finally lysosomes and also losing molecules no longer required via sorting and recycling endosomes, creating a dynamic phagosome membrane (Kinchen and Ravichandran, 2008). The molecular detail involved in progressing from early and late stages through to the

mature phagolysosome has been the topic of much research for many years. However, finding suitable control particles to study "normal" phagosome maturation is challenging. For example, latex beads have been used for this purpose for many reasons, including the range of particle sizes available and the ability to covalently link molecules to their surface. Indeed, they have been invaluable in probing the early stages of phagosome maturation (Desjardins *et al*., 1994). However, since these particles are essentially indestructible by phagocytes, they are of limited value in investigating the later phases of phagolysosome development. In particular, it is now clear that phagosomes containing living (or once living) things are processed in a different way to those containing inert particles. For example the tetraspanin CD63 is now used as a marker of mature phagolysosomes (although its exact role is still being determined) yet this marker is not found on phagosomes containing latex beads (Artavanis-Tsakonas *et al*., 2006). To address this, most research efforts now use non-pathogenic microbes alongside latex beads as targets and this dual approach has now led to a comprehensive model for normal phagosome maturation (Figure 1).

Despite this highly-coordinated process that is intended to kill invading microbes, many pathogens have evolved mechanisms to enable their survival within macrophages. Below, we consider the different classes of survival mechanism and highlight some outstanding questions in the field.

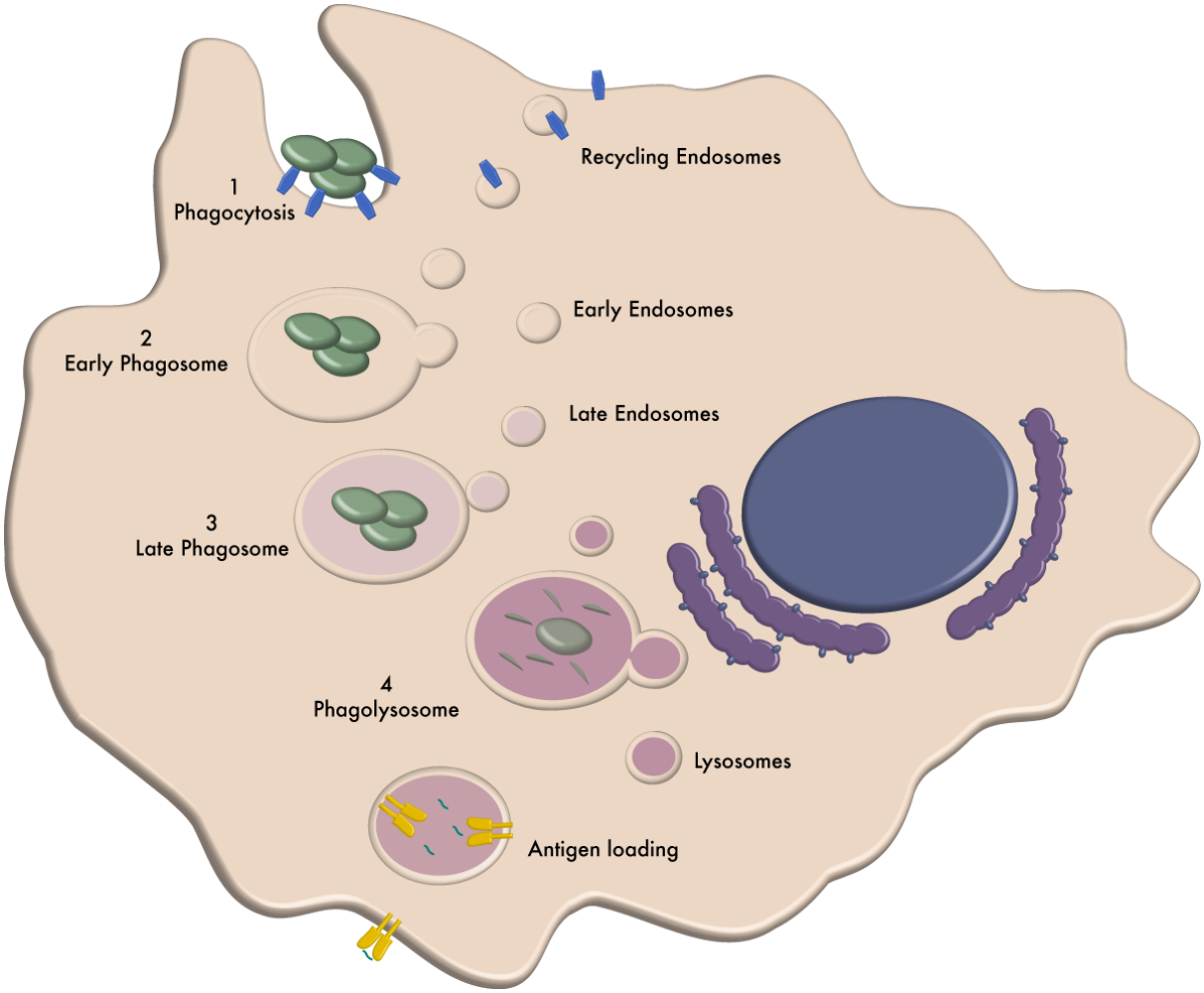

Figure 1. Phagosome maturation.

1, Phagocytosis of microbes via phagocytic receptors. Receptor engagement initiates cytoskeletal rearrangements and pseudopod extensions to surround and eventually fuse around the microbe, creating an internal phagosome. **2**, the early phagosome rapidly loses plasma membrane associated actin and PI(4,5)P2. Early phagosomes interact with early endosomes and recycling endosomes, allowing delivery of PI3P, Syntaxin-13 and the GTPase Rab5. After Rab5 activation the Rab5 effectors Vps34, Mon1 and Rab7 are recruited. Vps34 recruits proteins with FYVE or PX domains such as sorting Nexins, Hrs, p40phox and the early endosome marker EEA1. EEA1 is a bridging molecule that aids vesicle fusions. The Rab5-Mon1-Ccz1 complex recruits Rab7 and displaces the Rab7 GDI. **3**, the late phagosome becomes more acidic internally (~pH5.5), sheds early phagosome markers and acquires late markers, such as Rab7, Mon1, RILP and HOPS (Vps11, 16, 18 and 33). RILP and ORPIL (recruited by Rab7) are dynein adaptors that direct late phagosomes towards the microtubule organising centre, the collecting place for lysosomes. **4**, the phagolysosome is finally formed when late phagosomes fuse with lysosomes that are packed with hydrolases (nucleases, lipases, glycosidases, proteases and cathepsins) and a highly acidic content (~pH4.5). Lysosomes also deliver membrane proteins LAMP1 and the vATPase hydrogen ion pump.

Strategies of Diversion and Modification of Phagosome Maturation – Making the Environment More Comfortable.

Many pathogens, whether obligate intracellular pathogens or faculative, have evolved mechanisms to pause, arrest or redirect entirely the phagosome maturation pathway (Figure 2). If successful, pathogens are then able to lie dormant within a protected 'Trojan horse', disguised from the immune system. The most widely studied example of such a process occurs in *Mycobacterium tuberculosis*, the causative agent of human tuberculosis. Not only is this organism able to arrest normal phagosome maturation (Via *et al*., 1997), but *M. tuberculosis* may be able to escape the phagosome (Myrvik *et al*., 1984; Smith *et al*., 2008). The *M. tuberculosis*-containing phagosome retains the characteristics of an early phagosome, such as Rab5 (Via *et al*., 1997) and recycling endosome communication (Clemens and Horwitz, 1996), however, acidification is blocked by excluding vATPase (Sturgill-Koszycki *et al*., 1994). *M. tuberculosis* phagosomes also avoid lysosome fusion by disrupting host calcium signaling (Jha *et al*., 2010; Malik *et al*., 2003). *Mycobacterium marinum,* a close relative of *M. tuberculosis*, is able to escape the phagosome and use actin based motility within the cytosol (Stamm *et al*., 2003). This huge field of research has recently been comprehensively reviewed (Gengenbacher and Kaufmann, 2012; Soldati and Neyrolles, 2012; Welin and Lerm, 2012) and will not be considered further here.

Phagosome maturation is also manipulated by the food-borne pathogen *Salmonella enterica,* which arrests phagosome maturation at a late but not fully mature stage, creating a *Salmonella* containing vacuole (SCV)(Beuzon *et al*., 2000; Oh *et al*., 1996; Scott *et al*., 2002). Recent data suggests that this process may be dependent on the host cell

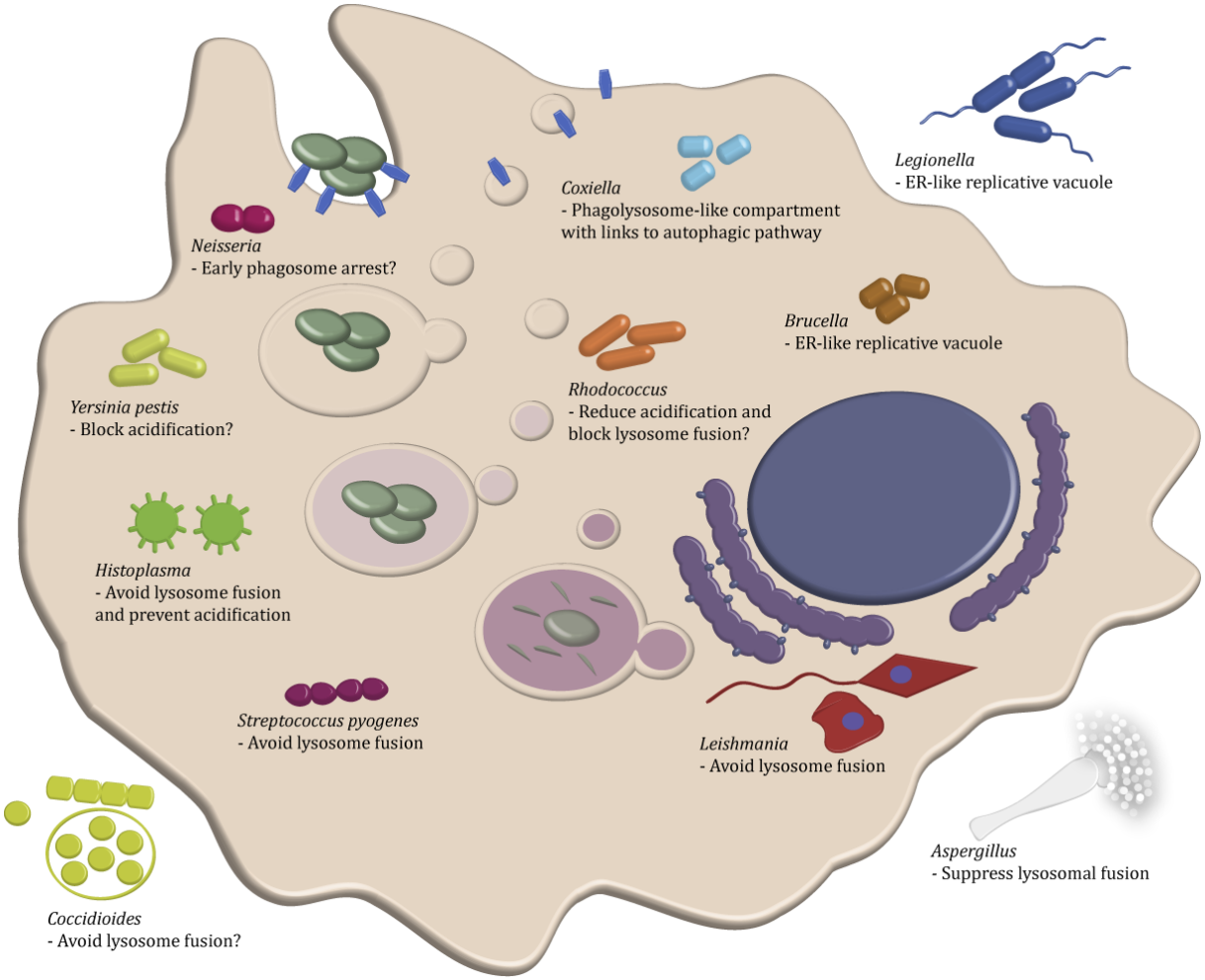

Figure 2. Strategies used by pathogens to modify phagosome maturation. Many pathogens are able to manipulate the phagosome. The pathogens discussed in this review and a summary of the modification they exert on the phagosome is illustrated here. Endoplasmic reticulum (ER).

type. For instance, novel *Salmonella* spacious phagosomes have recently been described for B cell infection (Rosales-Reyes *et al*., 2012). Phagosome escape (Brumell *et al*., 2002; Knodler *et al*., 2010) and autophagy (Birmingham and Brumell, 2006; Kageyama *et al*., 2011) have also been implicated in *Salmonella* infection. Because the intracellular *Salmonella* field goes beyond the scope of this introduction, I will not be covering this pathogen. However, I direct readers to recent in-depth reviews (Malik-Kale *et al*., 2011; Monack, 2012). This section will continue exploring some pathogens that are less well known for their ability to alter phagosome maturation.

Neisseria gonorrhoeae

Neisseria gonorrhoeae is a Gram-negative bacterium and the causative agent of gonorrhea in humans. *N. gonorrhoeae* is best known for its infection of epithelial cells, however, once gonococci have traversed this cellular layer they are likely to be engulfed by professional phagocytes such as macrophages and neutrophils. Indeed, gonococcal infection is characterised by inflammation and neutrophil recruitment.

Although relatively poorly studied, it is clear that *N. gonorrhoeae* can manipulate phagocytes in several diverse ways. *Neisseria* gonococcal porin is able to reduce reactive oxygen species (ROS) produced by primary human neutrophils and monocytes, thus limiting their antimicrobial activity (Lorenzen *et al*., 2000). The same porin has also been implicated in blocking phagosome maturation (Mosleh *et al*., 1998), since treatment of phagocytes with purified porin (PorB), causes latex bead phagosomes to retain increased amounts of early markers (such as Rab5) and reduced late markers (such as Rab7). Furthermore, *N. gonorrhoeae*-containing phagosomes are able to block

fusion with primary granules (protease storing vesicles) of neutrophils (Johnson and Criss, 2013). This fusion blocking appears to be orchestrated by a surface component on the bacteria as dead *N. gonorrhoeae* are also able to exert the effect (Johnson and Criss, 2013). Neutrophil reactive oxygen species (ROS) may play a controversial role in aiding *N. gonorrhoeae* pathogenicity. Recent research has found opacity proteins (Opa) of the bacteria play a role in host NADPH oxidase assembly, therefore contributing to ROS production (Smirnov *et al*., 2014). One explanation for this unusual activity could be that host ROS is used to initiate the activation of bacterial genes required for intracellular survival within neutrophils. The production of ROS being used against the host phagocyte to activate a pathogenicity program has also been seen for other pathogens. For example, it has recently been found that macrophage ROS can be used as a trigger for mitochondrial tubularisation and intracellular survival of the fungus *Cryptococcus gattii* (Voelz *et al.,* 2014).

N. gonorrhoeae also secretes proteases that digest the lysosomal associated membrane protein, LAMP1. This process appears important in mediating gonococcal survival within epithelial cells and fibroblasts, although has yet to be investigated in phagocytic cells (Binker *et al*., 2007).

Yersinia pestis

Yersinia pestis is the infamous bubonic plague causing Gram-negative bacterium. Upon engulfment, *Y. pestis* up-regulates a putative stress induced operon. Deletion of the stress induced operon, or the previously unidentified gene, *orfX*, leads to down-regulation of Type III Secretion, higher intracellular replication and a filamentous

morphology of internal bacteria (Fukuto *et al*., 2010). Transposon mutagenesis has also recently revealed a role for glucose-1-phosphate uridylyltransferase (galU) and the UDP modifying enzymes WecBC in the intracellular survival of *Y. pestis* (Klein *et al*., 2012). Once intracellular, *Y. pestis* containing vacuoles seem to fuse with lysosomes (Straley and Harmon, 1984), acquiring cathepsin D and LAMP1 (Grabenstein *et al*., 2006), and in some cases are positive for the LC3 autophagosome marker. Despite this, the vacuoles maintain a neutral pH (Pujol *et al*., 2009), suggesting modification of the *Y. pestis*-containing phagosome is likely to occur, although the precise stages of maturation interrupted are currently unknown.

The above relates to the *Y. pestis* – macrophage interaction. Less is known about the interaction of this pathogen with neutrophils. *Y. pestis* is able to replicate within macrophages, perhaps explaining why more attention has been given to this line of research. Within neutrophils, however, *Y. pestis* are generally killed with the exception of a small resister population recently found to still be viable at 22 h after phagocytosis (Spinner *et al*., 2014). Interestingly, after 12 h, infected neutrophils express phosphatidylserine (PS), a marker of apoptosis. This marker acts as a signal to macrophages to engulf apoptosing neutrophils by a process termed efferocytosis (Spinner *et al*., 2014). The authors hypothesise this as a 'Trojan horse' mechanism for a small percentage of *Y. pestis* to gain entry to the more hospitable niche within macrophages.

Legionella pneumophila

Legionella pneumophila is a Gram-negative facultative intracellular bacterium. Like many of the microbes discussed in this review, *L. pneumophila* primarily causes respiratory infections and is known to interact with, and replicate within, human alveolar macrophages. Infection with *L. pneumophila* can lead to an acute form of pneumonia known as Legionnaires' disease. *L. pneumophila* is naturally adapted to an intracellular lifestyle as it parasitises fresh water amoebae, hence outbreaks of Legionellosis are often associated with contaminated water supplies (Roy *et al*., 1998; Swanson and Sturgill-Koszycki, 2000). Early work by Horwitz and colleagues demonstrated that the *L. pneumophila* phagosome is less acidic than other non-pathogen phagosomes and does not fuse with lysosomes (Horwitz, 1983b). Additionally, within two hours of uptake by human monocytes, *L. pneumophila* phagosomes associate with mitochondria and the rough endoplasmic reticulum (RER), with the phagosome membrane becoming studded with ribosomes (Horwitz, 1983a).

More recent data have shown that the diversion of *L. pneumophila* - containing phagosomes away from the normal route of maturation, preventing them from acquiring LAMP1 or Rab7, occurs rapidly after uptake and requires the *L. pneumophila* product DotA. DotA is an inner membrane protein believed to be required for the formation of a macromolecular complex to direct the phagosome towards a replicative vesicle form (Roy *et al*., 1998).

Additionally, the fate of the *L. pneumophila* phagosome seems to be determined within minutes of uptake, as DotA negative bacteria were seen to already be LAMP1 positive at this premature stage (Roy *et al*., 1998). DotA is part of the Dot/Icm T4SS transporter

(Coers *et al*., 1999), and acts to export a series of effector proteins, including RalF. RalF acts as a Guanine exchange factor for the host ARF-1 protein, which regulates vesicle traffic between the ER and Golgi. In addition, active ARF-1 is only found on phagosomes containing *L. pneumophila* with the wild type *dot/icm genes* (Nagai *et al*., 2002). Further evidence for the rapid determination of phagosome fate comes from Stephen Weber and colleagues. Using live-cell imaging of the *L. pneumophila* phagosome within the amoebae *Dictyostelium discoideum*, the authors demonstrate that *L, pneumophila* cells are able to manipulate phosphoinositide (PI) lipids on the phagosome membrane. Phagosomes containing wild type *L. pneumophila* are able to speed up the removal of PI(3)P and yet accumulate PI(4)P (Weber *et al*., 2014). This manipulation appears to hold the vacuole in a quiescent state, allowing avoidance of degradation.

Once *L. pneumophila* has modified the phagosome appropriately, it then converts to a replicative form. This form is unable to secrete virulence factors (such as RalF) and thus the replicative vacuole slowly acquires lysosomal markers and becomes nutrient depleted after several rounds of bacterial replication (Byrne and Swanson, 1998). However, the accumulation of guanosine tetraphosphate (ppGpp) within this organelle eventually triggers a return to stationary phase and re-expression of virulence factors (Hammer and Swanson, 1999; Swanson and Sturgill-Koszycki, 2000). After approximately 24 h of replication, cells then burst open, although it is currently unclear whether this is due to the physical limits of phagocyte membranes or host cell apoptosis (Roy *et al*., 1998).

Streptococcus pyogenes (GAS)

Streptococcus pyogenes, or Group A *Streptococcus*, is a Gram-positive bacterial pathogen most commonly thought of as an extracellular infectious agent. However, *S. pyogenes* is able to cause serious deep tissue infections (such as necrotising fasciitis) in which macrophage reservoirs of persisting bacteria are likely to play an important role in disease progression. A well-known virulence factor of this particular *Streptococcus* is the M1 protein. Recent findings indicate a role for M1 protein in controlling vesicle trafficking and preventing lysosomal fusion of *S. pyogenes* phagosomes, as well as in suppressing the macrophage inflammatory response via NFκB signaling (Hertzen *et al*., 2010). Additionally, the regulon of the transcription factor Mga has been linked to vATPase acidification blocking by reducing vATPase delivery to the phagosome by an as yet undetermined mechanism (Nordenfelt *et al*., 2012).

Microarray studies looking for altered expression of *S. pyogenes* genes have identified 145 genes that are significantly altered following uptake. These included the genes encoding the CovS/CovR two-component system that has also been implicated in intracellular survival of the related pathogen *S. agalactiae* (Cumley *et al*., 2012; Nordenfelt *et al*., 2012).

Histoplasma capsulatum

The dimorphic fungus, *Histoplasma capsulatum* is the causative agent of the life-threatening infection histoplasmosis. During infection, *H. capsulatum* resides within a modified phagosome of macrophages, considered to be the primary infected cells within hosts. *H. capsulatum* survives within macrophages by modifying the vacuole and keeping

it at pH 6.5 by blocking acidification via the vATPase and lysosomal fusion with the phagosome (Strasser *et al*., 1999). This incomplete block of acidification is likely to reflect a compromise between enabling fungal utilisation of iron from transferrin (at low pH), whilst still reducing the hydrolytic activity of acid-dependent proteases. Interestingly, *H. capsulatum* actively retains a slightly acidic phagosomal pH even when normal phagosomal acidification is blocked by the vATPase inhibitor Bafilomycin (Strasser *et al*., 1999). Suggesting the pathogen itself creates the slightly acidic phagocytic lumen rather than allowing partial vATPase activity.

Histoplasma capsulatum is thought to replicate within macrophages and eventually lyse the host cell (Newman *et al*., 1991). A recent screen identified a mutant lacking the gene encoding 3-hydroxy-methylglutaryl coenzyme A (HMG CoA) lyase (*HCL1*) was unable to lyse host cells (Isaac *et al*., 2013). Further investigation revealed that *HCL1*, which is required for leucine catabolism, allows the fungus to multiply within a phagosome (Isaac *et al*., 2013). Without *HCL1*, acidic products accumulate due to the block of leucine metabolism. Therefore, without the gene, *H. capsulatum* find themselves unable to replicate and cause host cell lysis. The *HCL1* mutant shows partial attenuated virulence in an intranasal mouse model of infection, highlighting the potential relevance of leucine metabolism to infection.

Studies into *H. capsulatum* with human and mouse macrophages have highlighted differences between these two cell types; human macrophages do not need to induce phagosome acidification for effective fungal killing, whereas a low phagosomal pH is critical for the antifungal activity of mouse macrophages (Newman *et al*., 2006).

Phagosome manipulation by *H. capsulatum* can be altered by pre-treatment of the fungus with monoclonal antibodies. Non-opsonised *H. capsulatum* is typically engulfed via CR3. Opsonisation with IgM monoclonal antibody (mAb) to *H. capsulatum* cell surface histone 2B, increases phagocytosis via CR3 (Nosanchuk *et al*., 2003), but simultaneously decreases the ability of *H. capsulatum* to survive and replicate within macrophages (Nosanchuk *et al*., 2003). A further study revealed the reduction in intracellular viability was due to a decrease in the ability of the pathogen to modify the pH of the phagosome (Shi *et al*., 2008). Thus providing an interesting example of antibody opsonisation altering the intracellular fate of a pathogen, without changing the entry route. Moreover, this monoclonal antibody against histone 2B also improves mouse survival in a histoplasmosis infection model by reducing fungal burden and pulmonary inflammation (Nosanchuk *et al*., 2003). A potential explanation for the attenuated virulence could be that the H2B mAb alters cell wall biosynthesis and re-modeling, affecting growth of the fungus (Nosanchuk *et al*., 2003).

***Leishmania* spp.**

Leishmania are parasitic protozoa that are spread by sand fly vectors in tropical and subtropical regions of the world. *Leishmania* can exist in two life cycle stages, the promastigote and amastigote, both of which can be phagocytosed by means of receptor mediated phagocytosis (Sacks and Sher, 2002). The receptors used for uptake vary depending on species, stage and serum quality but generally involve CR2, CR3 or the mannose receptor (Alexander and Russell, 1992; Rodriguez *et al*., 2011). Upon internalisation, promastigotes insert lipophosphoglycan (LPG) into the phagosome

membrane, which inhibits the depolymerisation of F-actin and thus delays lysosomal fusion (Holm *et al*., 2001; Rodriguez *et al*., 2011; Winberg *et al*., 2009). This delay possibly provides enough time for the parasite to convert to the amastigote form, which is more resistant to hydrolases and thus able to replicate within the phagosome. It is worth bearing in mind that the dynamics of phagosome maturation are dependent on the route of uptake. For example, phagocytosis mediated via CR3 and FcγR results in a five hour delay in phagosome maturation, while CR3/FcγR-independent phagocytosis only results in a one hour delay in phagosome maturation. Surprisingly, the difference in phagosome maturation time does not impact on the eventual viability of intracellular *Leishmania donovani* (Polando *et al*., 2013).

Recent investigations into the mechanism used by *L. donovani* to alter the fusogenic properties of the phagosome have discovered a surface anchored metalloprotease GP63 (Matheoud *et al*., 2013). This protease has been found to cleave the soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein VAMP8, a molecule involved in orchestrating fusion of vesicles, such as the lysosome with the phagosome. By disrupting SNARE proteins, the promastigote is able to alter NADPH oxidase assembly and antigen presentation, potentially having substantial affect on this pathogen's ability to avoid the immune response (Matheoud *et al*., 2013).

The amastigote, in contrast to the promastigote, is specialised for survival within an acidic and hydrolytically active environment with much of its metabolism requiring low pH for optimal efficiency (Antoine *et al*., 1990). Persistence and replication in the mature phagolysosome is also aided by cell surface and secreted glycoconjugates such as glycoinositol phospholipids (GIPLS) and proteophosphoglycan (PPG) and by active

inhibition of protein kinase C, which blocks assembly of the NADPH oxidase, lowering the phagocyte oxidative burst (Descoteaux *et al*., 1992; Sacks and Sher, 2002).

Sub-cutaneous infection with *L. major* will recruit large quantities of neutrophils (Sunderkötter *et al*., 1993). The usual response of neutrophils is to phagocytose invading pathogens. In some cases, if neutrophils become overwhelmed, they undergo apoptosis, exposing the pathogen to other cells of the immune system, but also releasing tissue damaging molecules. This apoptosis usually happens within 6-12 h. However, *L. major*, although not replicating, is able to delay neutrophil apoptosis up to 42 h (Aga *et al*., 2002). It is at this time that macrophages arrive at the site of infection (Sunderkötter *et al*., 1993). Studies have therefore looked at the interaction between infected neutrophils, undergoing apoptosis, and macrophages. Infected neutrophils released macrophage chemo attractants such as MIP1-β. The infected and apoptotic neutrophils are then efferocytosed by macrophages, after which, *L. major* is then able to enter the replicative amastigote form (van Zandbergen *et al*., 2004). The work describes an astonishing manipulation of the host immune system to allow the parasite to gain entry into its final replicative niche.

Coxiella burnetii

The Q fever agent, *Coxiella burnetii*, replicates within a modified phagosome in many host cell types. It is a Gram-negative obligate intracellular bacterial pathogen able to create a large phagolysosome-like compartment. The mature *C. burnetii* phagosome looks like large phagolysosome but is actually a modified compartment (Ghigo *et al*., 2012), that recruits the autophagosome marker LC3. If autophagy, or the host signaling

factors cAMP and protein kinase A, are pharmacologically inhibited, *C. burnetii* replication is reduced (Beron *et al*., 2002; MacDonald *et al*., 2012). Additionally, the Dot/Icm T4SS has recently been found to be required for recruitment of autophagosomal membranes at later stages of intracellular infection (Winchell *et al*., 2014). It is currently thought that the autophagosome membrane contributes to the phagosome, allowing expansion of the *C. burnetii* – containing vacuole.

This modified phagosome still recruits the small GTPases Rab5 and Rab7, yet inhibits accumulation of antimicrobial proteases (as measured by the presence of cathepsin D) (Graham *et al*., 2012), although at present the link between autophagy and phagosomal modification remains unclear. By use of its lipopolysaccharide (LPS), *C. burnetii* is able to antagonistically bind TLR4, therefore blocking p38α-MAPK signaling and homotypic fusion protein vps41 recruitment to the vacuole, thus blocking lysosome fusion (Barry *et al*., 2012).

Rhodococcus

Rhodococcus equi, causes severe pneumonia in horses and tuberculosis-like symptoms in AIDS patients. Following uptake, this Gram-positive facultative intracellular pathogen transiently acquires maturation markers such as PI3P, EEA1 and Rab5, but not cathepsin D or vATPase (Fernandez-Mora *et al*., 2005; Toyooka *et al*., 2005). This non-acidic phagosome can be maintained for 48 h in a manner that is dependent on the presence of the bacterial VAP virulence plasmid and the gene encoding β-ketoacyl-(acyl carrier protein)-synthase A (KasA), which is essential for long chain mycolic acid synthesis (Sydor *et al*., 2012).

Coccidioides spp.

Coccidioides immitis and *C. posadasii* are dimorphic fungi found in regions of southern USA and pockets of South America where valley fever (coccidioidomycosis) is endemic. Coccidioidomycosis mostly remains asymptomatic, but can cause serious complications and disseminated disease (such as meningitis) in immunodysfunctional individuals (Borchers and Gershwin, 2010).

Coccidioides can be found in the soil in a mycelial form that produces infectious arthoconidia. Arthoconidia transform into large spherules (containing 100-300 endospores) rapidly after inhalation by humans (Huppert *et al*., 1982). Both arthoconidia and endospores can be phagocytosed by alveolar macrophages, although phagocyte activity against them appears to be fungistatic rather than fungicidal (Beaman and Holmberg, 1980). Even in immunocompetent individuals neutrophils will kill only up to 30% of invading *C. immitis* arthoconidia (Ampel *et al*., 1992). Spherules, on the other hand, are too large to be phagocytosed (with a 60-100 μm diameter), so no defense can be utilised until the endospores are released. Human monocytes and macrophages do, however, mount an inflammatory response to spherules, consisting of an increased production TNF-α, IL-1β, and IL-6 (Dooley *et al*., 1994).

Following uptake, endospores appear to inhibit lysosome fusion (Beaman and Holmberg, 1980) and can eventually lyse the host cell if endospores continue to develop into new spherules whilst within the phagosome (Borchers and Gershwin, 2010). Interestingly, if macrophages are primed by immune T-cells before infection, these cells then become capable of effectively killing *C. immitis* (Beaman *et al*., 1981; Beaman *et al*., 1983).

The avoidance of killing when *Coccidioides* encounters macrophages is likely helped by the pathogens ability to reduce nitric oxide (NO) production. Macrophages primed with IFN-γ and LPS then co-incubated with *C. immitis* spherule initials, produced less NO and iNOS mRNA compared to activated cells without pathogen. The suppression did not require phagocytosis of spherule initials and could be achieved with only spherule culture supernatant, indicating an as yet unidentified secreted fungal factor (Gonzalez *et al*., 2011).

Brucella spp.

The Gram-negative facultative intracellular pathogens of the genus *Brucella* cause the zoonotic bacterial disease brucellosis, mostly seen in developing countries where it has a high prevalence. There are many pathogenic species of *Brucella* although *B. melitensis* is the most common cause of human infection. Infection can cause abortion, infertility and septicaemia in animals and fever or debilitation in humans.

B. melitenis can invade and replicate within phagocytic and non-phagocytic cells of humans. This ability is crucial to disease progression. Ninety percent of *B. abortus* are ingested and killed by macrophages, but the small proportion left alive is sufficient to replicate with macrophages, allowing persistence within the host niche (Celli *et al*., 2003; von Bargen *et al*., 2012). The past ten years has seen an explosion of *Brucella* research that has greatly advanced our understanding of this host-pathogen relationship. The O-chain of the non-classical *Brucella* LPS appears to be necessary for *B. melitensis* to enter cells via lipid rafts and prevent lysosome fusion (Castaneda-Ramirez *et al*., 2012). In the mouse macrophage cell line J774, clinical strains of *B.*

melitenis do manipulate vesicle trafficking but this does not involve the autophagy pathway as seen in epithelial cell models (Arenas *et al*., 2000; Arenas *et al*., 2010; Pizarro-Cerda *et al*., 1998). This manipulation creates a modified phagosome known as a *Brucella*-containing vacuole (BCV) that eventually fuses with the ER (Celli *et al*., 2003; Starr *et al*., 2008). Intracellular survival of *B. abortus* requires a number of virulence factors such as the Type III secretion system VirG and cell envelope components such as osmoregulated periplasmic glucan (OPG) and cyclic β-1,2-glucans($C_{\beta}G$) (Arellano-Reynoso *et al.,* 2005). Interestingly, other $C_{\beta}Gs$ are used by legume endosymbionts to enable intracellular survival (Bhagwat *et al*., 1999). It has now been shown that *B.* abortus and *B. melitenis* $C_{\beta}G$ affects BCV maturation (including blocking lysosome fusion in epithelial cells) by modulating lipid rafts in pathogen (and possibly host) membranes (Arellano-Reynoso *et al*., 2005; von Bargen *et al*., 2012).

As with *Coxiella*, *B. melitensis* also manipulates host TLR-MAPK signaling. It has been found that *B. melitensis* requires activated host p38 and JNK MAPK family members for intracellular replication within primary human macrophages (Dimitrakopoulos *et al*., 2013). The sequence of events leading to becoming an ER-like vacuole are complex and key issues (such as whether lysosomal fusion occurs) remain unresolved.

***Aspergillus* spp.**

Several species of fungus within the genus *Aspergillus* are capable of causing serious human disease. The most common by far is *A. fumigatus,* a species that aids the decomposition of organic matter in the environment, but which is also an opportunistic pathogen causing several different human infections. Infections typically start following

inhalation of asexual conidia into the lungs. If not successfully cleared, this initial infection can progress into invasive aspergillosis, the most severe and life-threatening form of aspergillosis, often associated with high mortality in immunocompromised individuals (Mansour *et al*., 2012).

Following inhalation, most *A. fumigatus* conidia are efficiently phagocytosed and killed by alveolar macrophages. However, recent data have shown that a minority of conidia are capable of suppressing fusion between phagosomes and lysosomes, allowing the fungus to germinate and ultimately lyse the macrophage (Morton *et al*., 2012). The underlying mechanism of this process is currently unknown, although the species variant dihydroxynaphthalene (DHM)-melanin coating on *A. fumigatus* and *A. flavus* conidia appears important for diverting maturation away from lysosome fusion (Thywissen *et al*., 2011). Moreover, the fungal hydrophobin RodA appears to suppress the immune response to inhaled conidia, including macrophage maturation. Hydrophobin molecules are believed to mask the conidia of many inhaled environmental fungi (Aimanianda *et al*., 2009).

The related species *A. terreus* is a less common cause of invasive bronchopulmonary aspergillosis (IBPA). However, once established, *A. terreus* infection is often fatal. Exposure to alveolar macrophages results in rapid phagocytosis, possibly due to the high surface exposure of β-1,3-glucan and galactomannan. Despite rapid uptake, conidia show long-term persistence in macrophages, even in immunocompetent hosts. Unlike *A. fumigatus*, the phagosome becomes acidified both in cell culture and *in vivo*, indicating that *A. terreus* probably persists despite the harsh phagolysosome conditions (Slesiona *et al*., 2012).

Strategies of Escaping Phagosomes and Phagocytes – Knowing When to Bail Out

Many microbial pathogens are able to manipulate the phagosome. Far less pathogens are able to manipulate the host cell to such an extent to allow escape from the phagosome (Figure 3). In this section the surprising variety of mechanisms that pathogens use to escape the phagosome, and even the phagocyte, are described.

Cryptococcus neoformans

Cryptococcus neoformans is a lethal fungal pathogen of immunocompromised individuals infecting approximately one million people worldwide per year, with an overall mortality rate of 60% (Park *et al*., 2009). *C. neoformans* can be found primarily in soil, on some trees (such as Eucalyptus) and in pigeon excreta (Casadevall *et al*., 1998). The disease, cryptococcosis typically starts with the inhalation of infectious particles (desiccated yeast or spores) followed by survival and proliferation within the lung, dormancy within the host, reactivation, dissemination and infection of the central nervous system and subarachnoid space (Chen *et al*., 2014). During cryptococcosis, macrophages are vital in the innate immune response. However, cryptococci are well adapted to parasitise these phagocytic effector cells (Ma and May, 2009). It is well known that cryptococci are able to replicate within the macrophage phagosome and escape in a non-lytic manner (vomocytosis) (Alvarez and Casadevall, 2006; Ma *et al*., 2006). The molecular detail that regulates and allows this replication and escape is yet to be defined.

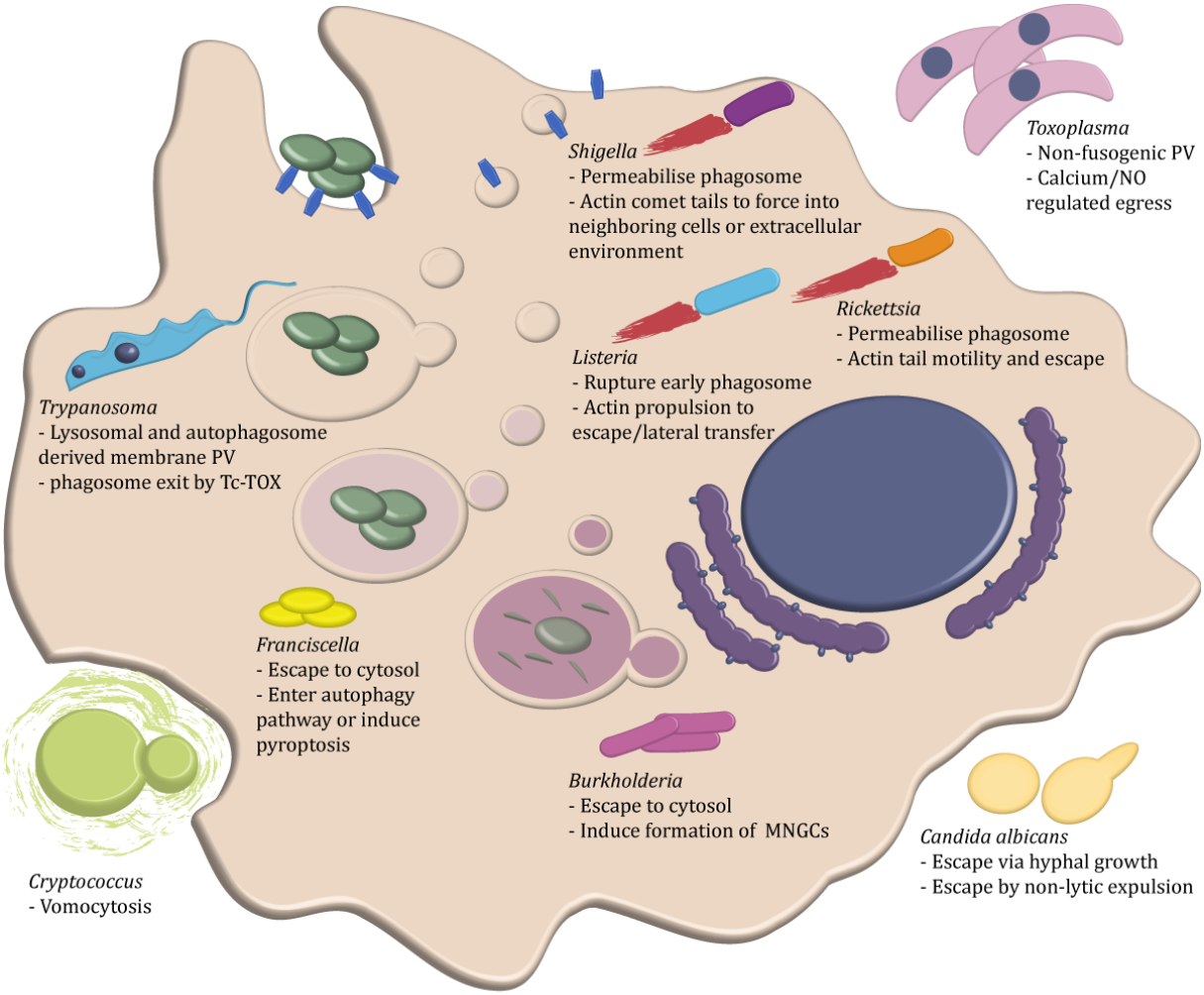

Figure 3. Strategies used by pathogens to escape the phagosome and phagocyte. There is a surprising number of pathogens able to escape either the phagosome or the phagocytic cell entirely. Summarised here are the mechanisms of escape used by the pathogens discussed in this review. Parasitophorous vacuole (PV). Nitric oxide (NO). Multi-nucleated giant cell (MNGC).

Intracellular replication *of C. neoformans* in macrophages was first observed in 1973 (Diamond and Bennett, 1973). Early hypotheses to explain this suggested that the cryptococcal phagosome was diverted from becoming a phagolysosome (Vecchiarelli *et al*., 1994), a model that has not yet been extensively tested. A detailed examination of the cryptococcal phagosome over the duration of infection has still not been completed, although some markers have been investigated. Cryptococcal phagosomes acquire EEA1 within 10 min of uptake by dendritic cells or macrophages (Qin *et al*., 2011; Wozniak and Levitz, 2008) and subsequently acquire LAMP1, MHCII and the tetraspanin CD63 (often used as a mature phagosome marker)(Johnston and May, 2010; Levitz *et al*., 1999; Qin *et al*., 2011; Wozniak and Levitz, 2008). CD63 recruitment requires acidification of the phagosome (Artavanis-Tsakonas *et al*., 2006) which is also, surprisingly, a prerequisite for intracellular survival of cryptococci (Levitz *et al*., 1999; Wozniak and Levitz, 2008). The presence of some late phagosome markers has led to the hypothesis that cryptococci persist within a normal phagolysosome, rather than modifying maturation processes. However, the presence of membrane markers is not in itself evidence for normal phagosomal contents and further work is required in this area.

C. neoformans is well equipped to avoid phagocytosis and to persist within a phagolysosome. The thick polysaccharide capsule (Bose *et al*., 2003), antiphagocytic protein 1(App1) (Stano *et al*., 2009), the transcription factor Gat201 (Chun *et al*., 2011; Liu *et al*., 2008) and the formation of enormous titan cells (Okagaki and Nielsen, 2012; Zaragoza *et al*., 2010) have all been implicated in phagocytosis prevention. Once internalised, *C. neoformans* are able to utilise many virulence factors for persistence and replication within phagocytes. Reactive oxygen and nitrogen species are absorbed or

detoxified by the capsule (Zaragoza *et al*., 2008). Protective factors such as Sod1, Aox1, Fhb1, Tsa1 and Ure1 are also known to aid resistance to oxidative stress (Brown *et al*., 2007), whilst the pigment melanin has been linked to antimicrobial peptide neutralization (Doering *et al*., 1999). Interestingly, it is likely that *C. neoformans* acquired many of these adaptive virulence traits to combat digestion by amoeboid predators in their natural environment (Casadevall, 2012), in an analogous way to *L. pneumophila*.

Vomocytosis is the unique non-lytic escape mechanism of cryptococci from host cells (Figure 3) (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Only recently have similar processes been described for *Candida albicans* (Bain *et al*., 2012) and *C. kruzei* (Garcia-Rodas *et al*., 2011). An escape mechanism that leaves both the host cell and the pathogen alive has major implications for infection progression and is likely to be important for tissue dissemination and possibly also as a mechanism for re-activation after latency. Vomocytosis has been confirmed *in vivo* within mice and possibly occurs more frequently *in vivo* than *in vitro* (Nicola *et al*., 2011). The current model for vomocytosis implies cryptococci escape via exocytic fusion of the phagosome with the plasma membrane, thus releasing the fungus (Johnston and May, 2010). Moreover, vomocytosis requires microtubule activity, but not actin polymerisation. However, actin and the WASP-Arp2/3 nucleating complex are involved in prevention of vomocytosis by the formation of dynamic actin cages, or "flashes", around the fungal phagosome. Although strains with high rates of vomocytosis induce more actin flashes, vomocytosis still occurs, suggesting that actin flashes may actually be a reaction invoked by vomocytosis attempts that will hold off expulsion for a short period but will eventually fail to contain this pathogen (Johnston and May, 2010).

C. neoformans phagosomes are also seen to permeabilise rapidly after phagocytosis (phagosomes lose fluorescently labelled dextran cargo) . This permeabilisation seems necessary and possibly even triggers actin flashes (Johnston and May, 2010; Tucker and Casadevall, 2002). The secreted phospholipase Plb1 is essential for vomocytosis (Chayakulkeeree *et al*., 2011). The exact role of this phospholipase is still under investigation, but it is tempting to predict a role in permeabilisation of the cryptococcal phagosome. The reason for permeabilising the phagosome is currently unknown, however, it is likely to aid neutralisation of the phagosome, thus inactivating antimicrobial proteases and allowing nutrients from the host cell to enter. One possible role for actin flashes is thus in resealing of the phagosome after pathogen-induced permeabilisation.

The pH of the phagosome has a major impact on intracellular parasitism by cryptococci. Chloroquine or ammonium chloride treatment to increase the phagosomal pH increases the rate of vomocytosis, but if acidification is blocked from occurring in the first instance (by using V-ATPase inhibitors) vomocytosis is suppressed (Ma *et al*., 2006; Nicola *et al*., 2011). Thus, it seems that *C. neoformans* either requires acidification to initiate vomocytosis (which seems to be true for replication) or vomocytosis only occurs from phagosomes that have been appropriately matured.

The molecular details that precede vomocytosis are still under investigation. The most likely explanation is 'hijacking' of the exocytic pathway normally used to expel indigestible material from host cells. In the amoeba *Dictyostelium discoideum*, exocytosis of digested material requires that the phagosome be devoid of vATPase, which is achieved by vesicle budding from the mature phagosome. This budding is controlled by

the cytoskeletal protein WASH. Blocking WASH activity inhibits this exocytosis from amoebae and, intriguingly, also reduces cryptococcal vomocytosis rates from macrophages (Carnell *et al*., 2011).

C. neoformans is also able to move from one cell to another via the process lateral transfer (Ma *et al*., 2007), a rare event that has not been extensively investigated. Transfer requires live cryptococci and may reflect either a vomocytosis event followed by rapid phagocytosis or another mechanism requiring a transient membrane tunnel between the two host cells in question (Johnston and May, 2012)

Listeria monocytogenes

Listeria monocytogenes is a Gram-positive bacterium capable of replication within many types of host cell (Radtke *et al*., 2011), by escaping from the phagosome into the cytoplasm. Before escaping the phagosome (approx 90 min after phagocytosis by macrophages), *L. monocytogenes* is also able to modify the phagosome. Early endosomal markers, but not late markers, such as cathepsin D or LAMP1, were found on phagosomal membranes containing live *L. monocytogenes* (Alvarez-Dominguez *et al*., 1997). Furthermore, this delay in maturation before escape could be partly due to disruption of the recruitment and activity of the early endosomal GTPase Rab5. *L. monocytogenes* disrupts Rab5 activity via a secreted and membrane inserted glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which recruits Rab5a and then induces Rab5a-specific ADP ribosylation and blocks the GDP/GTP exchange activity (Alvarez-Dominguez *et al*., 2008). To aid survival within the phagosome, the ArcA protein provides acid tolerance, as discovered by recent investigations (Cheng *et al*.,

2013). The authors hypothesise the acid tolerance is not only critical for intracellular infection, but also for surviving the low pH of the stomach in the initial stages of infection.

Phagosome escape requires multiple steps, proceeding from phagosome permeabilisation to phagosome rupture. Listeriolysin O (LLO) is a pore-forming toxin that permeabilises the phagocytic vacuole and is essential for phagosome escape (Cossart *et al*., 1989). Cross talk between the host cell and *L. monocytogenes* is required to complete phagosome rupture. For instance, the host γ-inducible lysosomal thiol reductase (GILT) is required for activation of the pathogen's LLO (Singh *et al*., 2008). Host cystic fibrosis transmembrane conductance regulator (CFTR) is used to escape into the cytosol by increasing calcium levels, likely to aid LLO pore formation and possibly inducing calcium dependent activation of host caplains via LLO (Lopez-Castejon *et al*., 2012; Radtke *et al*., 2011). LLO must be tightly regulated and its expression restricted to the intraphagosomal phase as over expression can lead to host cell lysis and exposure to the immune system. As such, LLO is induced by the low pH and high calcium conditions in the *L. monocytogenes* modified phagosome (Radtke *et al*., 2011).

It is by a combination of host (activated by bacterial factors) and bacterial phospholipases that the phagosome membrane is completely degraded and ruptures to allow *L. monocytogenes* escape into the cytosol (Goldfine *et al*., 2000; Portnoy *et al*., 2002). But the activity *Listeria* is probably best known for is its ability to transfer between cells once in the cytosol via characteristic 'actin rocketing', initiated by the *Listeria* surface protein ActA (Kocks *et al*., 1992; Pistor *et al*., 2000; Welch *et al*., 1997), a

phenomenon that has provided remarkable insights into host actin dynamics (Lambrechts *et al*., 2008; Stevens *et al*., 2006; Theriot *et al*., 1992; Welch *et al*., 1997).

Shigella flexneri

The Gram-negative bacterium *Shigella flexneri* is able to invade many host cell types by use of a Type 3 Secretion System (T3SS) (Marteyn *et al*., 2012; Roehrich *et al*., 2013). Once internalised, *S. flexneri* disrupts the phagosome membrane via the combined efforts of the bacterial effectors IscB (Allaoui *et al*., 1992), IpaB and IpaC (Page *et al*., 1999). Once in the cytoplasm, *S. flexneri* are also able to multiply and induce actin "comet tails" in an analogous way to *L. monocytogenes*, by using the bacterial protein IcsA to initiate actin polymerization and propel themselves around the cytosol and eventually into a neighbouring cell. IscA mimics the Cdc-42 dependent activation of N-WASP to initiate actin polymerization via Arp2/3 (Egile *et al*., 1999). Lateral transfer in this way results in a double membraned vacuole surrounding the *S. flexneri* cells once in the next cell. This double membrane is also ruptured, by IscB, and the cycle begins again (Allaoui *et al*., 1992). If all else fails, *Shigella* cells are also able to cause MxiI-induced pyroptosis of macrophages (a form of necrosis) triggering inflammation and enabling invasion of more epithelial cells (Suzuki *et al*., 2007).

Rickettsia

The genus *Rickettsia* was first recognised after the type species *R. ricketsii* was identified as the cause of Rocky Mountain spotted fever (Ricketts, 1906). All *Rickettsia* species are obligate intracellular pathogens that primarily inhabit endothelial cells. Once internalised, they rapidly escape the phagosome by using the secreted phospholipase A2

to destabilise the phagosome membrane (Walker *et al*., 2001). Once in the cytoplasm, *R. conorii* cells are able to replicate and produce actin tails via the activity of the bacterial protein RickA, which has domains homologous to the Wiskott-Aldrich Syndrome Protein (WASP)-family proteins, enabling Arp2/3 complex activation (Gouin *et al*., 2004). As with other pathogens, this actin-based motility allows direct cell to cell transfer of *R. conorii* cells without exposure to the host's immune system. For a recent review on spotted fever group (SFG) and typhus group (TG) *Rickettsia* see (Sahni and Rydkina, 2009).

Burkholderia pseudomallei

Burkholderia pseudomallei is a Gram-negative bacterial pathogen causing melloidosis in southeast Asia and northern Australia. The bacteria are able to induce their own uptake by phagocytic and non-phagocytic cells by manipulation of the host cytoskeleton (Stevens *et al*., 2002). Once internal, *Burkholderia* cells replicate in the cytosol of cells and can also induce cell-cell fusion to create multi nucleated giant cells (MNGC).

The molecular detail of *Burkholderia* intracellular survival is still poorly understood. To enable invasion and exit from the phagosome, *B. pseudomallei* utilizes an Inv/Mxi-Spa-like T3SS apparatus (Stevens *et al*., 2002), but otherwise does relatively little to perturb normal phagosome maturation, instead residing within a mature phagosome (Stevens *et al*., 2005). To survive in the harsh phagosome environment the bacterial sigma factor RpoS is used to initiate MNGC and inhibit iNOS to enhance intracellular replication (Utaisincharoen *et al*., 2006). After escape into the cytosol, *B. pseudomallei* uses RpoS and its T3SS to induce actin associated membrane protrusions. The superoxide

dismutase SodC is required for intracellular survival and virulence by offering protection from ROS produced by macrophages and neutrophils. However, there is a difference in susceptibility of the SodC mutant to extracellular ROS and ROS produced within cells (Vanaporn *et al*., 2011).

Francisella tularensis

The causative agent of tularemia, *Francisella tularensis,* enters macrophages via pathogen induced, CR3-dependant asymmetric spacious pseudopod loops (looping phagocytosis) (Clemens *et al*., 2005). *F. tularensis* can survive and replicate within many different cell types and host species. Entry via looping phagocytosis subverts the oxidative burst, although the phagosome still acquires EEA1, Rab5, CD63, LAMP1, LAMP2 and Rab7 (Clemens *et al*., 2004; Jones *et al*., 2014; Santic *et al*., 2006). Cathepsin D is not present within *F. tularensis*-containing phagosomes and so it is presumed lysosomal fusion is minimal. Escape into the cytosol occurs rapidly (30-60 min) followed by swift replication. Although, how rapid escape occurs depends on the receptor employed for uptake (Checroun *et al*., 2006; Geier and Celli, 2011). Vacuolar ATPase acidification also occurs quickly and is essential for phagosomal escape (Santic *et al*., 2008). If acidification is inhibited, phagosomal escape is delayed, further indicating a pH orchestrated signal/trigger for escape (Chong *et al*., 2008). Once in the host cytosol, *F. tularensis* can re-enter the endosomal autophagic pathway (24-48 h) (Checroun *et al*., 2006). The relevance of this link to autophagy is still undetermined as *F. tularensis* are known to repress several autophagy related proteins (Butchar *et al*., 2008). Interestingly, the route of initial uptake has a significant bearing on *F. tularensis,* with

complement-dependent uptake leading to slower escape from the phagosome (Santic *et al*., 2008). By manipulation of the macrophage inflammasome, *F. tularensis* are able to eventually induce pyroptosis and thus release from the host cell (Henry and Monack, 2007). The past decade has seen an explosion of research into this pathogen as researchers try to identify the pathogenic components required for the survival and escape from the phagosome. As a result, the *Francisella* Pathogenicity Island (FPI) genes (encoding a T6SS) have been found, required for phagosome escape (Weiss *et al*., 2007), the glutamate transporter GadC neutralises ROS (Ramond *et al*., 2014) and the necessity for capsule and O-antigen production for survival within monocyte derived macrophages (Lindemann *et al*., 2011). For an in depth review of the *F. tularensis* virulence factors required for infection, see (Jones *et al*., 2014).

Toxoplasma gondii

Toxoplasma gondii is a globally distributed obligate intracellular parasite that causes typically mild infections but can lead to significant pathology *in utero* or in immunocompromised individuals (Carruthers, 2002). *Toxoplasma* resides within a parasitophorous vacuole (PV) made by invaginating the host cell membrane as it actively invades many different types of host cell (thus keeping plasma membrane characteristics) and resists phagosome-lysosome fusion (Kinchen and Ravichandran, 2008; Sacks and Sher, 2002). *Toxoplasma* is able to replicate within this non-acidic vacuole, unless it has been previously opsonised by antibody that would lead to normal maturation, fusion and killing. This is a prime example of phagocytic entry route having huge implications on the intracellular fate of pathogens (Sibley *et al*., 1985). The non-

fusogenic PV also associates with host cell mitochondria and ER (Scott *et al*., 2003). Intracellular replication can generate between 32-128 parasites in one cell, at which point *T. gondii* exits cells either by orchestrated egress or simple mechanical lysis of host cells. Which of the two exit routes dominates under natural conditions is still under debate (Carruthers, 2002). Since *T. gondii* exit from macrophages seems to be related to preceding active invasion, it too is affected by Ca^{2+} and appears to be morphologically similar to invasion when microscopically recorded (Endo *et al*., 1982). It has also been demonstrated that by inducing the activity of *T. gondii* nucleoside triphosphate hydrolase, host cell ATP is depleted which then acts as a trigger for parasite exit. More recently, a *T. gondii* calcium dependent protein kinase 1 (TgCDPK1) has been identified as a key regulator of invasion, replication and egress. TgCDPK1 regulates the secretion of specialised organelles of the parasite, the micronemes, in response to increased intracellular calcium levels. Micronemes store many virulence factors, including the perforin-like protein TgPLP1 that facilitates permeabilisation of the PV (Kafsack *et al*., 2009; Lourido *et al*., 2010). TgCDPK3 also plays a role in microneme secretion, permeabilisation of the PV and initiation of gliding motility, but is able to sense when the parasite is intracellular from the higher potassium ion concentration (McCoy *et al*., 2012). Gliding motility via the unique actomyosin motor (glideosome) aids invasion, egress and travel through tissue. In the past two years a flurry of papers on calcium dependent *T. gondii* egress (Garrison *et al*., 2012; McCoy *et al*., 2012) and NO induced egress (Ji *et al*., 2013) have been published. Exciting new research has uncovered a novel mechanism for creating a PV. Instead of forming a PV from initial invasion of host cells, some *T. gondii* (preferentially avirulent) are instead phagocytosed and *then* invade from within to create a PV (Zhao *et al*., 2014). This new phagosome to vacuole invasion (PTVI)

route leads to a 'hypermotile' host cell phenotype that may provide an infectious advantage when encountering macrophages. New methods for separation of intracellular parasites, and parasites that have egressed from cells (Coleman and Gubbels, 2012) are likely to open new doors for the genetic exploration of other *T. gondii* factors responsible for egress from host cells.

Trypanosoma cruzi

The flagellated protozoan pathogen, *Trypanosoma cruzi*, causes American trypanosomiasis or Chagas' disease. This protozoan has many morphological forms in its lifecycle and can penetrate any nucleated cell type, although the host receptor is still unknown. Even though many can be killed in the human host, a small population will persist and stay with the host for their life-time (Fernandes *et al*., 2011). This persistent infection seems reliant on *T. cruzi*'s ability to replicate within cells and avoid immune discovery. The mechanisms of entry and phagosome manipulation are unusual. Whilst *T. cruzi* can be phagocytosed by macrophages it can also induce uptake from non-phagocytic cells. Remarkably, *T. cruzi* enters cells in an actin-independent manner, involving the recruitment of lysosomes to the plasma membrane entry point (Tardieux *et al*., 1992). Entry seems to involve *de-novo* microtubule polymerisation (possibly explaining lysosome recruitment) and LC3 decorated autophagosome membranes may contribute to the PV (Romano *et al*., 2009; Tyler *et al*., 2005). Lysosome recruitment could be a result of *T. cruzi* hijacking the plasma membrane lesion repair mechanism. Lysosome exocytosis releases acid sphingomyelinase, an enzyme whose function is to repair plasma membrane lesions and which has been found to be required for *T. cruzi*

entry (Fernandes *et al*., 2011; Sacks and Sher, 2002). In addition, the parasite requires TLR2 and activated Rab5 (via PI3K) for proper entry and PV establishment (Maganto-Garcia *et al*., 2008).

Once within a PV, *T. cruzi* rapidly escapes the PV by using Tc-TOX, a pore-forming molecule that is active at low pH. Tc-TOX activity is mediated by a *trans*-sialidase present on the trypomastigote form of *T. cruzi* (Hall *et al*., 1992; Ming *et al*., 1995). New data suggests that IL-17A exposed macrophages favour the phagocytic entry route of *T. cruzi*, resulting in pathogens becoming trapped in the endocytic pathway long enough to succumb to antimicrobial effectors (Erdmann *et al*., 2013).

***Candida* spp.**

Several species within the ascomycete fungal genus *Candida* are able to cause invasive infections of humans. The most common, *C. albicans*, can be engulfed by host phagocytes but rapidly undergoes filamentation, growing and eventually lysing the host cell (Lorenz *et al*., 2004). The mechanism for initiating this hyphal growth and escape is not fully determined, but is known to involve pH. With amino acids as the sole source of carbon, *C. albicans* is able to alkalinise its surrounding media by the production of ammonia. The neutral pH is a trigger for the morphogenic switch to hyphal growth and this appears to be replicated within the phagosome. The amino acid permease Stp2p is required for the utilisation of amino acids and neutralisation of the phagosome, resulting in hyphal lysis of the host cell (Vylkova and Lorenz, 2014). In contrast, the mechanism of lytic *C. albicans* escape has not been documented in the zebrafish *in vivo* model (Brothers *et al*., 2011). Visualising the infection intravitally, the authors found *C. albicans* internalised

by macrophages remained as yeast and replicated via budding rather than hyphal germination and lysis of the host cell (Brothers *et al*., 2011). Furthermore, the restriction of filamentous growth requires host NADPH oxidase activity, confirming previous neutrophil *in vitro* findings. Continued experimentation revealed the NADPH oxidase requirement was not primarily for oxidative stress to prevent germination but more so for ROS to be produced as a chemotactic signal (Brothers *et al*., 2013). The importance of ROS for the early recruitment of phagocytes to the site of infection and containment of the fungus is a discovery that is likely to only have been possible in such an *in vivo* model.

Mature *C. albicans* phagosomes show only minimal acquisition of late-stage markers such as lysobisphosphatidic acid and vATPase. In addition, *C. albicans* appears to induce the removal, by recycling vesicles, of LAMP1 and cathepsin D from the phagosome (Fernandez-Arenas *et al*., 2009). *C. albicans* also expresses catalase, which plays an important role in virulence and hydrogen peroxide resistance (Enjalbert *et al*., 2007). Whilst internalised, *C. albicans* is able to inhibit ROS generation by macrophages, although the mechanism is unknown (Wellington *et al*., 2009). Although it is notable that *C. albicans* still requires some ROS to act as a signal to initiate arginine biosynthesis and hyphal growth escape (Jimenez-Lopez *et al*., 2013; Nakagawa, 2008; Nasution *et al*., 2008). *C. albicans* has very recently been discovered to be capable of non-lytic escape, in a similar way to *Cryptococcus*, although how this expulsion is achieved is as yet unknown (Bain *et al*., 2012).

In contrast, to its polymorphic relative, *C. glabrata,* (the second most common cause of candidiasis) grows as a yeast but remains able to replicate within macrophages (Kaur *et*

al., 2007). As an emerging opportunistic infection of humans, little is known about its pathogenicity. Although non-lethal in animal models, *C. glabrata* is not efficiently cleared from immunocompetent animals, still detectable weeks after infection (Jacobsen *et al*., 2010). It is likely therefore, that this yeast hides within host cells to avoid immune stimulation and killing. Supporting this, *C. glabrata* does not induce apoptosis of the macrophages it infects, thus protecting itself from immune detection (Seider *et al*., 2011). How *C. glabrata* is able to survive within the macrophage phagosome is currently under investigation. It has been found that the *C. glabrata*-containing phagosome recruits LAMP1 and low levels of EEA1, but not Rab5 or cathepsin. Despite large amounts of vATPase on the phagosome, the contents are only weakly acidic (Seider *et al*., 2011). Microarray analysis to determine the transcriptional profile of *C. glabrata* during infection of J774 murine macrophages uncovered a role for glycosylphosphatidylinositol (GPI)-linked aspartyl proteases (Kaur *et al*., 2007). Related to the yaspins (*YPS* genes) of *S. cerevisiae*, the 11 Yps proteases of *C. glabrata* had varied roles in cell wall integrity and response to macrophages. A *yps1-11*Δ strain induced higher levels of macrophage NO, indicting a role for Yps proteases in macrophage activation. These observations could be attributed to Yps processing of GPI-anchored components of the fungal cell wall (Kaur *et al*., 2007). In an effort to further decipher the mechanisms employed by *C. glabrata* to evade killing within the phagosome, a transposon mutant library was screened to identify genes required for survival within the human monocyte cell line THP-1 (Rai *et al*., 2012). The study unveiled 56 genes required for survival or replication, including, intriguingly, several involved in chromatin remodeling and DNA repair. It appears that for intracellular replication, and virulence in a mouse model, chromatin remodeling allowing altered energy metabolism

and DNA damage repair are crucial (Rai *et al*., 2012). A more recent study found that fungal mannosyltransferases are involved in *in vitro* alkalinisation of the environment and for reducing the pH of the phagosome (Kasper *et al*., 2014). This activity did not depend on macrophage species, differentiation or activation. However, the modification of the phagosome did involve Syk activation, as opposed to MAPK or NFκB signaling (Kasper *et al*., 2014).

Strategies to Persist within phagosomes – Making the Best of a Bad Situation

In this final section, microbial pathogens known to persist within host cells, rather than actively manipulate the phagosome, are discussed (Figure 4).

Streptococcus agalactiae

The Gram-positive bacterium, *Streptococcus agalactiae,* or Group B *Streptococcus* (GBS), is the most common infectious agent of neonatal pneumonia, sepsis and meningitis (Valenti-Weigand *et al*., 1996). However, GBS infections are also becoming more prominent in elderly and diabetic adults (Henneke and Berner, 2006).

Phagocytosis of opsonised GBS by professional phagocytes provides a crucial clearance mechanism for invasive GBS. Non-opsonised bacteria are poorly killed following uptake (Valenti-Weigand *et al*., 1996). As such, GBS have a myriad of factors to mimic host molecules and interrupt the complement cascade (Maisey *et al*., 2008). As seen with other pathogens, the route of entry GBS takes into a macrophage has consequences on its intracellular survival. GBS opsonised with human serum before exposure to J774 macrophages are less able to persist intracellularly compared to non-opsonised bacteria (Valenti-Weigand *et al*., 1996). It is believed that non-opsonised bacteria are able to be recognised by macrophages in a C3 independent, but CR3-dependent manner (Antal *et al*., 1992). These non-opsonic interactions may be crucial in determining disease progression in neonates, a host with few active opsonic molecules against GBS.

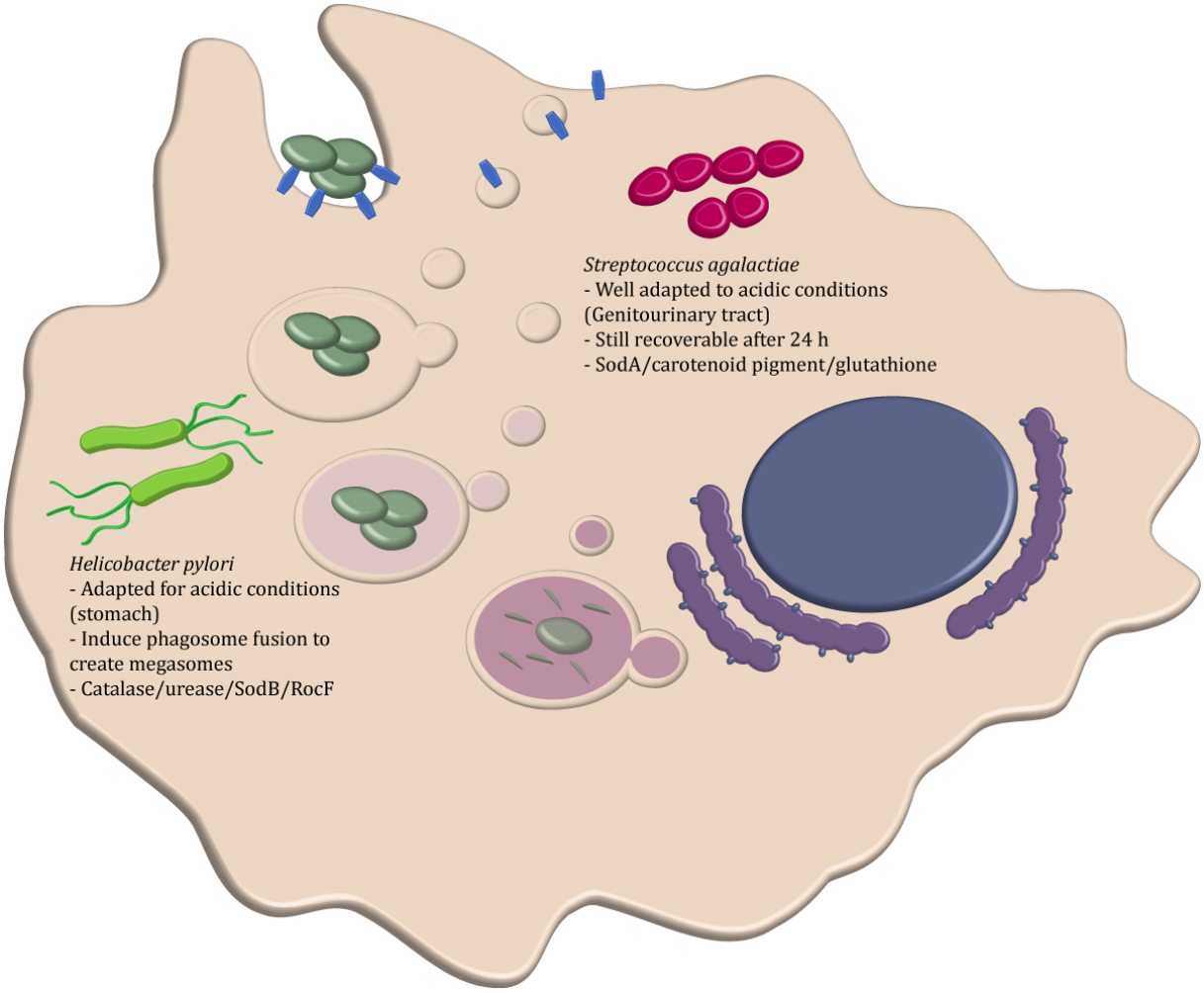

Figure 4. Pathogens that persist within the phagosome.
Pathogens that are able to persist within the phagosome are presented here. What is known about the mechanisms of this persistence has been summarised next to the illustration of each pathogen.

It has long been documented that GBS are able to survive within macrophages for a prolonged period of time (Valenti-Weigand *et al*., 1996), despite the fact that GBS appear to reside within a mature, LAMP1 positive phagosome (Cumley *et al*., 2012; Teixeira *et al*., 2001). Interestingly, phagosomal acidification seems to be necessary for the survival of internalised GBS. The acidic environment likely acts as a stress signal to induce survival genes, since there is a marked drop in the survival of GBS when macrophages are treated with Concanamycin A (an inhibitor of vATPase acidification). Additionally, the two-component system CovS/R is required for this acid sensing and intracellular survival of GBS (Cumley *et al*., 2012).

GBS have many virulence factors that can attribute to their ability to persist intracellularly (Figure 4). One such virulence factor is the GBS superoxide dismutase (SodA). By catalysing the conversion of oxygen radicals into oxygen and hydrogen peroxide, products of the macrophage oxidative burst can be fully metabolised by peroxidases (Poyart *et al*., 2001). This action of SodA protects GBS from oxidative damage (Poyart *et al*., 2001). Further protection is provided by large quantities of glutathione. GBS are able to use glutathione as an oxygen-metabolite scavenger (Wilson and Weaver, 1985). The streptococcal caratenoid pigment is also able to offer protection form oxidative damage by free radical scavenging (Liu *et al*., 2004). Interestingly, neonatal phagocytes are less able to mount a severe oxidative burst (Wilson and Weaver, 1985). This is most likely to further aid the intracellular survival of invading GBS.

The β-haemolysin of GBS is considered a key virulence factor. This reputation is mainly due to the cytolytic pore forming ability of this β-haemolysin/cytolysin. The structural

gene for the toxin has been determined to be *cylE* of the *cyl* operon (Pritzlaff *et al*., 2001). The *cylE* gene is also responsible for the production of the caratenoid pigment, however, the regulatory basis of the link is still unknown (Spellerberg *et al*., 1999). Interestingly, at sub-lytic concentrations β-haemolysin activates macrophage p38 MAPK signaling, promoting anti-inflammatory IL-10 and suppressing IL-12 and NOS2 (Bebien *et al*., 2012). Targeting MAPK activation in murine macrophages was able to provide resistance to invasive GBS infection (Bebien *et al*., 2012). Although, how this modulation of host cytokines impacts on the maturation or antimicrobial activity of GBS-containing phagosomes is currently unknown. Despite this, a recent study found that a serotype Ia non-haemolytic mutant (Δ*cylA*) was able to survive within macrophages better than the isogenic wild type (Sagar *et al*., 2013). A possible explanation could lie in the specific genes manipulated in the studies, *cylA* is the ATP binding component of the transporter where as *cylE* encodes the β-haemolysin/cytolysin itself that when mutated renders GBS attenuated for various virulence attributes.

The two-component response regulator CiaR has been found to affect intracellular survival of GBS strain COH1 (Quach *et al*., 2009). This regulator was found to increase resistance to not only phagocytic killing, but also exposure to anti-microbial peptides (AMPs), lysozyme and ROS. The function of CiaR is currently unknown although it appears to act as part of the two-component system CiaR/H to sense and respond to environmental cues in phagosomes.

Despite this ability to survive intracellularly, replication within the phagosome has not been documented for GBS, suggesting that GBS are able to persist rather than multiply within phagocytic cells. A novel vaginal colonisation model in mice might be able to

provide key answers in the ability of *S. agalactiae* to persist within this vaginal niche and also maintain virulence determinants for opportunistic infection (Carey *et al*., 2014).

Helicobacter pylori

Helicobacter pylori is a micro-aerophilic, spiral shaped motile bacterium infamous for causing gastric and duodenal ulcers. *H. pylori* is able to persist in the gastric mucosa and avoid killing by resident phagocytic cells. If engulfed by patrolling macrophages, *Helicobacter* is able to persist in macrophages and form "megasomes", by homotypic fusion of multiple *H. pylori*-containing phagosomes (Allen *et al*., 2000).

The tools *Helicobacter* has to enable persistence within the phagosome are numerous. Many are directed towards defending against the oxidative burst of macrophages. The *H. pylori* urease is required for megasome formation, ROS defense and acid neutralisation. Urease deletion can also alter EEA1 acquisition in J774 macrophages (Schwartz and Allen, 2006). Another important molecule produced by *H. pylori* is catalase that has been found to be partly responsible for the intracellular longevity seen in macrophages. Catalase (KatA) and superoxide dismutase (SodB) work together to convert superoxide to water and oxygen via hydrogen peroxide (Basu *et al*., 2004; Borlace *et al*., 2012). Protection from reactive nitrogen species is achieved in several ways. The arginase RocF converts the host iNOS substrate arginine to urea, which can then be converted to ammonium by urease, thus depleting arginine required for NO production (Borlace *et al*., 2012; Gobert *et al*., 2001). Neutrophil activating protein (NapA) and Alkyl hydrogen peroxide reductase (AphC) are also used in RNS defense (Bryk *et al*., 2000; Evans *et al*., 1995).

H. pylori is also able to delay phagocytosis by activating alternative protein Kinase C-ζ (PKCζ), possibly to allow for up-regulation of virulence factors required for phagosome survival and reducing respiratory burst activation by PKCα (Allen and Allgood, 2002).

When *H. pylori*-containing phagosomes were compared to those of *Escherichia coli,* Rab7 and EEA1 were retained on phagosomes and CD63, LAMP1 and LAMP2 were acquired normally. Cytotoxin associated gene A (CagA) negative *H. pylori* seem to acquire more EEA1 (relative to Rab5). There also seem to be strain differences in timing of Rab7 acquisition and megasome formation (Borlace *et al*., 2011). It is proposed that the persistence of EEA1 and Rab7 could be due to altered ability to remove them from the phagosome surface via vesicle budding (Borlace *et al*., 2011). Earlier studies hinted at diversion from phagosomal fusion with lysosomes, but as detailed above, the lysosomal markers do seem to be present, however, this LAMP1 and LAMP2 acquisition is apparently not always seen (Schwartz and Allen, 2006; Zheng and Jones, 2003). Past recordings of low LysoTracker co-localisation could be due to the many factors that *H. pylori* produces to neutralise its immediate environment. Maturation is disrupted but the relevance of these observations to pathogen survival is unclear.

Summary

The clearance of pathogens by phagocytosis and subsequent digestion is the primary role of macrophages. Despite this, many pathogens have evolved ways to arrest, divert or escape the phagosome. The list of microbes that are not completely eradicated by macrophages is constantly growing. Advances in live cell imaging, fluorescent microscopy and molecular probes have all aided research of these host – pathogen interactions. The need to carefully consider the phagosome chemistry when choosing molecular probes and fluorescent dyes is becoming more apparent (Nusse, 2011). The phagosome maturation field is a vibrant and growing one, unlikely to slow any time soon. The rest of this thesis will concentrate on the survival strategies employed specifically by *Streptococcus agalactiae* and *Cryptococcus neoformans*.

Thesis outline

Many microbial pathogens are able to evade killing by phagocytes of the innate immune system. Some examples of these pathogens and the mechanisms they use to manipulate phagocyte behaviour are described in the introduction to this thesis. The rest of the thesis focuses on two pathogens: *Cryptococcus neoformans* and *Streptococcus agalactiae*. *C. neoformans* is a fungal pathogen able to cause severe cryptococcal meningitis in mostly immunocompromised hosts, such as those with HIV infection. In contrast, the bacterial pathogen *S. agalactiae* is the leading cause of neonatal sepsis and meningitis. In both cases the lungs are likely to be the initial organ exposed to pathogen after inhalation of infectious particles. Thus, alveolar macrophages are thought to be the first cells that will encounter these pathogens and make attempts to clear the infection. Therefore, the interaction between macrophages and these two pathogens is likely to be critical in determining dissemination and outcome of disease in both instances.

In the first results chapter the *Cryptococcus*-macrophage interaction is explored. Previous research has revealed that cryptococci are not only able to avoid killing by phagocytes in a variety of ways, but also replicate within these cells. Cryptococci are also able to escape phagocytes by lysis of the host, non-lytic vomocytosis and lateral transfer between cells. Alterations in the maturation of the *Cryptococcus*-containing phagosome could contribute to the occurrence of such cryptococcal parasitic activities. The aim of the research undertaken was to determine if *C. neoformans* is able to modify the phagosome it resides within and, if so, to what extent can these modifications be correlated with proliferation or vomocytosis of the intracellular cryptococci. Firstly, the acquisition of Rab GTPases to phagosomes containing cryptococci were investigated using immunofluorescence for these markers of phagosome maturation. The results

reveal an altered recruitment of some GTPases to phagosomes containing live, but not heat killed cryptococci. Secondly, the acidification of these phagosomes was investigated with the use of the acidotropic dye LysoTracker Red and live-cell imaging. Phagosomes containing live cryptococci did not become highly acidic, in stark contrast to phagosomes containing heat killed cryptococci. In addition, this ability to modify the acidification of the phagosome was maintained in a variety of cryptococcal mutants. Suggesting an as yet unidentified virulence factor of cryptococci is responsible for this modification of the phagosome. Using the fluorescent dye MagicRed, the cathepsin activity within *Cryptococcus*-containing phagosomes was also determined. Again, phagosomes containing live cryptococci had an altered phenotype, lacking active cathepsin proteases in the phagosomes they reside in. Finally, testing the permeability of *C. neoformans* phagosomes with TRITC-Dextran revealed that cryptococci residing in permeabilised phagosomes are more likely to escape the macrophage by vomocytosis.

The second results chapter concentrates on the *Streptococcus*-macrophage relationship. *S. agalactiae* has been previously shown to persist within macrophages. We hypothesised that particular clinical isolates of GBS (such as those causing LOD) would be more able to survive within macrophages when compared to isolates found colonising women. Furthermore, that the 'hypervirulence' of MLST-17 GBS strains could be attributed to their ablity to survive within macrophages. To test if the ability to persist in macrophages was a feature of only disease causing strains, clinical isolates of *S. agalactiae* were assayed for this ability. It was discovered that all strains tested were able to persist within the murine macrophage cell line J774. The survival in macrophages did not correlate with particular serotypes, MLSTs or isolate origin. This chapter also reports experiments that investigated the acidification of the *Streptococcus*-

containing phagosome. It was hypothesised that to enable the persistence of GBS within macrophages, GBS could be modifying the phagosome maturation pathway to reduce the antimicrobial ability of the phagosome it resides within. The results showed that live *S. agalactiae* are rarely found in highly acidic phagosomes, suggesting GBS are able to modify phagosome acidification.

The final chapter of this thesis discusses the construction of a transposon mutant library in *S. agalactiae*.

Chapter II. *Cryptococcus neoformans* Alters Phagosome Maturation

Major parts of this chapter have been submitted for publication:

Smith, L.M., Dixon, E. May, R.C. (2014) The fungal pathogen *Cryptococcus neoformans* manipulates macrophage phagosome maturation. *Cellular Microbiology* .In Revision

Introduction

The phagocytosis of microbes and subsequent killing of these ingested particles by phagocytic cells is a vital component of the innate immune system. However, many microbial pathogens have evolved a variety of ways to avoid the intracellular killing mechanisms of host phagocytes (Smith and May, 2013). One example of such a pathogen is the lethal fungal pathogen *Cryptococcus neoformans*. *C. neoformans* can be found primarily in soil, some trees (such as Eucalyptus) and pigeon excreta (Casadevall *et al*., 1998). This opportunistic yeast is responsible for an estimated one million infections and approximately 600,000 deaths per annum (Park *et al*., 2009). A dramatic rise in the incidence of cryptococcosis over the last century coincides with an increasing number of immunocompromised individuals, especially in sub-Saharan Africa, the epicenter of the AIDS pandemic (Park *et al*., 2009). There are, however, rare cases of cryptococcosis caused by *C. neoformans* in individuals with no known immunodeficiency (Chen *et al*., 2008). The disease, cryptococcosis, is thought to start with the inhalation of infectious particles (desiccated yeast or spores) followed by survival and proliferation within the lung, dormancy within the host, reactivation, dissemination and infection of the central nervous system and subarachnoid space (Chen *et al*., 2014). Factors from both the host's immune system and the pathogen decide if disease progresses further than the lung. Of

particiular note, the *C. neoformans* polysccharide capsule and melanin pigment are key factors required for cryptococcal virulence and will be mentioned later in this chapter. The attributes of both host and pathogen that may affect disease progression, are discussed here.

Host associated risk factors for cryptococcosis

Cryptococcal infection usually affects individuals with underlying immunocompromisations or those that are undergoing treatment with immunosuppressive drugs (Mitchell and Perfect, 1995). For example, *C. neoformans* has been known to infect patients with chronic leukemia's and lymphomas and patients undergoing organ transplantation or treatment with corticosteroids (Mitchell and Perfect, 1995). However, *C. neoformans* infections, especially those escalating to cause cryptococcal meningitis, are most often associated with HIV-infected individuals (Powderly, 1993). In this section, the role of the immune system in determining the outcome of cryptococcal infection will be discussed.

The complement cascade can influence the clearance of cryptococcal infections. Proteins in serum bind pathogens in a sequential manner that eventually leads to the opsonisation of the pathogen. These complement components aid phagocytosis or act as chemokines for the recruitment of other phagocytes to the infected area. This response can be mediated via antibody (classical pathway), lectin molecules or the microbial surface directly (alternative pathway). Deficiencies in the complement pathway can exacerbate cryptococcal infections. One small study found that sufferers of cryptococcal fungaemia, when compared to patients with chronic cryptococcosis or healthy

individuals, have less complement component C3 and alternative complement factor B (Macher *et al*., 1978). Furthermore, the cryptococci residing in the brain of cryptococcal meningitis patients are not seen bound to complement C3 (Truelsen *et al*., 1992), suggesting cryptococci are able to avoid complement deposition, or are able to remove complement.

The adaptive immune response in the form of specific antibody production towards *C. neoformans* can aid fungal clearance. Interestingly, anti-*Cryptococcus* antibodies can often be isolated from individuals with no apparent infection (Abadi and Pirofski, 1999; Fleuridor *et al*., 1999; Houpt *et al*., 1994). This is likely due to childhood exposure to the fungus from the environment (Goldman *et al*., 2001). *Cryptococcus* specific antibodies offer an immune advantage to the host by providing opsonisation for phagocytosis and also activate the classical arm of the complement cascade (Fleuridor *et al*., 1999; Mukherjee *et al*., 1992). However, antibody alone is not always beneficial. For example, providing mice with excess anti-cryptococcal antibody before exposure can actually be detrimental to infection outcome (Taborda and Casadevall, 2001). Phagocytosis of *C. neoformans* was shown to increase with an increase in IgM dose, but phagocytosis was slightly reduced at IgM concentrations of over 100 μg/ml. High doses of IgM were also shown to enhance the resistance of cryptococci to killing by reactive nitrogen species (Taborda and Casadevall, 2001). Potential reasons to explain how increasing phagocytosis could be detrimental to infection clearance will be discussed later in this chapter.

For successful clearance of a cryptococcal infection, the host must be able to mount a complete cell-mediated immune response (Voelz and May, 2010). T-cells and natural

killer (NK) cells can be directly antimicrobial towards cryptococci (Levitz *et al*., 1994; Zheng *et al*., 2007). T cells are able to use granulysin to kill yeast (Ma *et al*., 2002; Zheng *et al*., 2007) with CD4+ T-cells being responsible for much of the antimicrobial activity towards cryptococci in the bloodstream (Zheng *et al*., 2007). NK cells are able to use the secreted protein perforin to lyse cryptococci and induce killing via the ERK1/2 signaling pathway (Ma *et al*., 2004; Wiseman *et al*., 2007). However, granulysin and perforin production is a process that is dysregulated in HIV positive patients, which partly explains the difficulty these patients have in clearing extracellular *C. neoformans* from the blood (Zheng *et al*., 2007).

The host cytokine profile has also been shown to affect the outcome of cryptococcal infection. An analysis of the effect that Th1, Th2 and Th17 cytokines have on the *C. neoformans*-macrophage interaction has been conducted (Voelz *et al*., 2009). This study found that Th1 (IFNγ and TNFα) and Th17 (IL-17) cytokines help drive the anti-cryptococcal response of macrophages. In contrast, macrophages treated with Th2 cytokines, such as IL-4 and IL-13, were unable to control intracellular replication of cryptococci (Voelz *et al*., 2009). Interestingly, the IFNγ-induced reduction of cryptococcal replication was more efficient in the mouse J774 cells than in primary human macrophages (Voelz *et al*., 2009). This highlights a potential difference in IFNγ pathways between the two species.

Further support for the importance of a Th1-biased response comes from studies where TNFα, IFNγ and IL-12 are associated with a good antifungal response (Decken *et al*., 1998; Kawakami *et al*., 1995; Milam *et al*., 2007; Wormley *et al*., 2007). Moreover, IFNγ-/- mice are unable to control intracellular cryptococcal growth (Arora *et al*., 2011) and

Th2 cytokines such as IL-13 (Muller *et al*., 2007) and IL-4 (Blackstock and Murphy, 2004) are detrimental to infection.

The importance of a Th1-focused immune system becomes relevant in HIV-infected individuals. As during viral infection progression, the regulators of Th1 immunity become progressively reduced (Wadhwa *et al*., 2008). The depletion of Th1 responses also coincides with an increased incidence of cryptococcal infections (Altfeld *et al*., 2000). To make matters worse, *C. neoformans* is able to suppress the Th1 response with the production of immunomodulatory eicosinoids via a cryptococcal phospholipase (PLB1) (Noverr *et al*., 2003).

A variant of the FcγRIIIA phagocytic receptor that has a higher affinity for *Cryptococcus* has recently been identified as a risk factor for cryptococcal disease (Rohatgi *et al*., 2013). The FcγRIIIA 158V polymorphism is positively correlated with cryptococcal infection in HIV-positive men. Subsequent studies suggest that this susceptibility is due to the increased affinity of the Fc receptor for cryptococci (Rohatgi *et al*., 2013). This finding implied that cryptococcal binding and phagocytosis can be a risk factor for disease. Recent studies have also shown that these attributes can make the disease more severe. This was discovered in a study involving 65 clinical, HIV-associated isolates of *C. neoformans* (Sabiiti *et al*., 2014) . In this study the authors identified correlations between *in vitro* virulence measures of cryptococcal virulence and patient outcomes. Surprisingly, high macrophage phagocytosis rates, rather than increased intracellular proliferation, correlated with high fungal burdens in the CSF. These isolates with high uptake rates were hypocapsular. More unusual though, was the discovery that the isolates displayed elevated laccase activity, which enhanced survival in CSF. The laccase

augmented production of melanin was not the cause of the difference seen between high and low uptake strains (Sabiiti *et al*., 2014). This suggests that either another function of laccase is important for increasing uptake and CSF survival, or the correlation is not causal.

Thus, both host factors and cryptococcal factors can affect the amount of initial phagocytosis of cryptococci. These initial phagocytosis events, and the outcome of this interaction are crucial for determining the severity of disease, as will be discussed further in this chapter.

Cryptococcal mechanisms of immune evasion

As with many microbial pathogens, *C. neoformans* has developed a vast number of strategies to avoid alerting the host immune system. The processes of cryptococcal immune evasion will here in be described.

Cryptococcus species are unique amongst pathogenic fungi, due to their thick polysaccharide capsule that surrounds the yeast cell body (Srikanta *et al*., 2013). Rabbit polyclonal anti-sera have been used to define 4 different capsule serotypes A to D (Wilson *et al*., 1968). The species *C. neoformans* is split into the variants *grubii* (serotype A) and *neoformans* (serotype D). Serotypes B and C categorise the lesser-known species *C. gattii*, a species of *Cryptococcus* that is able to cause disease in immunocompetent individuals (Srikanta *et al*., 2013; Wilson *et al*., 1968). *C. neoformans* serotype A is believed to cause 95% of all cryptococcosis cases (Hull and Heitman, 2002; Mitchell and Perfect, 1995).

In addition, there are 8 molecular types to further categorise the genus *Cryptococcus*. Those concerning *C. neoformans* are the molecular types VNI and VNII for var*. grubii*, VNIV for var*. neoformans* and lastly VNIII for hybrids of the two variants (Meyer *et al*., 2009).

The capsule is one of the most studied virulence factors of *C. neoformans* (Doering, 2009). Strains without a capsule, or with low levels of capsule polysaccharide, are avirulent (Chang and Kwon-Chung, 1994; Perfect, 2005). The capsule is mostly comprised of two polysaccharides: glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (Doering, 2009; Heiss *et al*., 2013). The capsule is attached to the cell wall, forming the most exterior part of the cell, and is able to be shed into the surrounding environment. Both attached and shed capsule components are able to inhibit phagocytosis of cryptococci (Mitchell and Friedman, 1972; Syme *et al*., 1999).

In addition, the polysaccharides of the capsule are able to interfere with the migration of cells such as neutrophils. To inhibit neutrophils from leaving the blood stream and migrating to the site of infection, the polysaccharides are able to induce cross-desensitisation of chemokine receptors. Part of this migratory defect could be attributable to polysaccharide repression of C5a receptor expression (Ellerbroek *et al*., 2004). Moreover, both GXM and mannoproteins are able to induce L-selectin shedding from neutrophil cell surfaces, which is likely to affect neutrophil rolling on the endothelium. Finally, the polysaccharides of the cryptococcal capsule are able to not only induce the release of anti-inflammatory IL-10, but also reduce the expression of TNF-α receptors on the surface of neutrophils (Ellerbroek *et al*., 2004). Overall, the

components of the cryptococcal capsule are able to significantly reduce the inflammatory response.

Whilst causing infection, *C. neoformans* is likely to encounter many different environments within the body. Some of these situations will induce the induction of a virulence program within the pathogenic yeast. The host environment is able to induce alterations in the size and composition of the *C. neoformans* capsule (Charlier *et al*., 2005; Zaragoza *et al*., 2010). Of note is the capsule enlargement seen during murine pulmonary infection (Feldmesser *et al*., 2001a). The increased capsule size offers several immune evasion advantages: Firstly, the large capsule is thought to reduce complement-mediated phagocytosis (Zaragoza *et al*., 2003). Secondly, the capsule offers protection from ROS mediated intracellular killing (Zaragoza *et al*., 2008).

Furthermore, the capsule has been proven to have an immunomodulatory role. Acapsular strains have been shown to induce the expression of dendritic cell genes involved in antigen presentation and the production of cell surface receptors, chemokines and cytokines. Conversely, an encapsulated strain did not induce the same response and even down-regulated genes encoding chemokines (Lupo *et al*., 2008). Remarkably, the GXM from all four serotypes of *Cryptococcus* is able to impair T-cell proliferation (Yauch *et al*., 2006).

In an effort to develop a long-term model of cryptococcal infection, Siddiqui *et al*. used human peripheral monocytes pre-treated with heat killed *Cryptococcus* (Siddiqui *et al.,* 2006). The method aims to reveal components required for a good immune response towards *C. neoformans*. Indeed, pre-treated peripheral blood monocytes were better able to restrict the growth of cryptococci during subsequent infection with live yeast

(Siddiqui *et al*., 2006). For optimal growth restriction, CD4+ and CD8+ T cells were required, along with pre-stimulation of monocytes with acapsular yeast. Pre-stimulation with acapsular yeast increased IL-6 production compared to encapsulated yeast. The mechanism of this IL-6 cryptococcal protection is currently under further investigation (Siddiqui *et al*., 2006).

The GXMGal of the capsule has also been shown to aid modulation of the immune response to *C. neoformans*. Purified GXMGal is able to impair T cell proliferation and induce apoptosis of T cells. The apoptosis of T cells appears to be induced via caspase-8 activation (Pericolini *et al*., 2006). These results demonstrate the capsule is not only able to mask antigens of the cryptococcal cell wall, but is also able to dampen the cell-meditated immune response.

Cryptococci are not only able to increase the size of their capsule, but also the size of the yeast cell body. The average size of *C. neoformans* when grown in laboratory media ranges from 4 -10 μm. After challenging mice, 10-80% of cryptococci recovered had a cell body size of up to 30 μm in size; these were described as giant cells or 'titan cells' (Zaragoza *et al*., 2010). Their size is believed to make phagocytosis by host cells impossible (Okagaki *et al*., 2010) and this morphology has been shown to promote survival and dissemination of cryptococci (Crabtree *et al*., 2012).

Titan cell formation is ageing dependent and potentially involves cell replication without fission, due to resultant polyploidy (Zaragoza *et al*., 2010). Additionally, the capsule becomes more cross-linked and resistant to complement deposition (Zaragoza *et al*., 2010). The cell wall of titan cells also becomes enlarged and melanised (Feldmesser *et al*., 2001a; Zaragoza *et al*., 2010). The titan cells are, however, still able to

bud and produce normal sized daughter cells. This ability to remain viable has led to the hypothesis that titan cells play a role in cryptococcal latency. Titan cells potentially provide a resistant and persistent population, able to seed new infection challenges via budding.

The pathways that regulate the phenomenon of titan cell formation are yet to be fully characterised. To date, the role of the cAMP/PKA pathway and the transcription factor Rim101 has been implicated (Okagaki *et al*., 2011; Zaragoza *et al*., 2010), along with the Ste3a pheromone receptor (Okagaki *et al*., 2010) and the G-protein coupled receptor Gpr5 (Okagaki *et al*., 2011). The ligands required for triggering titan cell formation via these receptors are unknown, although recent data suggests that cryptococci might be sensing the phospholipid content of macrophages to trigger the titan cell switch (Chrisman *et al*., 2011).

Breaching the blood-brain barrier (BBB) is a key yet poorly understood component of cryptococcosis. To cause meningoencephalitis the fungus must first be able to traverse the barrier that protects the central nervous system. The mechanism of traversal is currently unknown but could involve either trancytosis through the endothelial cells (Chang *et al*., 2004), crossing between the cells by degrading the tight junctions (paracellular route)(Kim *et al*., 2012) or via carriage in phagocytes (Trojan horse route) (Charlier *et al*., 2009). Invasion of human brain microvascular endothelial cells (HBMEC) requires the cryptococcal gene *CPS1*, encoding a hyaluronic synthase, to allow attachment to surface exposed CD44 (Jong *et al*., 2008b). Consequently, cells deficient in the hyaluronic acid receptor CD44 allow fewer cryptococci cells to invade (Jong *et al*., 2012). This HBMEC invasion is also thought to involve protein kinase C alpha (PKCα)

(Jong *et al*., 2008a). The activation of PKCα by cryptococci potentially induces actin cytoskeleton rearrangements, leading to induced engulfment of the *Cryptococcus* (Chen *et al*., 2003).

Despite the importance of capsule in other areas of cryptococcal pathogenesis, the capsule seems to have little effect on BBB migration. Intravital mouse imaging has shown transmigration across the BBB did not require capsule. However, transmigration did involve cryptococcal urease (Shi *et al*., 2010). This is in agreement with *in vitro* data which observed no difference in the binding and uptake of encapsulated *C. neoformans* versus acapsular *C. neoformans*, using the mouse brain endothelial cell line bEnd3 (Sabiiti and May, 2012). The uptake into brain endothelia was also opsonin independent, with similar results seen for the human cell line hCMEC/D3. Surprisingly, yeast are not required to be viable for binding and entry into either cell line (Sabiiti and May, 2012). However, the viability of yeast cells reduced over time, indicating that brain endothelial cells can kill cryptococci once internalised.

To aid dissemination from the initial infection site (the lungs) to the brain, *C. neoformans* is able to use a vast array of virulence factors. Some of which have already been described in detail in this section. What follows, is a brief description of the other virulence factors *C. neoformans* utilises during pathogenesis.

Melanins are high molecular weight, hydrophobic pigments found in many kingdoms of life (Nosanchuk and Casadevall, 2006). Laccase mediates production of cryptococcal melanin by catalysing the oxidisation of 3,4-dihydroxyphenylalanine (DOPA) (Eisenman *et al*., 2007). The production of melanin has been shown to enhance survival of cryptococci challenged by amoeba (Steenbergen *et al*., 2001; Steenbergen *et al*., 2003)

and macrophages (Wang *et al*., 1995). Melanin provides cryptococci with resistance to environmental stresses such as temperature, radiation and hydrolytic enzymes (Nosanchuk and Casadevall, 2003; Rosas and Casadevall, 1997; Rosas and Casadevall, 2001). Melanin production has also been implemented in reducing the phagocytosis of cryptococci (Wang *et al*., 1995). One potential hypothesis to explain this is that the increased negative charge exerted on the fungal cell surface reduces unspecific binding to host cells (Nosanchuk and Casadevall, 2006). Melanin has also been described as a scavenger of free radicals, therefore providing protection against reactive oxygen and nitrogen species produced by host cells (Wang and Casadevall, 1994). Moreover, melanin has been demonstrated to aid cryptococcal resistance to antimicrobial peptides, potentially via direct binding and sequestering of the peptides (Doering *et al*., 1999).

Superoxide dismutase (Sod1) is a metalloenzyme that is able to convert oxygen radicals to hydrogen peroxide and oxygen, offering protection from endogenous superoxides (Cox *et al*., 2003). The superoxide dismutase 1 (Sod1) of *C. neoformans* has been described as having a compensatory role for the loss of melanin. Despite its importance for environmental stress resistance, melanin production is reduced at 37 °C. It was therefore hypothesised that Sod1 would be induced in such conditions to allow for ROS and RNS resistance despite the lack of melanin. This was indeed shown to be the case, cryptococci grown at 25 °C have high melanin production and low *SOD1* expression and the opposite occurs at 37 °C (Jacobson *et al*., 1994). Deletion of *SOD1* results in increased susceptibility to ROS and reduced virulence in a mouse inhalation model of infection, potentially due to their attenuated survival in macrophages (Cox *et al*., 2003).

The cryptococcal alternative oxidase (Aox1) has also been implicated in protection from ROS and intracellular survival of cryptococci within phagocytes (Akhter *et al*., 2003).

Urease is used by many pathogens to aid survival in acidic environments and increase local host cell damage (Rutherford, 2014). Urease converts the urea, found throughout hosts, into ammonia and carbonic acid. Urease is required for cryptococcal virulence (Cox *et al*., 2000) and is able to polarise the host towards a Th2 immune response (Osterholzer *et al*., 2009). The ammonia produced by urease can aid disruption of the tight junctions holding brain endothelial cells together (Singh *et al*., 2013). This potentially explains the previously mentioned requirement of urease for BBB penetration (Shi *et al*., 2010). Due to the fact that no human enzymes require nickel as a cofactor, and urease does, urease has the potential to be targeted therapeutically (Rutherford, 2014).

Phophosphingolipid-phospholipase C (*ISC1*) is required for the metabolism of fungal inositol sphingolipids. Isc1 has been shown to aid resistance to acid, oxidative and nitrosative stress (Shea *et al*., 2006). *C. neoformans* Isc1 mutants are hyper-encapsulated and found only extracellularly in a mouse infection. Mutants also had severely reduced dissemination to the brain (Shea *et al*., 2006).

Cryptococcal interactions with macrophages

The interaction of *C. neoformans* with alveolar macrophages is thought to be crucial in determining disease progression (Diamond *et al*., 1972; Diamond and Bennett, 1973; Shao *et al*., 2005; Vecchiarelli *et al*., 1994). Indeed, the interaction of *C. neoformans* with macrophages has been found to correlate with disease outcome in humans (Alanio *et al*.,

2011; Mansour *et al*., 2011; Sabiiti *et al*., 2014). Furthermore, macrophages have been shown to potentially exacerbate infection and aid dissemination of this yeast (Kechichian *et al*., 2007). In line with this, intracellular cryptococci are able to disseminate to the CNS in greater numbers than extracellular cryptococci (Charlier *et al*., 2009).

After inhalation of desiccated yeast or spores, alveolar macrophages are the first cells of the immune system that cryptococci are likely to encounter. With the help of an appropriate cellular immune response, the cryptococci are cleared. In cases where an appropriate cellular response is not mounted (HIV-infected individuals), cryptococci are able to replicate within macrophages and develop an uncontrolled infection.

Opsonophagocytosis is the uptake route most likely to occur in the *in vivo* system, due to the lack of efficiency of *in vitro* phagocytosis without any opsonin (Johnston and May, 2012). Many of the non-opsonic phagocyte receptors are unlikely to gain access to the antigens they recognise, as they are buried under the thick cloak of the cryptococcal capsule. Non-opsonised phagocytosis has thus far been an under researched area of cryptococcal-host interactions. The most efficient phagocytosis rates are achieved with opsonic antibody (Levitz *et al*., 1991; Mukherjee *et al*., 1996; Shapiro *et al*., 2002). Although, cryptococcal specific antibody production is a slow process, most of the population are seropositive by late childhood (Goldman *et al*., 2001). In cases when antibody is not present, complement is able to aid phagocytosis (Levitz and Tabuni, 1991), although the efficiency is likely to vary with strain capsule size (Zaragoza *et al*., 2003).

Perhaps surprisingly, lung surfactant protein D is able to opsonise strains of *Cryptococcus* with low capsule production (Geunes-Boyer *et al*., 2009; Geunes-Boyer *et al*., 2012). Furthermore, this surfactant opsonisation can be used to increase the pathogenesis of acapsular strains. Potentially by initiating an altered phagosome maturation pathway, as LAMP recruitment to these phagosomes is reduced (Geunes-Boyer *et al*., 2012)

C. neoformans however, is able to reduce phagocytosis by host cells in a variety of ways. Firstly, the capsule is able to physically and chemically block receptor binding sites (Bose *et al*., 2003). Acapsular strains are known to be phagocytosed more, likely via mannose and β-glucan recognition (Cross and Bancroft, 1995). The aptly named antiphagocytic protein 1 (App1) binds CD11b of CR3 to block complement iC3b-mediated phagocytosis (Stano *et al*., 2009). Perhaps surprisingly, the Δ*app1* cryptococcal mutant was hypervirulent in mice deficient for T cells and NK cells. Possibly further demonstrating the importance of cellular responses to alveolar macrophages that may have phagocytosed large amounts of cryptococci (Luberto *et al*., 2003).

Gat201 is a global transcription factor also capable of enhancing the antiphagocytic property of cryptococci. The transcription factor Gat204 and Barwin-like protein 1 (Blp1) are regulated by Gat201 and are required to prevent phagocytosis (Chun *et al*., 2011).

Other than receptor blocking, *C.* neoformans is also able to deter phagocytosis by sheer size. Titan cells reaching and enormous 50-100 um (including enlarged cell body and capsule) are resistant to phagocytosis. Surprisingly, via a mysterious mechanism, titan

cells are able to also prevent the phagocytosis of neighbouring normal sized cryptococci (Okagaki and Nielsen, 2012; Zaragoza *et al*., 2010).

Once internalised, cryptococci are able to utilise many virulence factors for persistence and replication within phagocytes (Figure 5). Intracellular replication of cryptococci in macrophages was first observed in 1973 (Diamond and Bennett, 1973). Early hypotheses to explain this suggested that the cryptococcal phagosome was diverted from becoming a phagolysosome (Vecchiarelli *et al*., 1994), a model that has not yet been extensively tested.

To aid intracellular survival reactive oxygen and nitrogen species are absorbed or destroyed by the capsule (Zaragoza *et al*., 2008) as well as protective factors such as Sod1, Aox1 and Ure1 (Brown *et al*., 2007) as previously described in this chapter.

Induction of host cell lysis is also an option for *C. neoformans* to escape phagocytic death. Intracellular cryptococci are able to induce apoptosis of host cells via NK-κB pathway (Ben-Abdallah *et al*., 2012). Although, lysis of host cells, appearing morphologically distinct from apoptosis is also seen, suggesting other routes of cell death are able to be used for this mechanism of escape (Johnston and May, 2012). Interestingly, it is likely that *Cryptococcus* acquired many of these adaptive virulence traits to combat digestion by amoeboid predators in their natural environment (Casadevall, 2012).

Vomocytosis is the unique non-lytic escape mechanism of cryptococci from host cells (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Only recently have similar processes been described for *Candida albicans* (Bain *et al*., 2012) and *C. kruzei* (Garcia-Rodas *et al*., 2011). An escape mechanism that leaves both the host cell and the pathogen alive has

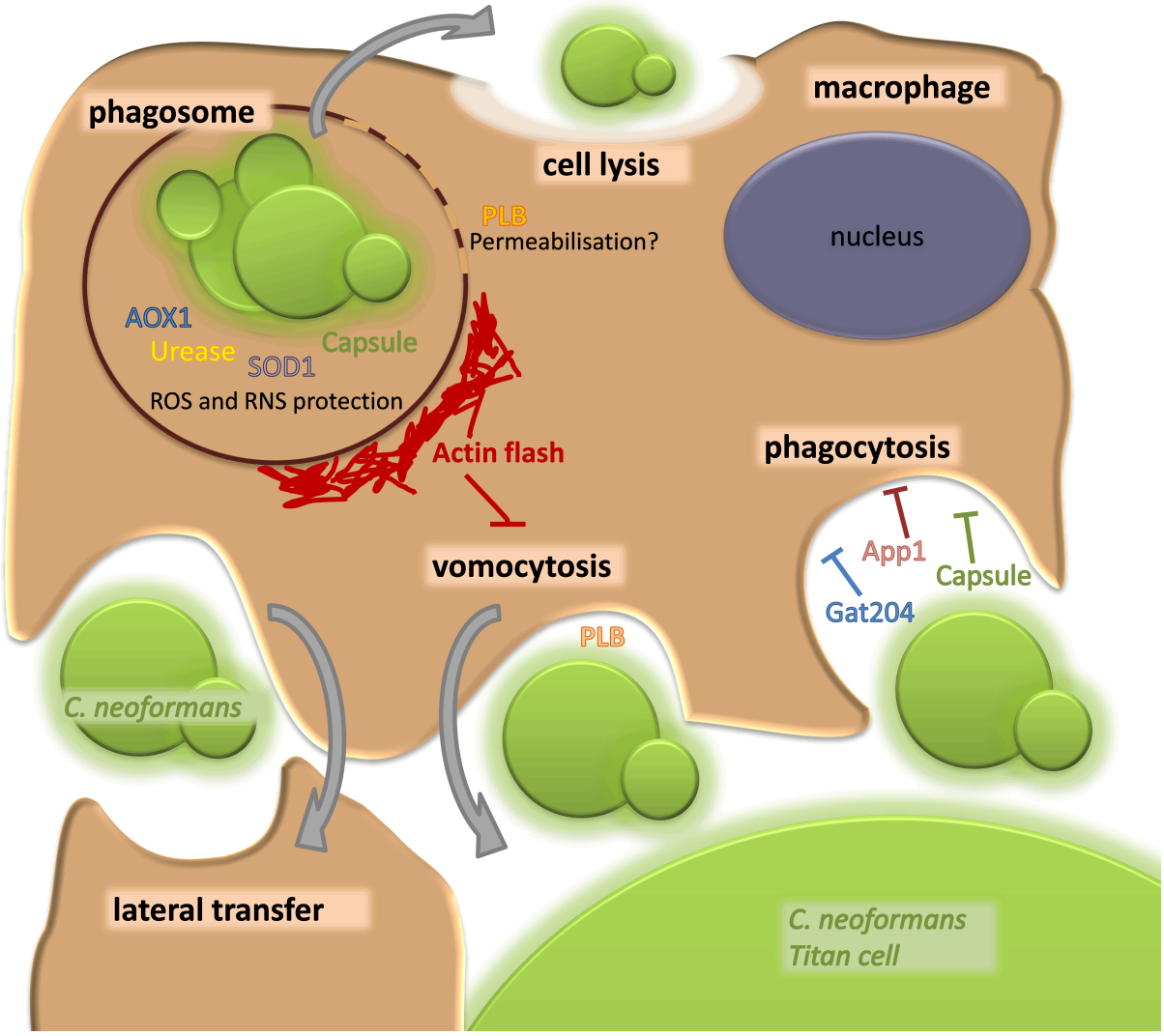

Figure 5. *Cryptococcus neoformans* interactions with macrophages.
The illustration summarises the most studied mechanisms of cryptococcal avoidance of intracellular killing. These include mechanisms to prevent phagocytosis: Gat204 regulator, Antiphagocytic protein 1 (App1), capsule and titan cell formation. Factors to aid intracellular survival: phospholipase B (Plb), capsule, alternative oxidase (Aox1), Urease and superoxide dismutase (Sod1). Mechanisms to escape the phagocyte include: cell lysis, lateral transfer and vomocytosis

major implications for infection progression and is likely to be important for tissue dissemination and possibly also as a mechanism for re-activation after latency. Vomocytosis has been confirmed *in vivo* within mice and possibly occurs more frequently *in vivo* than *in vitro* (Nicola *et al.*, 2011). The current model for vomocytosis implies cryptococci escape via exocytic fusion of the phagosome with the plasma membrane, thus releasing the fungus (Johnston and May, 2010). Moreover, vomocytosis requires microtubule activity, but not actin polymerisation. However, actin and the WASP-Arp2/3 actin-nucleating complex are involved in prevention of vomocytosis by the formation of dynamic actin cages, or "flashes", around the fungal phagosome. Although strains with high rates of vomocytosis induce more actin flashes, vomocytosis still occurs, suggesting that actin flashes may actually be a reaction invoked by vomocytosis attempts that aim to hold off expulsion for a short period but will eventually fail to contain this pathogen (Johnston and May, 2010).

The molecular details that precede vomocytosis are still under investigation. The most likely explanation is 'hijacking' of the exocytic pathway normally used to expel indigestible material from host cells. In the amoeba *Dictyostelium discoideum*, exocytosis of digested material requires that the post-phagolysosome be devoid of vATPase, which is achieved by vesicle budding from the mature phagosome (Clarke *et al*., 2002). Budding is controlled by WASP and SCAR homologue (WASH), an important regulator of actin polymerisation. WASH, Arp 2/3 complex and actin are thought to work together to disassemble vATPase, thus allowing the lumen of the post-phagolysosome to neutralise (Carnell *et al*., 2011; Clarke *et al*., 2002). Blocking WASH activity inhibits the exocytosis of indigestible material from amoebae and, intriguingly, also reduces cryptococcal vomocytosis rates from macrophages. The occurrence of

cryptococcal expulsion was reduced by almost half, in J774 cells expressing an inactive form of WASH, WASHΔVCA (Carnell *et al*., 2011). These results indicate that whilst actin flashes could be blocking vomocytosis (Johnston and May, 2010), actin polymerisation to aid vATPase removal might be required to occur before vomocytosis attempts can commence.

The macrophage response to *Cryptococcus* can also be affected by the cytokines that the macrophage has been exposed to. As previously mentioned, Th1 and Th17 cytokines (IFNγ and IL-17, respectively), are able to help control the intracellular replication of cryptococci. Cytokines are also able to affect the likelihood of vomocytosis. Th2 cytokine treatment of macrophages with IL-4 and IL-13, allowed higher rates of cryptococcal replication, but were able to reduce the rate of vomocytosis (Voelz *et al*., 2009).

Cryptococcal phagosomes are also seen to permeabilise rapidly after phagocytosis. This permeabilisation seems necessary and possibly even triggers actin flashes (Johnston and May, 2010; Tucker and Casadevall, 2002). The secreted phospholipase Plb1 has become recognised as another major virulence factor of *C. neoformans* (Cox *et al*., 2001; Noverr *et al*., 2003). Mostly due to *PLB1* knock out mutants having reduced dissemination to the CNS, and thus overall virulence, in animal models (Chayakulkeeree *et al*., 2011; Noverr *et al*., 2003). Plb1 is able to hydrolyse the ester linkages of membrane phospholipids by using its phospholipase and lysophospholipase activities (Chen *et al*., 2000). Plb1 has a third activity, lysophospholipase transacylase, which is thought to enable the use of host arachidonic acid for remodelling into cryptococcal lipids (Wright *et al*., 2007). Plb1 is also essential for vomocytosis and its secretion is augmented via the phosphatidylinositol transfer protein, Sec14 (Chayakulkeeree *et al*., 2011). The exact

role of this phospholipase is still under investigation, but it is tempting to predict a role in permeabilisation of the cryptococcal phagosome. The reason for permeabilising the phagosome is currently unknown, however, it is likely to aid neutralisation of the phagosome, thus inactivating antimicrobial proteases and allowing nutrients from the host cell to enter. One possible role for actin flashes is thus in resealing of the phagosome after pathogen-induced permeabilisation.

The pH of the phagosome has a major impact on intracellular parasitism by cryptococci. Chloroquine or ammonium chloride treatment to increase the phagosomal pH increases the rate of vomocytosis, but if acidification is blocked in the first instance (by using V-ATPase inhibitors) vomocytosis is suppressed (Ma *et al*., 2006; Nicola *et al*., 2011). Therefore, it seems that *C. neoformans* either requires acidification to initiate vomocytosis (which seems to be true for replication) or vomocytosis only occurs from phagosomes that have appropriately matured (although, these suppressive treatments did not block vomocytosis completely) (Ma *et al*., 2006). Additionally, the direct affects of chloroquine on cryptococcal replication make interpreting such results difficult (Harrison *et al*., 2000; Levitz *et al*., 1999).

C. neoformans is also able to move from one cell to another via a process termed "lateral transfer" (Ma *et al*., 2007), a rare event that has not been extensively investigated. The process of cryptococcal transfer between cells, is not dependent on uptake route (Ma *et al*., 2007). Cryptococci opsonised with either serum or specific antibody are both able to transfer between cells. In contrast to vomocytosis, lateral transfer requires actin polymerisation (Ma *et al*., 2007). Transfer requires live cryptococci and may reflect either a vomocytosis event followed by rapid phagocytosis or a more cooperative

mechanism requiring a transient membrane tunnel (Onfelt *et al*., 2004; Onfelt *et al*., 2006) between the two host cells in question (Johnston and May, 2012). The role of lateral transfer in disease is unknown, but could potentially allow cryptococci to remain undetected even after several transfer events between cells. These observations may explain how *C. neoformans* remains latent within the host for far longer periods of time than the average lifespan of a host phagocyte.

The disruption of phagosome maturation is a tactic adopted by many microbial pathogens as detailed in the introduction to this thesis. *C. neoformans* is well known to survive within, replicate (Feldmesser *et al*., 2001b) and escape from (Alvarez and Casadevall, 2006; Ma *et al*., 2006) the phagosome. This observation suggests that the fungus is either able to withstand the anti-microbial onslaught of the mature phagolysosome or is able to manipulate the host environment to reduce its anti-microbial activity.

Autophagy is a cellular process requiring a characteristic double membraned autophagosome to enable recycling and degradation of cell contents (Deretic, 2012). Proteins involved in host cell autophagy, such as Atg2a, Atg5, Atg9, Atg12 and LC3 have been indicated in aiding cryptococcal parasitism of *Drosophila* S2 cells. In the same study LC3 positive vesicles were seen to gather close to *Cryptococcus*-containing phagosomes (Qin *et al*., 2011). Conversely, blocking autophagy in the murine macrophage cell lines RAW and J774, help these cells to control growth of intracellular cryptococci (Nicola *et al*., 2012; Qin *et al*., 2011). Although, bone marrow derived macrophages from Atg5-/- mice, show little difference in their ability to control

cryptococcal growth (Nicola *et al*., 2012). The potential role for autophagy in the intracellular life of cryptococci is still debated.

Cryptococcal phagosomes acquire EEA1 within 10 min of uptake by dendritic cells or macrophages (Qin *et al*., 2011; Wozniak and Levitz, 2008) and subsequently acquire MHCII and the tetraspanin CD63 (Artavanis-Tsakonas *et al*., 2006). CD63 recruitment requires acidification of the phagosome (Artavanis-Tsakonas *et al*., 2006), which is also a prerequisite for intracellular survival of cryptococci (Levitz *et al*., 1999; Wozniak and Levitz, 2008). The lysosomal marker LAMP1 has been seen to associate with cryptococci-containing phagosomes from 1h to 3h post phagocytosis (Johnston and May, 2010; Levitz *et al*., 1999; Qin *et al*., 2011; Wozniak and Levitz, 2008). The presence of some late phagosome markers has led to the hypothesis that cryptococci persist within a normal phagolysosome, rather than modifying maturation processes. However, the presence of membrane markers is not in itself evidence for normal antimicrobial contents and further work is required in this area.

In this chapter, immunolabeling and live cell microscopy have been combined to demonstrate that *C. neoformans* subtly alters the maturation of the phagosome it resides within in order to generate a compartment that is amenable to cryptococcal replication.

Experimental Procedures

Cryptococcal strains, growth conditions and opsonisation

All reagents were acquired from Sigma unless otherwise stated. Strains used were *Cryptococcus neoformans var. grubii* serotype A strain H99 and mutants in this genetic background: *ure1*Δ (Cox *et al*., 2000), *ureG*Δ, *ureG*Δ:*UREG* (Singh *et al*., 2013), *plb1*Δ, *plb1*Δ:*PLB1* (Cox *et al*., 2001), *sec14-1Δ, sec14-1Δ*: *SEC14-1, sec14Δ*:*SFH1* (Chayakulkeeree *et al*., 2011) as well as the serotype D strain B3501 and its acapsular derivative CBS7931 *cap67*Δ (Jacobson *et al*., 1982) along with the wild type serotype A strain ATCC90112. Cryptococci were grown for 18 h at 25°C with rotating (20 rpm) in YPD medium (2% glucose, 1% peptone, and 1% yeast extract). Yeast from cultures were collected by centrifugation at 4,563 x g for 2 min and washed in triplicate with PBS. Yeast were then counted and their concentration adjusted to 10^7 yeast/ml. Where required, yeast were heat killed for 30 min at 55°C, and killing confirmed by CFU plating. Yeast were then opsonised with 10 μg/ml mouse IgG 18B7 (a monoclonal antibody to glucuronoxylomannan, a component of the cryptococcal capsule, kindly provided by Arturo Casadevall) for 1 h at room temperature. Alternatively, where stated, yeast were opsonised in 10% pooled human serum. When latex beads or zymosan were used, these were resuspended to the required concentration in PBS before use in infection assays.

Macrophage cell line culture

Murine macrophage-like cells J774A.1 were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, 100

μg/ml streptomycin and 100 U/ml penicillin. The cell line was incubated in a humidified environment at 37°C with 5% CO_2 and used between passage 5 and passage 15 after thawing. J774 cells ($1x10^5$) were seeded in 24-well plates (with 13 mm diameter glass coverslips for immunofluorecence experiments) 18 h before infection.

Human monocyte derived macrophages isolation and differentiation

Human peripheral monocytes were isolated from healthy volunteers. Blood was diluted at least 1:3 in sterile PBS before layering on to Ficoll plaque (GE Healthcare). This was then centrifuged for 30 min at 400 x g with no braking. White blood cells were then collected from the Ficoll interface and diluted in an excess PBS. Cells were then centrifuged for 10 min at 300 x g with braking and the resulting pellet resuspended in an excess of PBS and washed a further two times with centrifuging for 10 min at 200 x g. Cells were then resuspended in RPMI 1640 with 10% FBS and counted. Cells were then adjusted to 3 x 10^5 cells/ml and seeded into 48-well tissue culture plates. After 1 hour non-adherent cells were removed and RPMI 1640 with 10% FBS and 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) was added for differentiation of monocytes to human monocyte derived macrophages (HMDMs). Media was replaced every 3 days. Differentiated human monocyte derived macrophages (HMDMs) were used between 7 and 10 days after isolation.

Infection of macrophages with Cryptococcus

J774 cells were activated with 150 ng/ml PMA for 1 hour in DMEM without FBS prior to infection with cryptococci. HMDMs were activated 24 h prior to infection with 1 μg/ml

LPS and 1000 U/ml IFNγ (Immuno Tools) and then incubated in serum free RPMI 1 hour before infection. Macrophages were then infected with opsonised *Cryptococcus,* 3 µm latex beads, *Saccharomyces cerevisiae* or Zymosan A at a multiplicity of infection (MOI) of 10:1 for immunofluorescence experiments, or 5:1 for live cell imaging experiments. At the time of infection, serum free RPMI or DMEM (for HMDMs or J774s respectively) containing either 50 nM LysoTracker® Red DND-99 (Invitrogen, molecular probes), acidotropic dye for the visualisation of acidic cellular compartments, 5 µM MR-$(FR)_2$ Magic Red® (Immunochemistry Technologies), for fluorescent detection of the lysosomal cysteine protease cathepsin L or 1 mg/ml TRITC-Dextran (Sigma) as required. Cells were then taken directly for live cell imaging or incubated in a humidified environment at 37°C with 5% CO_2 for the stated time before fixation.

Labeling and imaging of fixed cells

For Rab GTPase labeling of cryptococcal-containing phagosomes, fixed coverslips were washed in PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were then treated with 50 mM NH_4Cl for 10 min and permeabilised in 0.1 % Triton X-100 for 4 min. Coverslips were then blocked with 5% goat serum for 1 hour and then washed in PBS before being treated with 0.5 µg/ml monoclonal rabbit anti-Rab5, Rab7, Rab9 or Rab11 (Cell Signaling) with 2.5 µg/ml human IgG for 30 min. After PBS washing, coverslips were then treated with 2 µg/ml goat anti-rabbit IgG-FITC (Sigma) with 10 µg/ml human IgG for 30 min

For fixed TRITC- Dextran experiments, J774 cells were washed with PBS and fixed for 10 min with 4% PFA in glass bottom plates. After fixing, cells were washed and left in PBS for imaging.

Coverslips were visualised with a Nikon Eclipse T*i*-S microscope, Plan Apo 60x/ 1.40 NA oil DIC objective (Nikon) and captured with QICAM Fast1394 camera (Q imaging).All image analysis was performed with NIS Elements AR software (Nikon).

Live cell imaging

Time lapse images were captured on a Nikon TE2000 enclosed in a temperature controlled and humidified environmental chamber (Okolabs) with 5% CO_2 at 37°C, with Digital Sight DS-Qi1MC camera (Nikon), Plan Apo Ph1 20x objective (Nikon), using NIS elements AR software (Nikon). Images were captured every 5 min for 18 h for LysoTracker and MagicRed experiments and every 2 min for 18 h for live TRITC-Dextran experiments. The Microscope was enclosed in a humidified environmental chamber (Okolabs) at 37°C with 5% CO_2.

Statistics

Statistical analysis of data was completed using GraphPad Prism version 6.04. Categorical data were analysed for statistical significance with a two-tailed Fisher's Exact Test. All data are presented as mean ± SEM from at least 3 independent experiments. **p<0.01, ***p<0.001.

Results

Acquisition of Rab GTPases is altered on Cryptococcus-containing phagosomes

Rab GTPases are a family of proteins that perform critical functions in coordinating vesicle maturation (Barr, 2013). Thus, we examined the distribution of Rab family GTPases on maturing cryptococcal-containing phagosomes. Rab5 is a well known marker of early endosomes and phagosomes, which is recruited rapidly to new phagosomes and activated by its guanine exchange factor (GEF). Once active, Rab 5 is able to recruit a host of effectors, including early endosome marker 1 (EEA1)(via hVPS34) and Rab7, which is considered a late phagosome marker thought to play an integral role in the fusion of the phagosome with lysosomes. Also present on early phagosomes is Rab11. Rab11 is located on recycling endosomes, aiding the recovery of phagocytic receptors back to the plasma membrane. Finally, Rab9 is a marker of endoplasmic reticulum derived membranes and often associated with vesicles of the late endocytic pathway (Kinchen and Ravichandran, 2008).

To determine which of these Rab GTPases are recruited to *C. neoformans*-containing phagosomes, J774 murine macrophages were infected with live *C. neoformans* H99, heat killed H99 or latex beads and immunolabeled at 15 and 120 min post infection for Rab5, 7, 9 and 11 (Figure 6A-D).

The early endocytic markers Rab5 and Rab11 were recruited to fewer phagosomes containing live cryptococci (Figure 6A and B). The GTPase Rab 9 recruitment was only altered between live and heat-killed cryptococcal-containing phagosomes at 2 h post infection (Figure 6C). Rab7 showed equivalent patterns of recruitment and loss across all three types of phagosome (Figure 6D). Subsequent examination of earlier time points

post phagocytosis revealed that Rab5 was recruited rapidly to all targets, but subsequently lost much more rapidly from phagosomes containing live cryptococci than from those containing killed yeast (Figure 7). A similar pattern was confirmed with another strain of *C. neoformans,* the serotype D strain B3501 (Figure 8).

Interestingly, when yeast were opsonised with human serum, as opposed to 18B7 antibody, Rab5 recruitment was slightly altered (Figure 9). Phagosomes containing live cryptococci appear to have enhanced initial recruitment of Rab5 that remains on the phagosome longer than if internalised via Fc receptors. Thus, early, but not late, endosomal maturation steps appear to be modified on phagosomes containing live cryptococci. Furthermore, this modification is not serotype specific. However, the route of phagocytosis can alter the timing of such modifications.

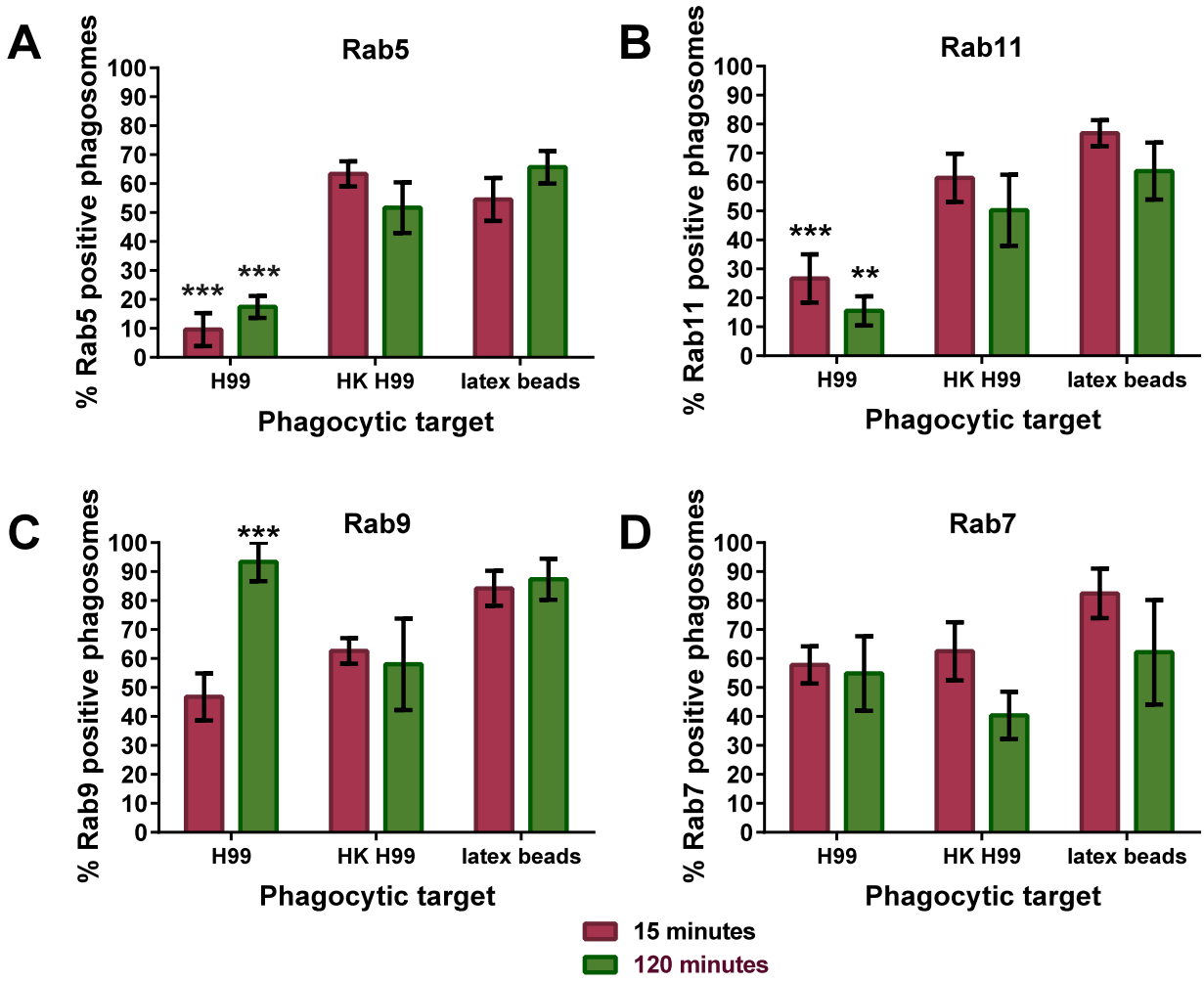

Figure 6. Acquisition of Rab GTPases onto phagosomes at 15 and 120 min post phagocytosis.

At 15 min and 120 min, Rab5 (A), Rab11 (B), Rab 9 (C) and Rab7 (D) recruitment was determined for phagosomes containing live *C. neoformans* H99, heat-killed H99 (HK H99) or latex beads. All data were collected from immunofluorescence analysis of J774 phagocytosed particles. All bars represent data collected from observing 186-664 phagosomes for each target at each time point over 3-5 biological repeats, mean ±SEM. *** $p<0.001$, ** $p<0.01$ Fisher's Exact Test.

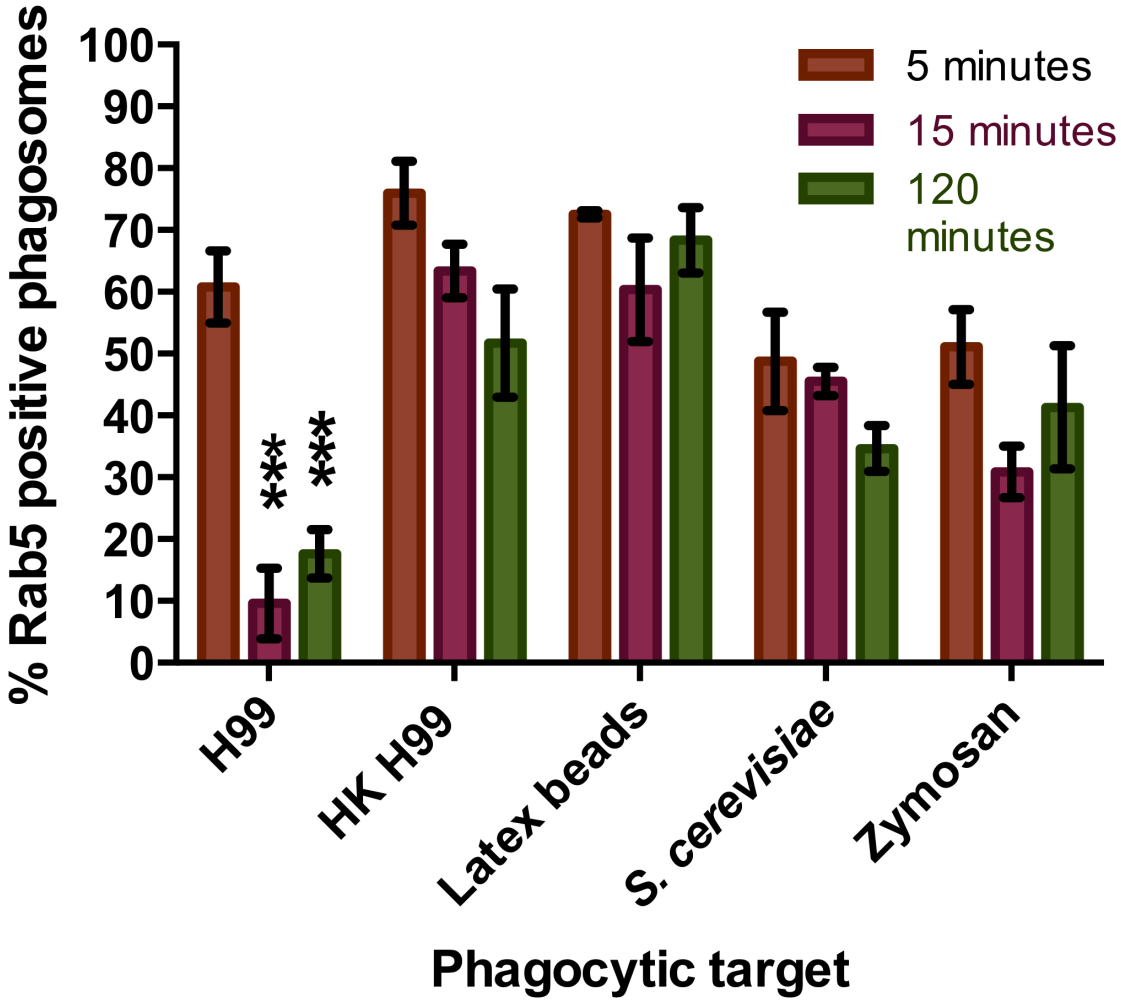

Figure 7. Acquisition of Rab5 GTPase onto phagosomes at 5, 15 and 120 min post phagocytosis

At 5, 15 and 120 min post phagocytosis Rab5 GTPase recruitment was determined for phagosomes containing live *C. neoformans* H99, heat-killed H99 (HK H99), latex beads, *S. cerevisiae* or zymosan. All data were collected from immunofluorescence analysis of J774 phagocytosed particles. Data for H99, HK H99 and latex beads at 15 and 120 min is replicated from data shown in Figure 6A. All bars represent data collected from observing 100-395 phagosomes for each target at each time point over 3-6 biological repeats, mean ±SEM. *** $p<0.001$, ** $p<0.01$ Fisher's Exact Test.

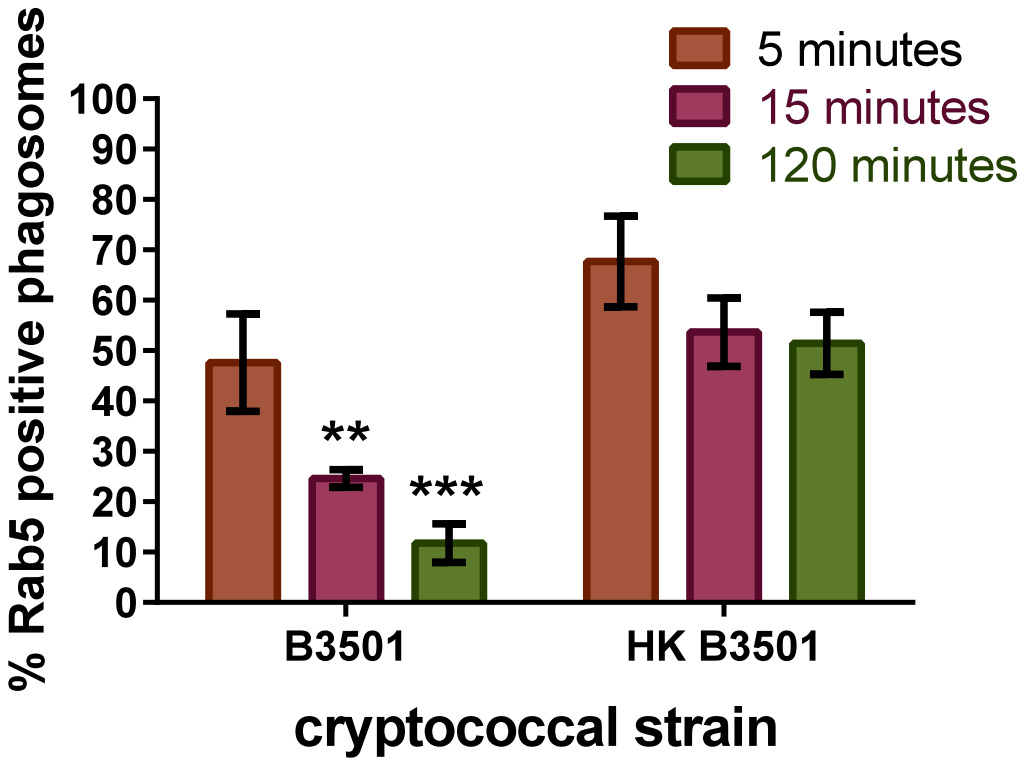

Figure 8. Acquisition of Rab5 GTPase to *C. neoformans* B3501 – containing phagosomes

At 5, 15 and 120 min post phagocytosis Rab5 GTPase recruitment was determined for phagosomes containing live *C. neoformans* B3501 or heat-killed B3501 (HK B3501). Data were collected from immunofluorescence analysis of J774 phagocytosed particles.. All bars represent data collected from observing 100-368 phagosomes for each target at each time point over 3-4 biological repeats, mean ±SEM. *** $p<0.001$, ** $p<0.01$ Fisher's Exact Test.

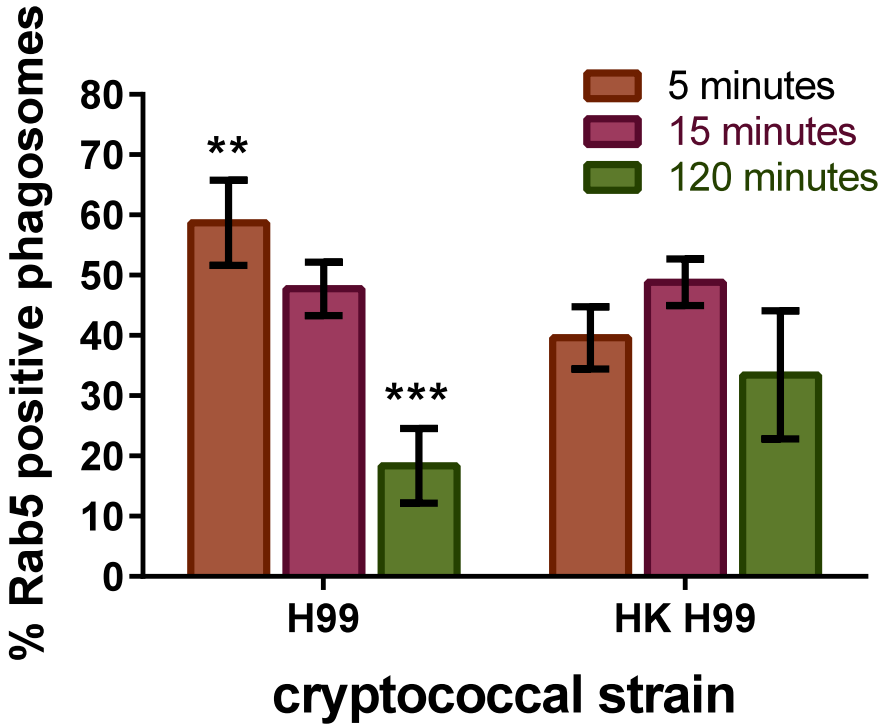

Figure 9. Serum opsonisation and Rab5 GTPase acquisition to CCPs
At 5, 15 and 120 min post phagocytosis Rab5 GTPase recruitment was determined for phagosomes containing live *C. neoformans* H99 or heat-killed H99 (HK H99) after opsonisation of yeasts with 10% human serum. Data were collected from immunofluorescence analysis of J774 phagocytosed particles.. All bars represent data collected from observing 66-654 phagosomes for each target at each time point over 4-5 biological repeats, mean ±SEM. *** $p<0.001$, ** $p<0.01$ Fisher's Exact Test.

Live cryptococci are able to block acidification of the phagosome

Normal phagosome maturation culminates in an acidic phagolysosome containing high levels of hydrolytic enzymes. Thus, subtle changes in Rab5/11 recruitment to the phagosome were investigated as they may lead to a modification of later stages of maturation. To determine if the *C. neoformans*-containing phagosome reaches a typical acidic pH (as low as 4.3-4.5 for J774 cells)(Chen, 2002), we used the acidotropic dye LysoTracker Red to monitor pH during live imaging of the macrophage – *Cryptococcus* interaction. Over the 18 h infection of J774 macrophages with live H99, very few cryptococcal-containing phagosomes became acidic (Figure 10A, 11A and movie 1). In contrast, heat killed H99-containing phagosomes always became highly acidic (acquired LysoTracker positive signal) over the course of the experiment, typically within 1 h of engulfment (Figure 10A, 11B and movie 2). These findings were confirmed in human peripheral blood monocyte-derived macrophages (Figure 10B) and with another strain of *C. neoformans,* ATCC 90112 (Figure 10D).

Since most phagosomes containing live cryptococci do not acidify, we wondered whether the rare instances in which acidification was seen might reflect variation in the yeast population within the inoculum. Reanalysis of the time-lapse movies demonstrated that budding or vomocytosis activity of the intracellular cryptococci was restricted exclusively to yeast resident in non-acidified (LysoTracker negative) phagosomes (Figure 10C). Thus, the rare acidification events (<10%) that are observed in the infections performed with live H99 probably represent dead cryptococci in the inoculum. To ascertain if this was the case the percentage of dead cryptococci in an

overnight culture was determined (Figure 12). The results confirmed that a small percentage of yeast in overnight cultures are no longer viable.

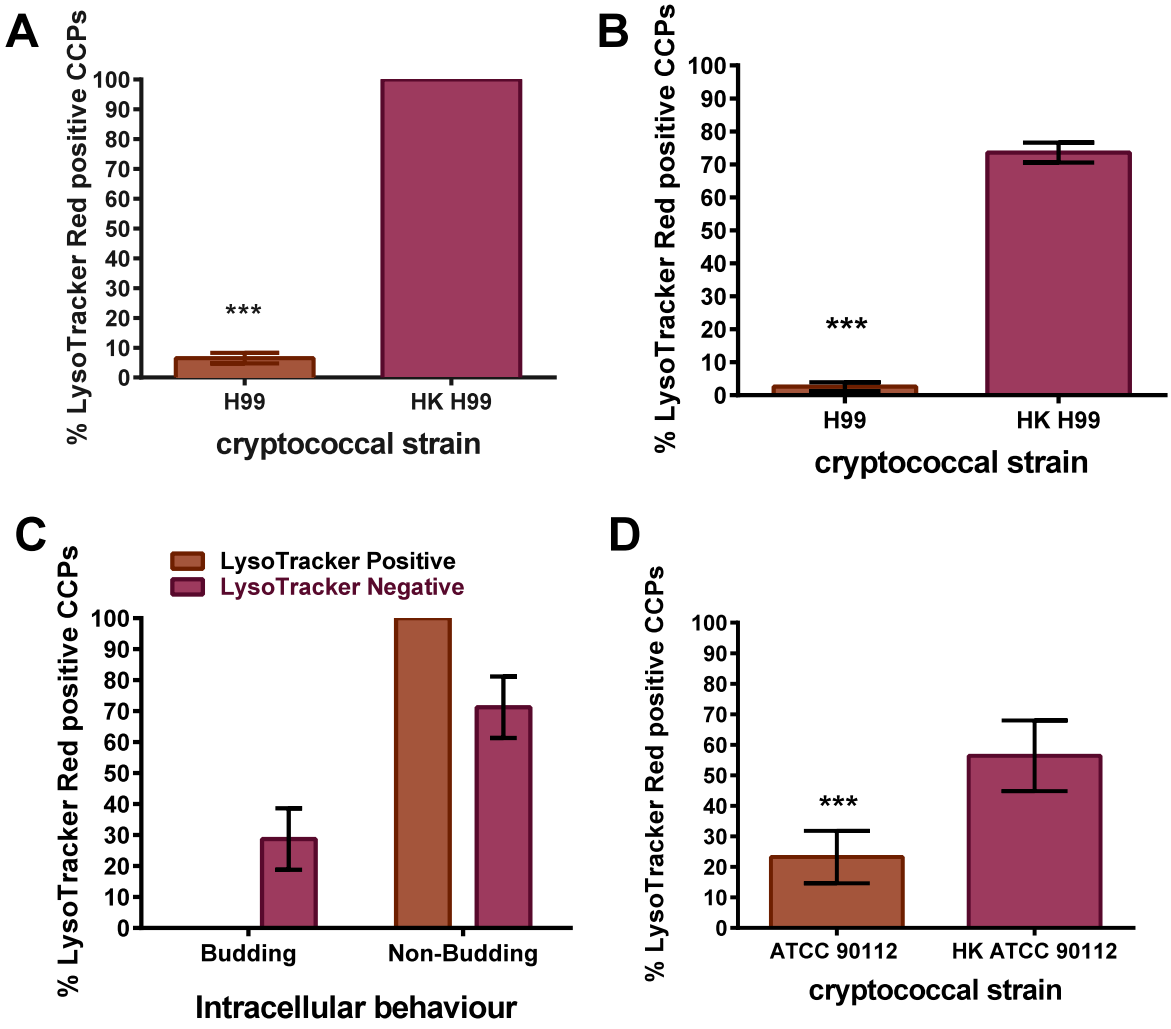

Figure 10. Acidification of the *C. neoformans*-containing phagosome.
Phagosomes containing live *C. neoformans* H99 do not acquire the acidification reporter LysoTracker Red in either J774 cells (A) or human monocyte derived macrophages (B). Phagosomes containing heat-killed cryptococci mature normally and become acidic in both cases. Only H99 yeast residing in non-acidified phagosomes are capable of budding (C). High vomocytosis strain ATCC 90112, displayed the same LysoTracker pattern in human monocyte derived macrophages (D). CCPs; *Cryptococcus*-containing phagosomes. Graphs display mean ±SEM of 95-202 phagosomes for each target across 3-4 biological repeats. Significance figures for A and B come from a Fisher's Exact Test of the raw data. *** $p<0.001$. ** $p<0.01$.

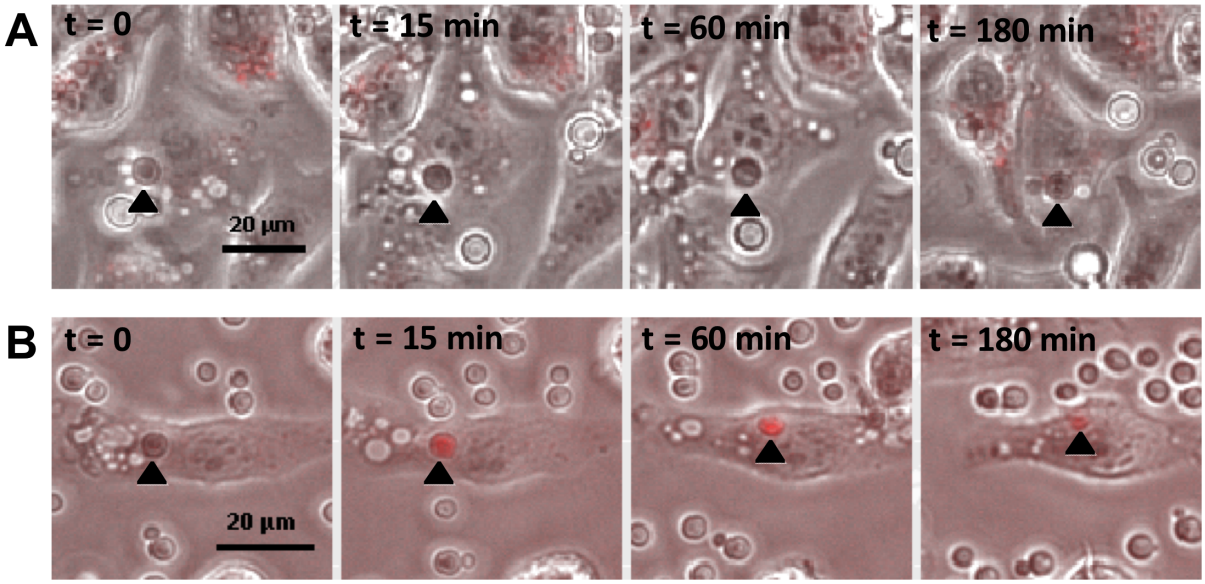

Figure 11. Acidification of heat killed cryptococcal-containing phagosomes
Panels A and B represent still images taken from time-lapse microscopy experiments with J774 cells(Movie 1, Movie 2) at the indicated times post phagocytosis, displayed is the merged Phase contrast and Red fluorescence (LysoTracker Red) images. Live H99 (A) reside in a LysoTracker negative phagosome, whilst heat-killed H99 rapidly acquire LysoTracker (B).

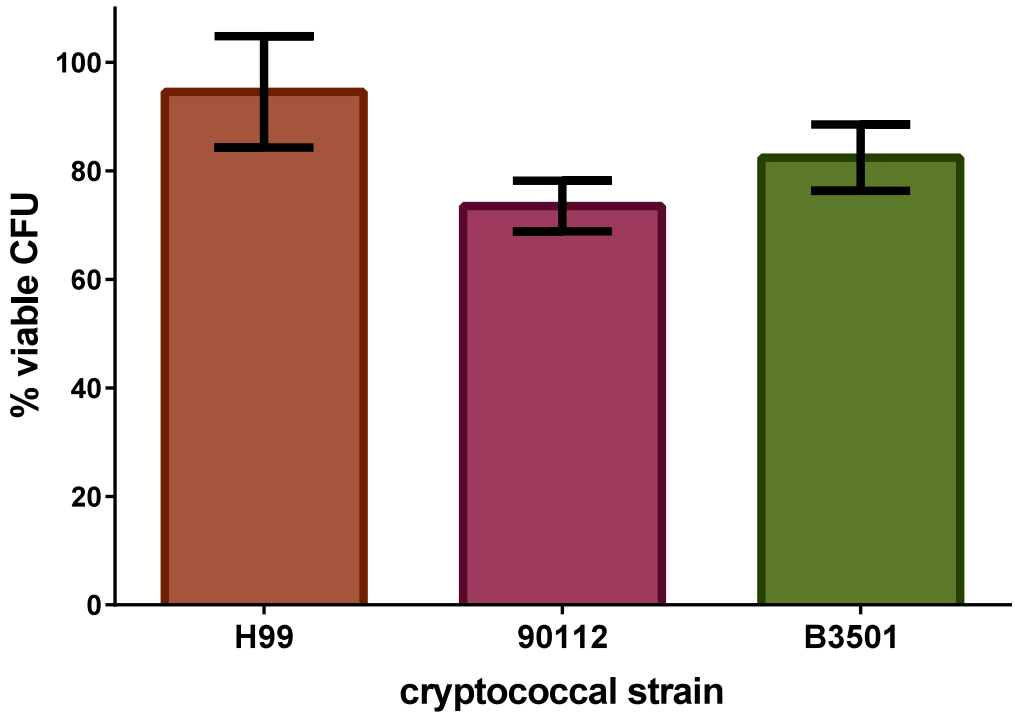

Figure 12. Percentage viable cryptococci in 18 hour cultures.
After overnight growth of cryptococci in YPD media at 25 °C rotating at 25 rpm, yeast were washed in PBS, counted with a haemocytometer and serial diluted for plating. By comparing haemocytometer counts and CFU counts obtained after incubation of serial dilution plates, a percentage of viable yeast was calculated. Data represents mean ± SEM, collected over 3 biological repeats.

Cryptococcus-containing phagosomes do not harbour cathepsin activity

The cathepsins are a family of degradative enzymes that accrue to high levels within mature phagosomes in order to aid digestion of the phagosomal cargo. Cathepsin L is most active in the latest stages of phagosome maturation in macrophages (Claus *et al*., 1998; Lennon-Dumenil *et al*., 2002). I therefore exploited the fluorescent reporter dye MagicRed that is activated by cathepsin L (and to a lesser extent cathepsin B) to monitor cathepsin activity within phagosomes containing cryptococci. As with LysoTracker, we saw very little evidence of cathepsin activity in phagosomes containing live cryptococci (Figure 13A, 14A and movie 3). In rare cases when cathepsin activity was detectable, these cryptococci failed to bud or vomocytose (Figure 13A, 14B and movie 4). Interestingly, time-resolved monitoring of Magic Red demonstrated that there is a relatively short (<30 minute) "window" within which phagosomes acquire cathepsins; phagosomes that are negative for Magic Red during this "window" remained negative throughout the remaining 17.5 h of imaging (Figure 13B). The same cathepsin activity was seen for the cryptococcal strain ATCC 90112 (Figure 15 and 16).

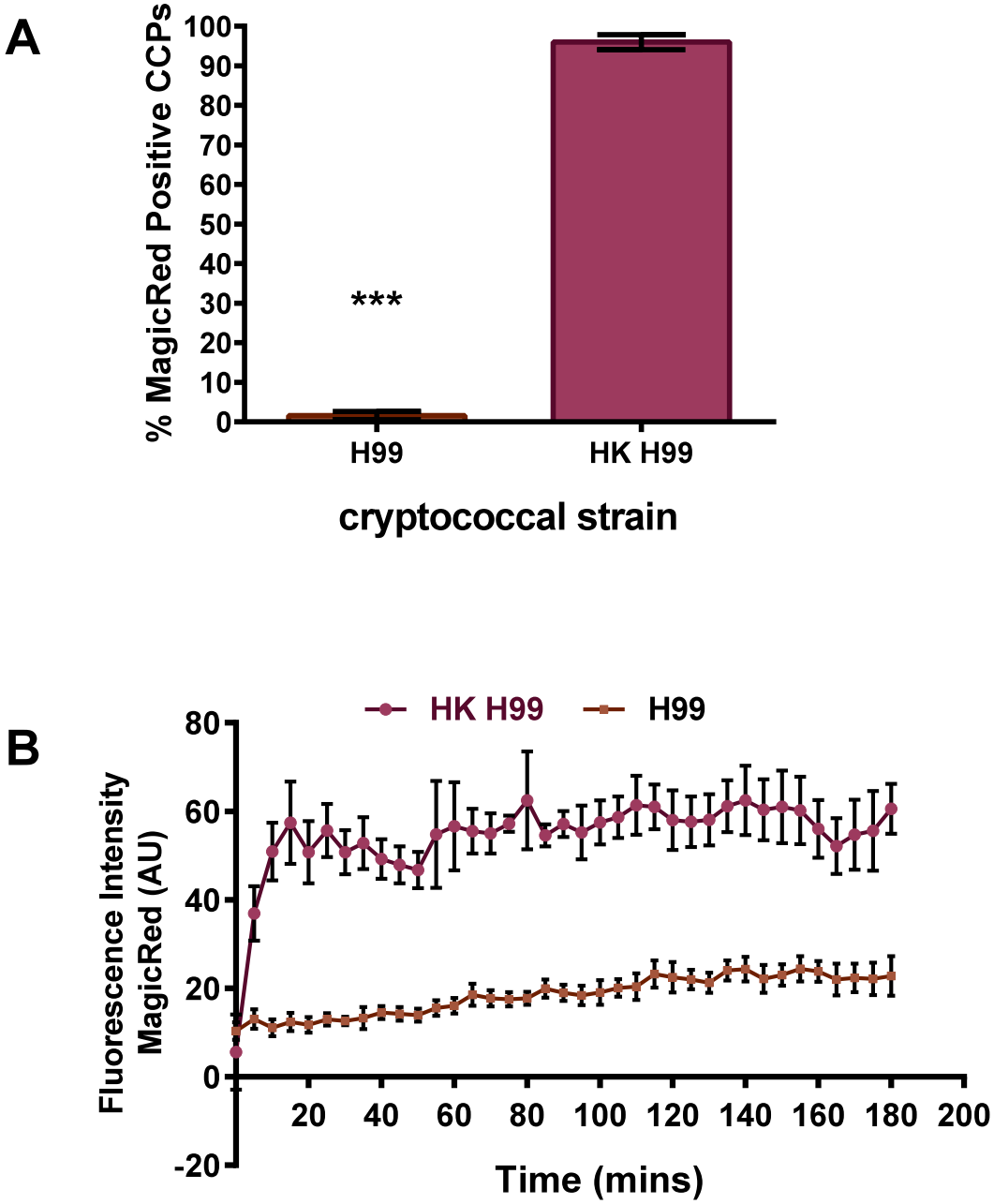

Figure 13. Cathepsin activity within the *C. neoformans*-containing phagosome. Cathepsin L activity was negligible in phagosomes containing live *C. neoformans* H99 (A). Graph represents mean ±SEM of n=4, 182-275 phagosomes at 30 min after infection. For analysis a Fisher's Exact Test was completed on the raw data. (B)Time-lapse imaging reveals that cathepsin L activity either develops rapidly (within 30 min post phagocytosis) or is never acquired. Graph represents mean fluorescent intensities from respective regions of interest ±SEM after background subtracting the mean intensity of 3 extracellular cryptococci selected from the same field of view (n=5 phagosomes). CCPs; *Cryptococcus*-containing phagosomes. ***p<0.001.

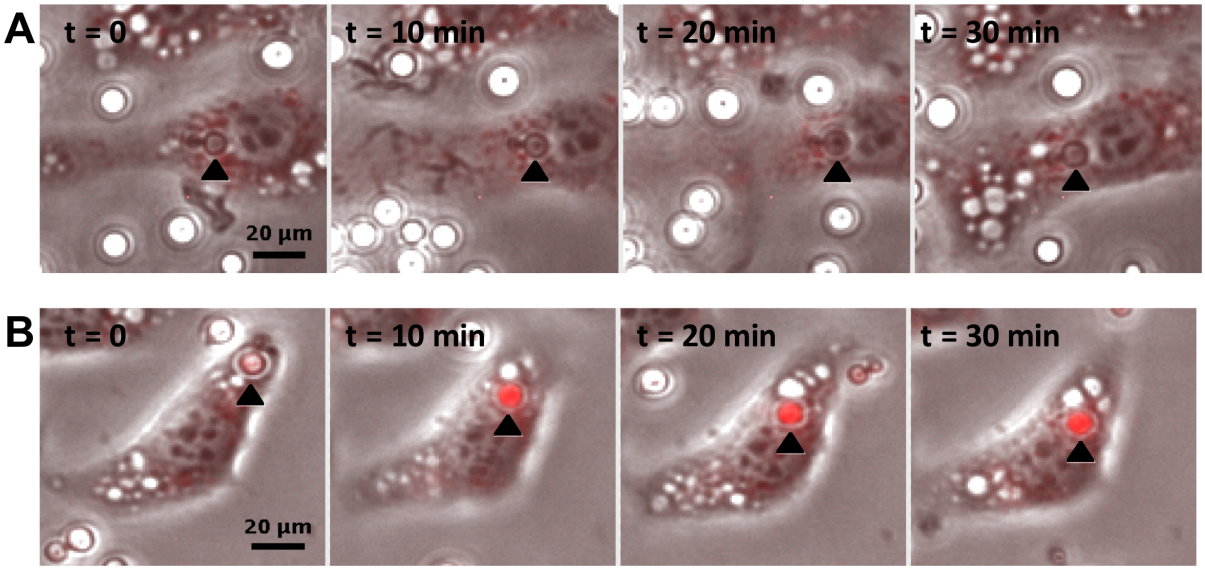

Figure 14. Phagosomes containing live cryptococci do not accrue cathepsin L activity

Panels A and B represent still images taken from time-lapse microscopy experiments with J774 cells (Movie 3, Movie 4) at the indicated times post phagocytosis, displayed is the merged Phase contrast and Red fluorescence (MagicRed) images. Live H99 (A) reside in a MagicRed negative phagosome, whilst heat-killed H99 rapidly acquire MagicRed (B). Showing lack of cathepsin L activity in live H99 containing phagosomes and cathepsin L activity in HK H99-containing phagosomes.

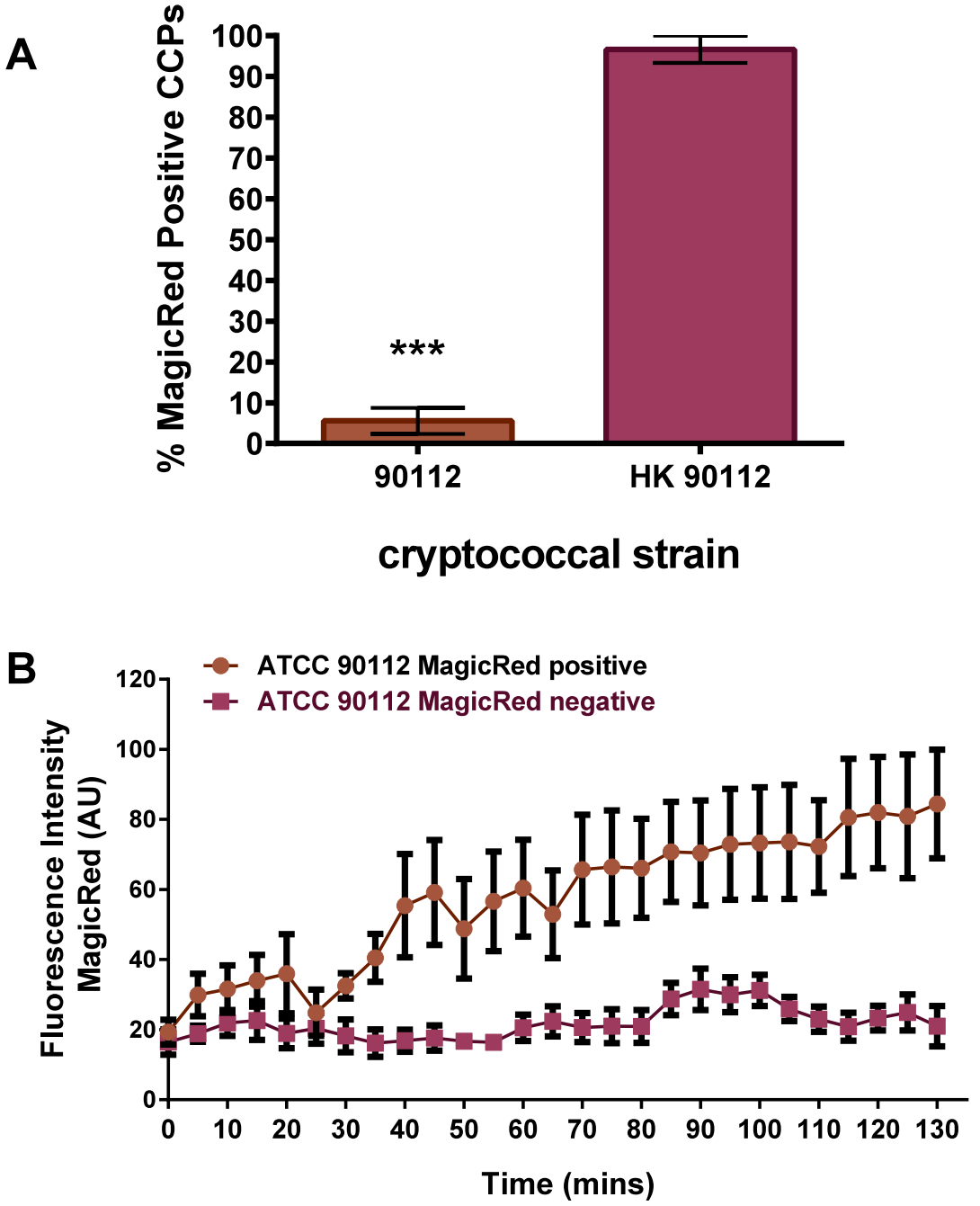

Figure15. Cathepsin activity in *C. neoformans* 90112-containing phagosomes
Cathepsin L activity was negligible in phagosomes containing live *C. neoformans* ATCC 90112 (A). (B) Graph represents mean fluorescent intensities from respective regions of interest ±SEM after background subtracting the mean intensity of 3 extracellular cryptococci selected from the same field of view (n=4 phagosomes). In (A) graph represents mean ±SEM of n=4, 166-222 phagosomes at 30 min after infection. For analysis a Fisher's Exact Test was completed on the raw data CCPs; cryptococcal-containing phagosomes. ***p<0.001.

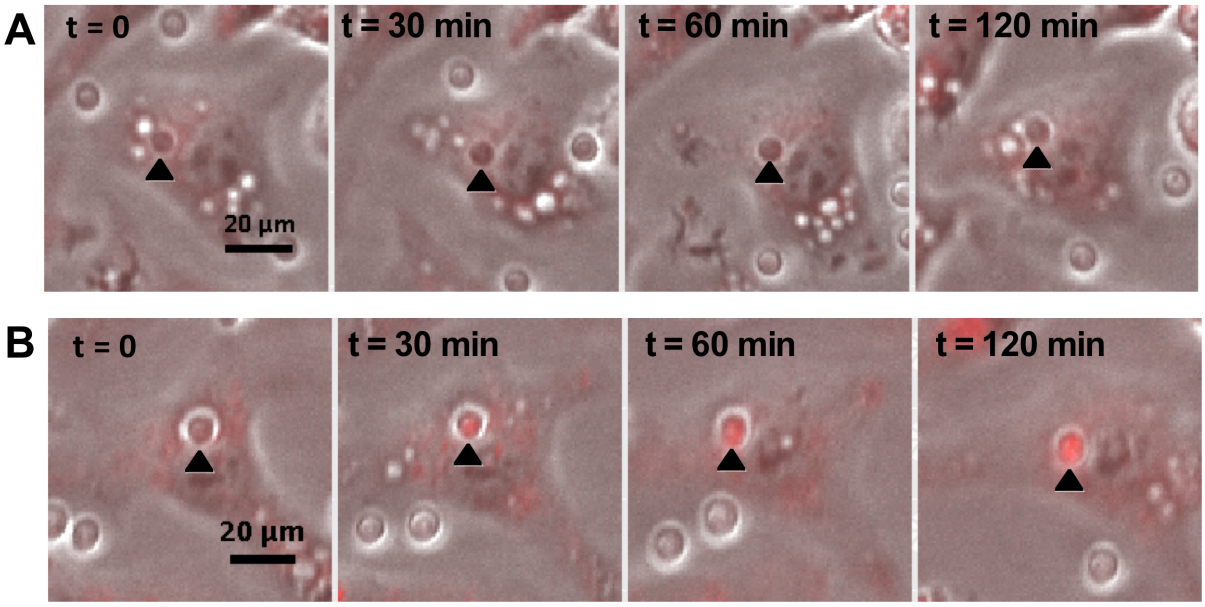

Figure 16. Phagosomes containing live *C. neoformans* ATCC 90112 do not accrue cathepsin L activity

Panels A and B represent still images taken from time-lapse microscopy experiments with J774 cells at the indicated times post phagocytosis, displayed is the merged Phase contrast and Red fluorescence (MagicRed) images. Live ATCC 90112 (A) reside in a MagicRed negative phagosome, whilst heat-killed ATCC rapidly acquire MagicRed (B). Showing lack of cathepsin L activity in live 90112 containing phagosomes and cathepsin L activity in HK 90112-containing phagosomes.

A variety of cryptococcal mutants are still able to block acidification

To begin to decipher the possible mechanism *C. neoformans* employs to alter the maturation of the phagosome, we monitored the behaviour of several mutant strains known to be attenuated in virulence in animal models of disease. These mutant strains included a knock out of the phospholipase B gene (*PLB1*), a tri-functional enzyme required for full virulence in mouse models and implicated in permeabilisation of the phagosome (Chayakulkeeree *et al*., 2011; Cox *et al*., 2001), as well as its associated Sec14 secretion system (Chayakulkeeree *et al*., 2011). We also tested mutants lacking functional urease gene expression (*URE1* and *UREG*) (Cox *et al*., 2000; Singh *et al*., 2013) since urease is thought to play an important role in intracellular parasitism (Rutherford, 2014). Lastly, we studied the acapsular mutant Δ*cap67* and its parental strain, B3501 (Jacobson *et al*., 1982), since the production of capsule is considered one of the most important virulence determinants of cryptococci with a key role in protecting the pathogen from the host attack (Vecchiarelli *et al*., 2013).

Interestingly, none of these mutants showed any alteration in their ability to prevent acidification of the phagosome (Figure 17). Thus, even attenuated cryptococcal mutants retain the ability to modify host phagosome maturation, suggesting the existence of hitherto unidentified virulence mechanisms in this pathogen.

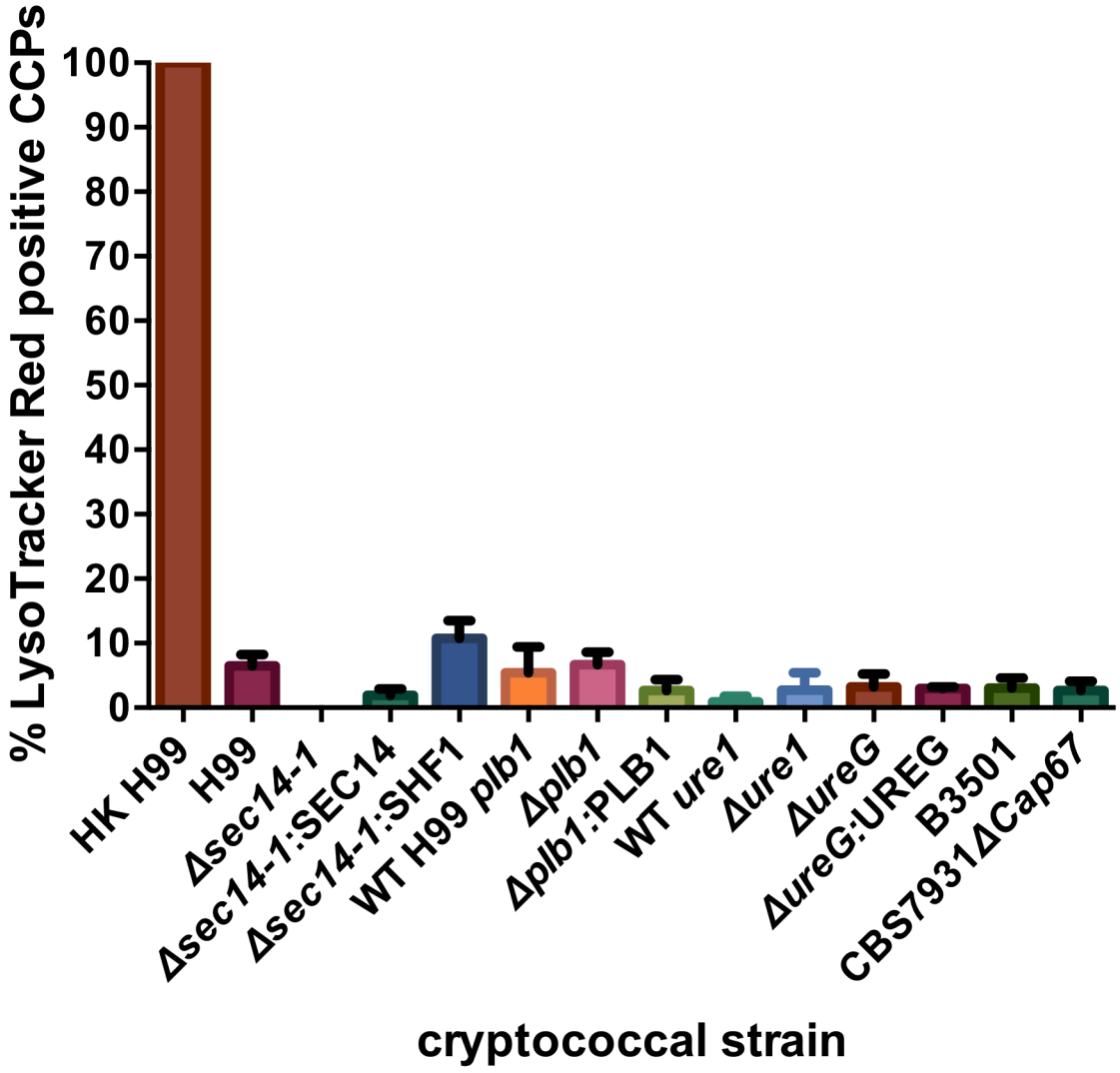

Figure 17. Several mutants of *C. neoformans* are able to alter phagosome acidification.

A variety of *C. neoformans* mutants are still able to modify the acidification of the phagosome in J774 cells. CCPs, *Cryptococcus*-containing phagosomes. Graph displays mean ± SEM of 100-176 phagosomes for each target from at least 3 biological repeats.

The cryptococcal-containing phagosome rapidly permeabilises

Permeabilisation of a phagosome can be measured by using TRITC-conjugated dextran. This fluorescently tagged molecule is added to the infection media and is therefore phagocytosed along with the *C. neoformans,* as well as being delivered to the phagosome via vesicle trafficking with the macrophage. When a phagosomal lumen looses this fluorescent marker it is presumed this is due to compromisation of phagosomal membranes, allowing the dextran to diffuse out of the phagosome (a phagosome permeabilisation event). The permeabilisation of cryptococcal phagosomes has been implicated with the inset of actin flashes to prevent vomocytosis. It is known that *Cryptococcus*-containing phagosomes permeabilise (Johnston and May, 2010), but how early after phagocytosis was unknown. To investigate permeabilisation further, J774 macrophages were infected with *C. neoformans* strains MT105, B3501, Wild type H99 *PLB1*, mutant H99 Δ*plb1*, reconstituted strain H99 Δ*plb1*:*PLB1* and latex beads. TRITC conjugated dextran was added to the media at the time of infection. This allowed fluorescent material to be phagocytosed with the cryptococci and also be macropinocytosed by macrophages, thus labeling internal vesicles as media is trafficked within cells. After 2 h of infection, most cryptococcal-containing phagosomes have permeabilised (transitioned from containing TRITC-dextran to being TRITC-dextran negative) (Figure 18). The Plb1 mutant and reconstituted strain was also able to permeabilise their phagosome, although both mutant and reconstituted strains were significantly different to wild type H99 (Figure 18).

Following this, live cell images previously created by Dr Simon Johnston (SAJ) were re-analysed for links between cryptococcal phagosome permeabilisation and vomocytosis.

In line with previous findings by SAJ (permeabilisation occurs before actin flashes)(Johnston and May, 2010), permeabilisation of the cryptococcal phagosome enables subsequent vomocytosis (Figure 19). Therefore, if phagosomes are still TRITC-dextran positive (not permeabilised) the cryptococci are unlikely to vomocytose.

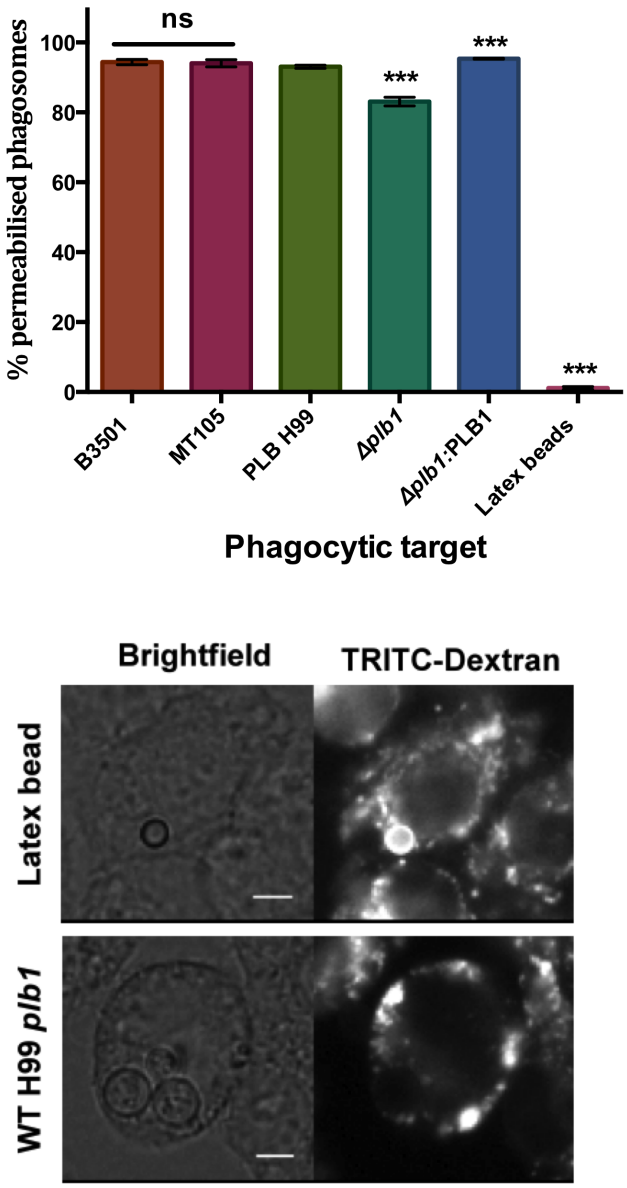

Figure 18. Permeabilisation of cryptococcal phagosomes.
J774 macrophages were seeded into glass bottom 96 well plates and infected with cryptococci. TRITC conjugated dextran was added to the media at the same time as infection. After 2 h of infection, extracellular cryptococci (or latex beads) were washed off and the cells fixed with 4% PFA. Fixed cells were left in 1xPBS and imaged for fluorescent dextran (A). Between 1022 and 9490 phagosomes scored for each strain over 3 biological repeats. Presented is percentage mean ± SEM. Bar represents 10 μm. Chi Squared test on categorical data. ***=p<0.001

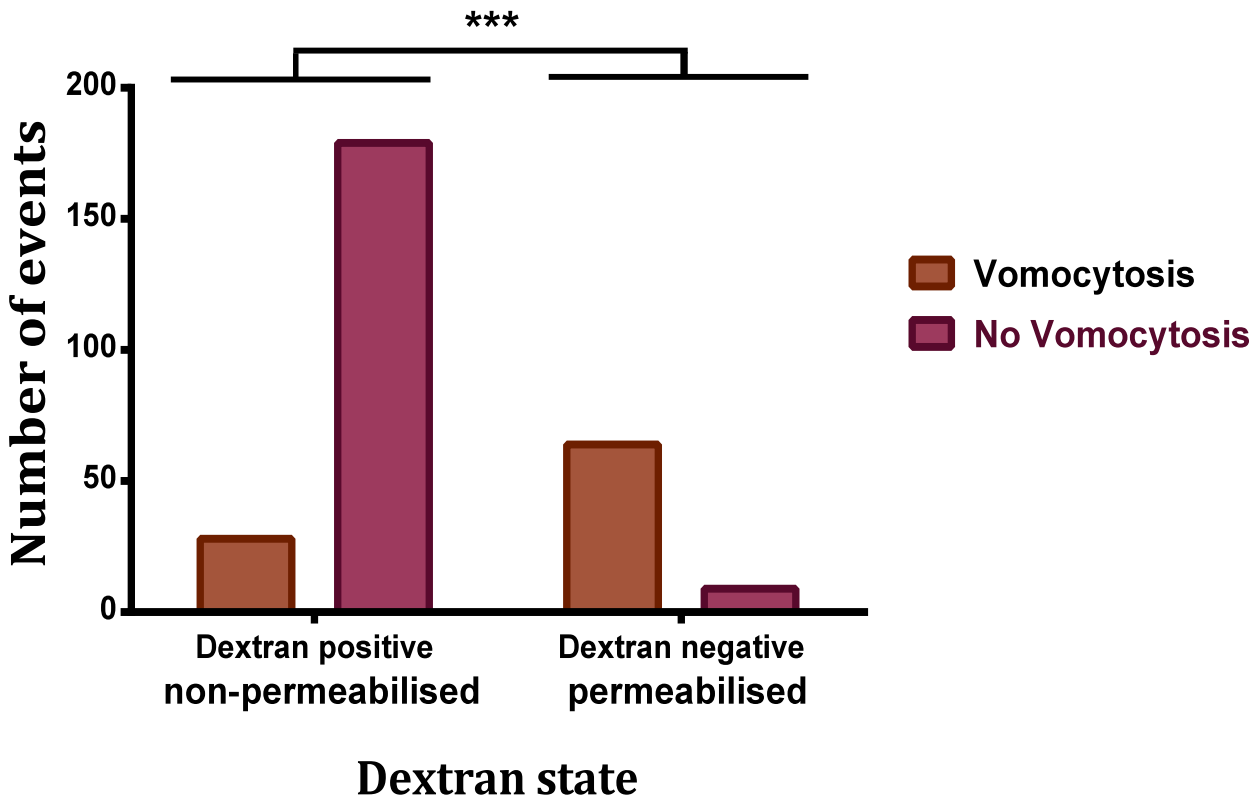

Figure 19. Permeabilisation and Cryptococcal vomocytosis.
Live microscopy videos for this data were created by SAJ and analysed by LMS. RAW 264.7 macrophages were pretreated with TRITC conjugated dextran. RAWs were then infected with *C. neoformans* strain H99. Infection was recorded for 18 h and the videos analysed for dextran state and vomocytosis A total of 280 cryptococci phagosomes were tracked and scored. Permeabilised and non-permeabilised phagosome populations were compared via a two-tailed Fisher's Exact test. *** =$p<0.001$. ns, non-significant.

Discussion

The relationship between macrophages and *Cryptococcus* species is known to be a complex one. Cryptococci are not only able to survive and replicate within the macrophage phagosome, but also escape the phagocyte by a non-lytic expulsion process termed vomocytosis (Alvarez and Casadevall, 2006; Ma *et al*., 2006). Previous research has indicated that the cryptococcal-containing phagosome acquires several markers of normal maturation. However, by examining very early events post-phagocytosis, I now show that cryptococcal-containing phagosomes display altered acquisition of the Rab family of small GTPases, in particular Rab5 and Rab11, within minutes of uptake. Such behavior is not seen with heat killed *C. neoformans*, latex beads, zymosan particles or *S. cerevisiae*, strongly implicating the existence of a pathogen-driven mechanism to disrupt phagosome maturation. This alteration in Rab5 GTPase recruitment was not serotype specific, as demonstrated by testing serotype A strain H99 and serotype D strain B3501. The timing of cryptococcal Rab5 modification was, however, altered slightly when cryptococci were first opsonised with human serum. This opsonisation is likely to have favoured complement receptor-mediated phagocytosis rather than via Fc receptor. The overall loss of Rab5 at 2 h post infection was, however, still far reduced in phagosomes containing live cryptococci.

Mechanisms that other pathogens use to manipulate Rab GTPases are varied, but include effector proteins such as SopB of *Salmonella*, which is able to reduce the levels of negatively charged $PI(4,5)P_2$ and phosphatidylserine on the phagosome, resulting in dissociation of Rab proteins and therefore a delay in phagosome fusion (Bakowski *et al*., 2010; Hernandez *et al*., 2004). Similarly, *Mycobacterium tuberculosis* manipulates

phagosome maturation (Fratti *et al*., 2001; Purdy *et al*., 2005) via at least two mechanisms; the dephosphorylation of Rab5-GTP and Rab7-GTP, resulting in inactivation of both critical GTPases (Sun *et al*., 2010) and via Lipoarabinomannan modification of the host calcium/calmodulin, EEA1, PI3K hVPS34 and PI3P production pathway (Vergne *et al*., 2003). Lastly, both *Listeria monocytogenes* and the lesser-known intracellular pathogen, *Tropheryma whipplei*, disrupt Rab5 activity via a glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which induces Rab5a-specific ADP ribosylation and blocks the GDP/GTP exchange activity (Alvarez-Dominguez *et al*., 2008). In the case of *T. whipplei* this leads to a chimeric phagosome displaying both Rab5 and Rab7 on its surface (Mottola *et al*., 2014). To date, it is unknown whether intracellular fungal pathogens have analogous routes to manipulate host endocytic trafficking, but recent advances in high-throughput genetics in cryptococci make this an exciting and promising avenue for future exploration.

The acidification of the cryptococcal-containing phagosome has been previously examined via ratiometric imaging (Levitz *et al*., 1999). This approach indicated an average phagosomal pH of 5, which was described as acidic. However, the macrophage phagosome is capable of reaching pH 4.3 (Chen, 2002) and data presented here suggests an alternative theory that the cryptococcal-containing phagosome actually does not fully mature but may reach only an intermediate pH that is insufficient for full antimicrobial activity. This modification of maturation is driven only by the live pathogen, indicating the existence of a currently unknown host-manipulation pathway in this organism that is independent of major virulence factors such as capsule, urease expression or phospholipase B production.

It was also observed that only cryptococci residing within a non-acidified phagosome were capable of budding and/or vomocytosis, strongly suggesting that cryptococci within acidifying phagosomes are dead or are in a quiescent/non-metabolically active state. The possibility of infecting macrophages with dead cryptococci from overnight cultures was investigated. Indeed, not all cryptococci are viable after overnight incubation and this could explain the rare acidic phagosomes seen in this experimental set up.

Other fungal pathogens have also been shown to alter the pH of the macrophage phagosome. The *C. glabrata*-containing phagosome, similarly to the *C. neoformans*-containing phagosome, recruits LAMP1, but not Rab5 or cathepsin. Furthermore, despite large amounts of vATPase on the phagosome, the contents are only weakly acidic (Seider *et al*., 2011). *C. glabrata* could be using mannosyltransferases, that are involved in *in vitro* alkalinisation of the environment and for reducing the pH of the phagosome (Kasper *et al*., 2014). The fungal pathogen *H. capsulatum* survives within macrophages by modifying the vacuole and keeping it at pH 6.5 by blocking acidification (Strasser *et al*., 1999). This incomplete block of acidification is likely to reflect a compromise between enabling fungal utilisation of iron from transferrin (at low pH), whilst still reducing the hydrolytic activity of acid-dependent proteases. Interestingly, *H. capsulatum* actively retains a slightly acidic phagosomal pH even when phagosomal acidification is blocked by the vATPase inhibitor Bafilomycin (Strasser *et al*., 1999), suggesting that the pathogen itself creates the slightly acidic phagocytic lumen rather than allowing partial vATPase activity. If such mechanisms exist for *C. neoformans* is unknown, but definitely warrants consideration in future investigations into the cryptococcal-containing phagosome.

The cryptococcal-containing phagosome has previously been shown to permeabilise soon after phagocytosis (Chayakulkeeree *et al*., 2011; Tucker and Casadevall, 2002). Interestingly, the data presented here indicates that cryptococcal phospholipase, Plb1, is not required for the majority of phagosome permeabilisation seen, despite the fact that Plb1, and permeabilisation are required for vomocytosis (Chayakulkeeree *et al*., 2011; Johnston and May, 2010). Therefore, Plb1 contributes to, but is not the sole driver of, phagosome permeabilisation.

The permeabilisation of the *C. neoformans*-containing phagosome could allow for dilution of phagosomal content and increase in the luminal pH throughout phagosome biogenesis. This would mean that markers of the phagosomal membrane would still be present (as seen in many other studies), but that the antimicrobial activity of the lumen would not be maximal. An alternative, or additional, mechanism could be avoidance of fusion with lysosomes. A lack of cryptococcal killing by human alveolar macrophages in a previous study was hypothesised to be due to a lack of phagosome-lysosome fusion (Vecchiarelli *et al*., 1994). A similar lack of acidification has been seen in microglia cells (specialised phagocytes of the central nervous system), in which only approximately 35% of phagosomes underwent full acidification (Orsi *et al*., 2009).

Previous studies of mouse macrophages determined that phagosome maturation is highly cargo-dependent (Oh *et al*., 1996). All the targets examined in this study (erythrocytes, poly-e-caprolactone beads, polystyrene beads and polyethylene glycol-conjugated beads) fused with lysosomes initially, but the amount of fusion with lysosomes after this differs. The retention of some markers, such as cathepsin L, differed dramatically between these targets and the authors suggest that some pathogens that do

fuse with lysosomes initially may still alter the further interactions with other vesicles (Oh *et al*., 1996). It is thus possible that such a situation occurs for the cryptococcal-containing phagosome, leading to a partially, but not completely, mature phagosome. The cryptococcal Plb1 is known to have maximum activity at low pH (Chen *et al*., 2000). Therefore, it could be that partial acidification is required to allow Plb1 augmented permeabilisation of the phagosome, resulting in dilution of the lumen content and neutralisation. Further interactions with lysosomes would lead to a cyclic process of acidification, Plb1 activity, permeabilisation and neutralisation. The precise role of Plb1 in permeabilisation of the phagosome and subsequent vomocytosis of cryptococci requires further investigation.

By using a combination of immunofluorescence and live-cell microscopy, the data presented here describes various markers of phagosome maturation (Rab GTPases, acidification and intraphagosomal cathepsin activity) are subtly altered by live cryptococci. These findings support the theory that *C. neoformans* does in fact alter the phagosome it resides in.

Chapter III. Intracellular persistence of *Streptococcus agalactiae*

Parts of this chapter have been previously published:

Cumley, N.J., **Smith, L.M**., Anthony, M., May, R.C. (2012) The CovS/CovR Acid Response Regulator Is Required for Intracellular Survival of Group B *Streptococcus* in Macrophages. *Infection and immunity*, **80** (5): 1650-1661.

Introduction

Streptococcus agalactiae, more commonly known as Group B *Streptococcus* (GBS), is the leading cause of neonatal pneumonia, sepsis and meningitis (Depani *et al*., 2011). GBS is able to colonise a variety of animal hosts and is commonly responsible for cases of bovine mastitis (Brochet *et al*., 2006; Keefe, 1997). GBS is a Gram-positive commensal of the human gastrointestinal and genitourinary tract. This relationship with humans means that approximately one third of female adults are colonised with GBS (Doran and Nizet, 2004; Hansen *et al*., 2004). Moreover, GBS is the most common infectious agent of neonatal pneumonia, sepsis and meningitis (Valenti-Weigand *et al.,* 1996). Infants born to colonised women will subsequently become infected in 70% of instances. Post-colonisation, 1% of colonised neonates will not control this infection and develop serious GBS disease (Becker *et al*., 1981; Melin, 2011). The current rate of invasive GBS infections in the UK for infants aged ≤90 days is 0.7 per 1000 live births, an increase from the year 2000 figure of 0.44/1000 live births (Lamagni *et al*., 2013).

Interestingly, recent research indicates that the emergence of GBS pathogenesis in the 1960s was due to the selection of a small number of tetracycline resistant clones (after the initiation of tetracycline use in 1948). These clones were also well adapted to cause disease in humans and have since given rise to most of the disease causing GBS isolates

(Da Cunha *et al*., 2014). GBS infections in elderly and diabetic adults are also increasing; rising from 92/1000 population in 1991 to 239/1000 population in 2010 (Lamagni *et al*., 2013). Reasons for this shift of burden to adults are currently unclear but may indicate that common immune dysfunctions or dysregulations occur in these populations.

Neonatal Group B Streptococcus transmission and infection

After colonisation of the mother, GBS are able to be vertically transmitted to the neonate via inhalation of contaminated vaginal or amniotic fluid during birth (Maisey *et al*., 2008; Melin, 2011). The inhalation of fluid provides GBS with direct access to the upper and lower respiratory tract, where GBS is able to attach and establish infection. GBS are also able to colonise the placental membranes of the mother, which can lead to rupture of membranes and premature delivery (Doran and Nizet, 2004). If the mother has developed protective anti-GBS IgG antibodies, these can be placentally transferred to the feotus (Becker *et al*., 1981). The levels of maternal IgG transferred to the neonate act as a strong predictor for the clearance of GBS (Becker *et al*., 1981). In the majority of cases, initial colonisation of neonates does not result in serious infection. However, in some instances, GBS are able to persist and cause severe GBS infections. Infections with GBS that present rapidly after birth are described as Early Onset disease (EOD). Infections that have a delayed presentation of up to many months after birth are referred to as Late Onset Disease (LOD).

GBS infections that present within the first week after birth, usually as pneumonia, are cases of EOD (Valenti-Weigand *et al.*, 1996). This pneumonia, if not detected early, can

progress into bacteraemia and septicaemia. The most likely route of infection for EOD is the previously mentioned inhalation of GBS contaminated fluids, as this will expose the neonatal lung to GBS (Maisey *et al*., 2008). Despite good treatment availability, EOD still carries a high mortality and morbidity. Although, fatalities due to EOD have been reduced thanks to the initiation of intrapartum antibiotic prophylaxis (IAP)(Kaambwa *et al*., 2010; Levent *et al*., 2010; Melin, 2011). IAP is the administration of antibiotics (usually penicillin) to the mother during labour. This strategy is used after positive culture tests for GBS at the latter stages of pregnancy (weeks 35-37), in countries such as the USA. The UK, however, currently recommends IAP only in the presence of one or more of five risk factors associated with GBS disease (Jones *et al*., 2006). Whether this strategy really is the best way to reduce EOD cases and be the most cost effective, is currently under debate (Kaambwa *et al*., 2010).

Capsular serotypes of GBS causing EOD correlate well with serotypes found colonising pregnant women (Figure 20)(Melin, 2011; Remington *et al*., 2011). Fatality rates in pre-term infants are a staggering eight times higher than that seen for full-term infants (Melin, 2011). This could potentially be due to incomplete placental transfer of maternal IgG antibodies, making pre-term infants more vulnerable to bacterial infections (Melin, 2011; Remington *et al*., 2011).

Late Onset disease (LOD) occurs after the first week of life and usually up to a maximum of seven months old (Doran and Nizet, 2004). Infants do not present with lung infections, but instead with bacteraemia, of which approximately half will develop meningitis (Doran and Nizet, 2004). For infants that survive GBS meningitis, a further 50% will suffer long-term morbidities such as vision and hearing impairments, seizures

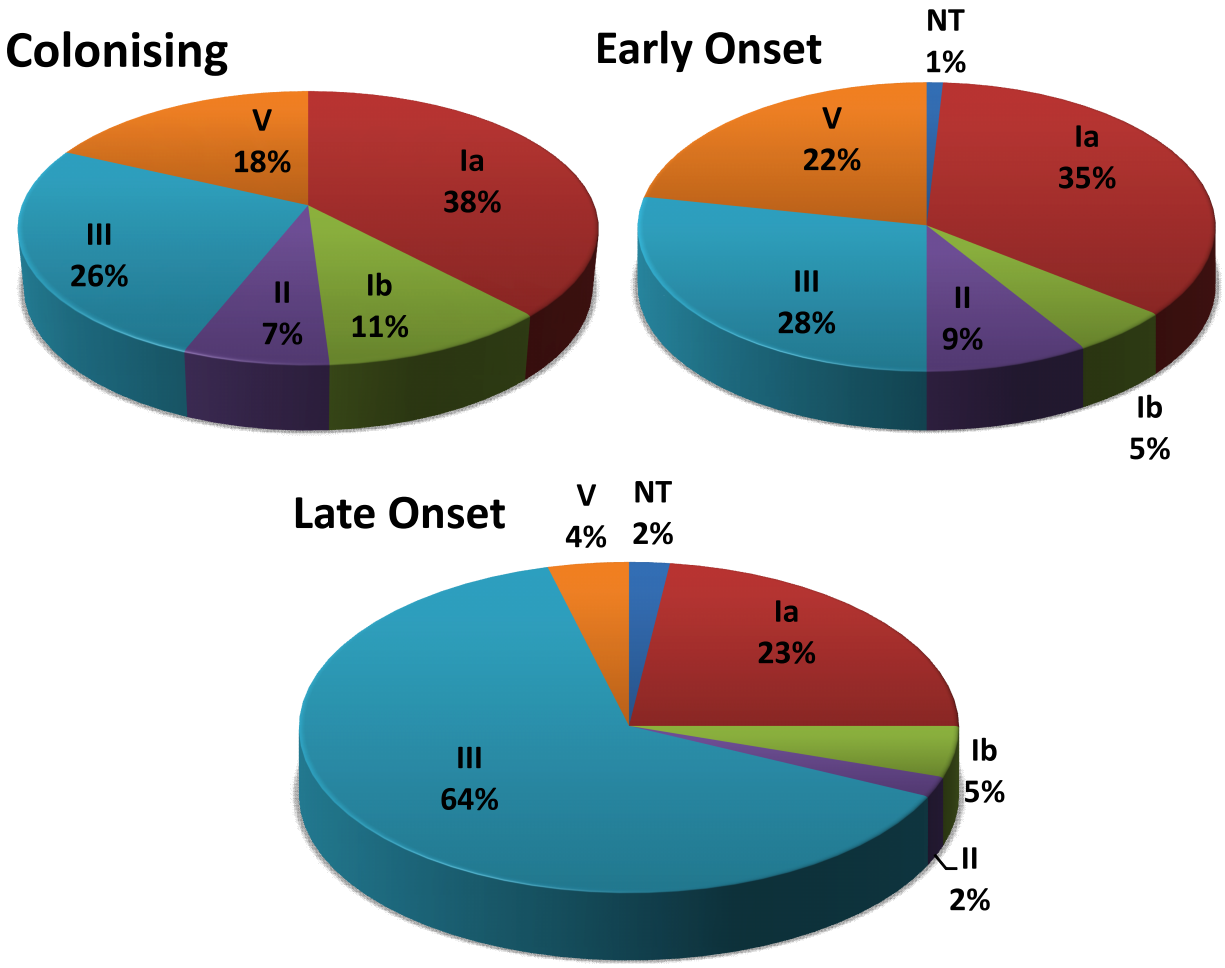

Figure 20. Serotypes of Group B *Streptococcus* that colonise women and cause neonatal disease.

Charts summarise data from several studies. Colonising, displays the percentage of GBS serotypes that are found colonising the female genitourinary tract of pregnant women. Early Onset, presents the proportions or GBS serotypes found causing neonatal early onset disease. Late Onset, presents the GBS serotypes found in cases of neonatal late onset disease. NT, non-typable. Adapted from (Remington *et al.*, 2011). Data from ((Blumberg *et al.*, 1996; Harrison *et al.*, 1998; Lin *et al.*, 1998)

and neurological sequelae (Johri *et al*., 2006; Melin, 2011). For the prevention of EOD there is now IAP, however, there are no prevention strategies in place for LOD.

The source of GBS for LOD is still unknown. Due to the lateness of infection it is unclear whether the GBS source is maternal or environmental. It would seem unlikely that the GBS for LOD originates from the GBS colonising the genitourinary tract of the mother as the serotype distributions do not match (Figure 20). The latest clue comes from research conducted by Tazi *et al.*, demonstrating that LOD causing GBS show increased adherence to gastrointestinal epithelia, potentially suggesting an oral route of LOD infection (Tazi *et al*., 2010). Once the source of LOD GBS is known, efforts can be targeted towards designing preventative strategies to combat this devastating form of streptococcal disease.

Infection initiation: Host contributing factors

The progress of many infections will rely upon virulence factors of the microorganism and individual immune factors of the host. In the case of GBS infections there are two hosts to consider: the mother and the neonate. Each mother has a unique set of risk factors that will influence the risk of neonatal infection. Young maternal age, black race, the use of foetal monitoring devices and the number of vaginal examinations during labour are all said to increase risk (Melin, 2011). There are also the previously mentioned five risk factors that the health care system will use to determine if IAP is required; previous baby affected by GBS; GBS urinary tract infection detected during the current pregnancy; preterm labour; prolonged rupture of the membranes; and fever in labour. Premature labour, rupture of membranes and bacterial infection are very

interlinked and difficult to extract which of these three events causes the others (Melin, 2011).

Of course, maternal colonisation is a prerequisite for GBS infection, but the immune response of the mother to her colonising GBS can make a substantial difference to the outcome of subsequent neonatal colonisation. Firstly, to have the potential to provide protection, the mother must be producing sufficient levels of anti-GBS antibody specific to the GBS strain she is carrying at the time of birth (Baker and Kasper, 1976). The risk of giving birth to a child with EOD is reduced by 91% if the mother is producing GBS specific antibody in quantities greater than 10 mg/ml (Lin *et al*., 2004). Secondly, these IgG molecules must be transferred across the placenta to the foetus. Finally, this transfer must be complete to provide maximum levels of protective IgG to the unborn infant. For pre-term infants this transfer has not completed and they are likely to have insufficient levels of maternal IgG to be protective (Wessels *et al*., 1998). It is currently unknown if colonisation is sufficient for an adult antibody response or if a more invasive exposure must occur.

Naturally occurring high concentrations of estradiol and progesterone are present during gestation and at birth. These steroid hormones have recently been found to impair the function of cord blood and newborn monocytes. Their cytokine production, when stimulated with endotoxin, bacterial lipopeptide, *E. coli* or GBS was markedly reduced, an affect not seen with adult monocytes. Neonatal cells are thought to be more susceptible to these steroids due to their increased expression of estradiol and progesterone receptors when compared to adult cells (Giannoni *et al*., 2011).

Lower levels of anti-GBS antibodies, of protective complement and of lung surfactant put premature and low birth weight neonates at higher risk of EOD (Doran and Nizet, 2004; Maisey *et al*., 2008). The lung surfactant is comprised mostly of dipalmitoylphosphatidylcholine (DPPC) (Nkadi *et al*., 2009). DPPC is able to protect the cells of the alveolus from the cytotoxic activity of the GBS virulence factor β-haemolysin/cytolysin (β-H/C) (Marchlewicz and Duncan, 1980). DPPC therefore prevents the cell damage and dissemination of GBS, normally achieved by the use of β-H/C (Liu *et al*., 2004).

There are many aspects of the immune system that have not reached full functionality in the neonate. Generally the neonatal immune system is complete (the cells are present), but it is the regulation and signaling that can be different to adults (Zaghouani *et al*., 2009). These differences are likely to account for the susceptibility of neonates to opportunistic infections, such as GBS disease.

One such difference can be found in the neonatal resident alveolar macrophages. Overall numbers of these macrophages present are reduced and their antibacterial activity is weakened (Sherman and Lehrer, 1985; Sherman *et al*., 1992). For pre-term (<32 weeks) neonates, the total mass of the neutrophil population is approximately 20% of that for term infants and adults (Carr and Huizinga, 2000). Furthermore, until 10 days of age, the chemotaxis abilities of neutrophils are significantly reduced; up to 50% compared to adult cells (Anderson *et al*., 1981; Carr, 2000).

More recent studies to investigate the deficiencies of neonatal phagocytes have used neonatal cord blood samples for experimentation. One such study has demonstrated that mononuclear cells and whole-blood cytokine responses of preterm infants

(gestational age, 26-33 weeks) to heat-killed GBS have a reduced production of tumour necrosis factor and IL-6 (Currie *et al*., 2011). For very preterm infants (gestational age <30 weeks), their monocyte cytokine responses to GBS could not be increased with the addition of complement, when compared to full term and adult monocytes (Currie *et al*., 2011). In addition, a restricted V_H gene repertoire and reduced IgG synthesis leave the neonate less able to opsonise pathogens and activate the adaptive immune response (Bauer *et al*., 2002). Thus, the immature neonatal immune system has vulnerabilities in both the innate and adaptive immune response.

Group B Streptococcus contributing factors: adherence and colonisation

GBS is capable of colonising various niches within the human body. These environments range from the lung epithelium to the acidic vaginal mucosa (Konto-Ghiorghi *et al*., 2009; Lalioui *et al*., 2005; Samen *et al*., 2007; Sheen *et al*., 2011; Tamura *et al*., 1994; Zawaneh *et al*., 1979). To initiate successful colonisation, GBS need to first attach to host cells. GBS is able to do this with both low affinity interactions via Lipoteichoic acid (LTA) and stronger attachment with various GBS surface proteins (Maisey *et al*., 2008). Some examples of such proteins will now be described.

GBS have a plethora of proteins to aid adhesion and colonization (Figure 21). ScpB is a GBS surface associated C5a peptidase, capable of interacting with fibronectin of the ECM (Cheng *et al* 2002). ScpB not only helps GBS attach to epithelial cells, but is also able to

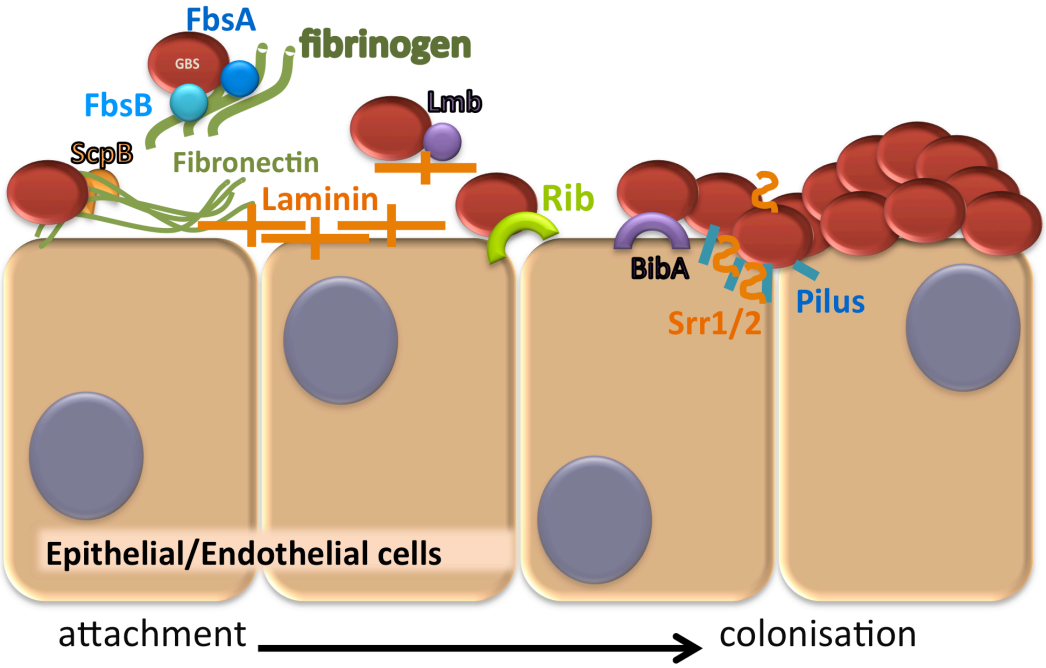

Figure 21. Group B *Streptococcus* mechanisms of attachment
Summarised are the main mechanisms that GBS uses to aid attachment and colonisation of host membranes. GBS are able to interact with components of the extracellular matrix (fibronectin, fibrinogen and laminin) and host cells directly. The host cell components recognised by Rib, BibA, Srr proteins and the GBS pilus are currently unknown. GBS, Group B *Streptococcus*. ScpB, streptococcal C5 peptidase B. Fbs, fibrinogen binding protein. Lmb, laminin binding adhesin. Rib, resistance to proteases, immunity, group B. Srr, serine rich repeat. BibA, Group B *Streptococcus* immunogenic bacterial adhesin.

reduce opsonisation. By only binding the immobilised form of fibronectin, ScpB does not risk opsonisation of GBS with soluble fibronectin (Hull *et al*., 2008). In addition, GBS is able to use the Lmb adhesin protein to interact with laminin of the ECM (Maisey *et al*., 2008). Moreover, the saliva component keratin 4 can be directly bound by the serine-rich repeat 1 (Srr-1) protein of GBS (Samen *et al*., 2007). However, only GBS of serotypes Ia, Ib, II, V and some strains of serotype III, express Srr-1. Srr-2, a non-homologous form of Srr-1, appears to be unique to serotype III strains of GBS (Seifert *et al*., 2006). Interestingly, Srr-2 has been associated with the heightened virulence in a neonatal sepsis murine infection model of serotype III strains (Seifert *et al*., 2006). Fibrinogen ECM attachment is also possible by *S. agalactiae*. This is achieved with the surface anchored proteins FbsA and FbsB (Doran and Nizet, 2004; Maisey *et al*., 2008).

The BibA adhesin increases adherence to human cervical and lung epithelial cells, and has been found to provide resistance to phagocytic killing (Santi *et al*., 2007). Although the human component recognised by BibA is yet to be determined. The Rib surface protein of GBS has also been shown to enhance binding to epithelial cells via attachment to a currently unknown host factor (Doran and Nizet, 2004; Maisey *et al*., 2008; Stalhammar-Carlemalm *et al*., 1993).

Many gram-positive bacteria express pili structures on their external surface. A recently identified pilus-encoding operon, *gbs1479-1474*, was characterised for strain NEM316 and named the PI-2A pilus (Dramsi *et al*., 2006; Lalioui *et al*., 2005). PilB is the major pilus component, forming the backbone of the pilus. PilA is the adhesin at the tip of the pilus and PilC is an accessory protein that is incorporated into the pilus backbone (Konto-Ghiorghi *et al*., 2009). This pilus structure is used by GBS not only for biofilm

formation, but also adherence to human alveolar epithelial cell line A549 (Konto-Ghiorghi *et al*., 2009). The biological relevance of these biofilm results may be limited as assays were performed on polystyrene wells rather than on cells or ECM components. The adhesin PilA has been found to work in combination with the Srr proteins to aid adhesion to vaginal epithelial cells (Sheen *et al*., 2011). Both mechanisms were found to be important for vaginal colonisation and retention in a mouse model (Sheen *et al*., 2011).

Group B Streptococcus contributing factors: evasion of the immune system

Many successful pathogens require the ability to evade stimulation of the host immune system. The surface of a microbe is often key in this evasion process, as it is exposed to and is constantly sampled by the host immune system. Using a combination of molecular mimicry and varying surface structures, GBS can do this remarkably well. The various ways GBS are able to avoid phagocytosis are discussed in this section (Figure 22).

Ten capsule serotypes exist for GBS; Ia, Ib, II through to the recently identified IX, dependent on the arrangement of capsule components (Maisey *et al*., 2008). The capsule is formed of varying levels of glucose, N-acetylglucosamine, galactose and sialic acid (Doran and Nizet, 2004). The capsule is a important virulence factor of GBS (Rubens *et al*., 1987). The α2-3 linked terminal sialic acid of the GBS capsule mimics host sialic acid linkages, thus reducing the likelihood of GBS being recognized as 'non-self' (Rajagopal, 2009). In addition to this molecular mimicry, the GBS capsule reduces the host inflammatory cytokine response (Doran *et al*., 2003).

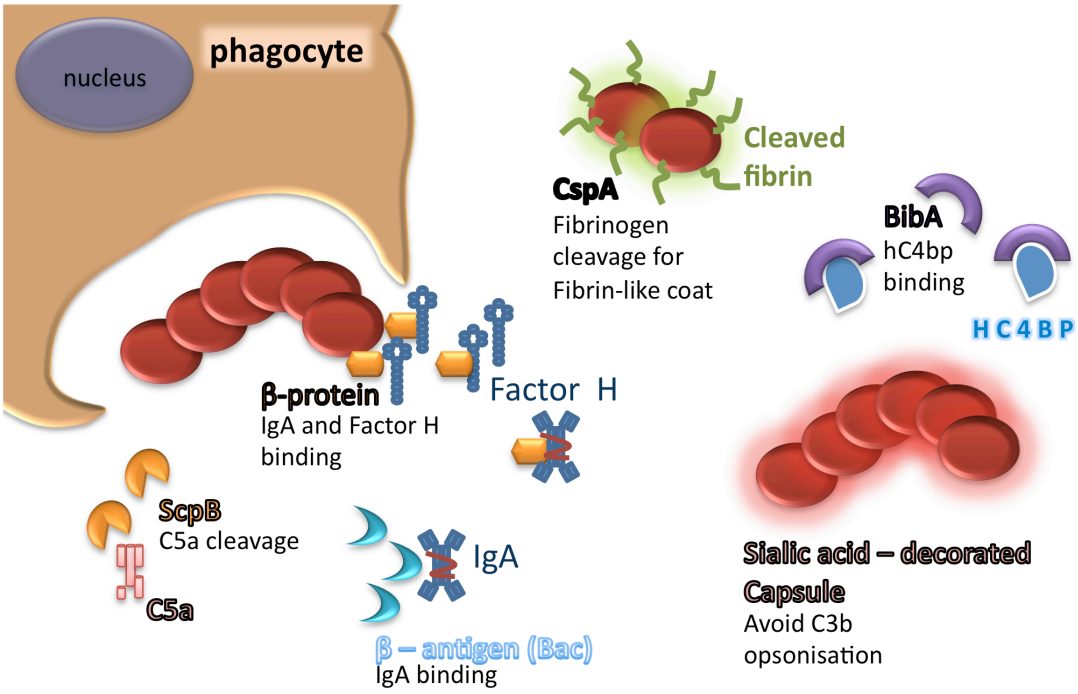

Figure 22. Group B *Streptococcus* mechanisms of phagocytosis avoidance
Using a combination of surface associated and secreted effectors, GBS are able to avoid opsonisation and/or phagocytosis. The main mechanisms GBS uses for this avoidance are summarised here. ScpB, streptococcal C5 peptidase B. BibA, Group B *Streptococcus* immunogenic bacterial adhesin. CspA, cell surface protease A.

Streptococcal C5 peptidase B (ScpB) is not only able to aid adhesion to the ECM component fibronectin, but also help to evade opsonisation (Bohnsack *et al*., 1991; Jarva *et al*., 2003). ScpB cleaves the complement component C5a, resulting in blocking the complement cascade and abolishing the chemoattractant activity of C5a for neutrophils (Bohnsack *et al*., 1997).

Cell-surface-associated protein A (CspA) offers further protection from opsonisation. By binding and cleaving fibrinogen, a fibrin-like coat surrounding the streptococci is produced (Harris *et al*., 2003). This fibrin-like coat provides a clever way for GBS to avoid recognition and stimulation of host responses. To further evade opsonisation, this time by antibodies, GBS β-antigen (Bac) is able to bind host IgA by its Fc domain. This binding orientation means phagocytes will not be able to use IgA for Fc receptor-mediated phagocytosis of GBS (Jerlstrom *et al*., 1996). Thus, the action of an antibody that is most associated with mucosal areas of the body, is abrogated. Moreover, Bac is able to block activation of the complement cascade by binding host factor H, a molecule that protects cells of the host from complement activation (Jarva *et al*., 2004). Bac binds the middle region of factor H, leaving the terminals available for prevention of opsonophagocytosis (Jarva *et al*., 2003). More recent research indentified that streptococcal histidine triad (SHT) can also be used for Factor H binding (Maruvada *et al*., 2009).

Select strains of GBS are able to produce the antigenically variable α component of C protein. This component increases the persistence of GBS within phagocytes (Madoff *et al*., 1996). In addition, α component variants (achieved by tandem repeat deletions) enhances the low immunogenic properties of the GBS surface (Madoff *et al*., 1996*)*. To

further reduce phagocytosis, GBS are also able to use the cell wall anchored adhesin BibA (Santi *et al*., 2009b). Secreted BibA potentially reduces phagocytosis by binding human C4-binding protein, a regulator of the classical complement pathway (Santi *et al*., 2007).

It is clear that GBS possess a vast array of mechanisms for host immune evasion.

Group B Streptococcus contributing factors: host cell invasion

GBS are able to induce the invasion of respiratory and pulmonary endothelial cells via a phagocytosis-like mechanism (Rubens *et al*., 1992). This mechanism initiates host phosphoinositide-3 kinase (PI3K) and Akt signaling cascades (Burnham *et al*., 2007). The actin cytoskeleton is subsequently reorganised to extend the cell membrane around streptococci and eventually engulf GBS (Doran and Nizet, 2004; Maisey *et al*., 2008). The GBS surface proteins Spb1 (Adderson *et al*., 2003) and α-C (Bolduc *et al*., 2002) have been found to aid this invasion into epithelial cells. Forced entry of GBS into cells of the host could provide a further mechanism of avoidance, by allowing GBS to 'hide' from immune surveillance.

GBS is able to cause severe tissue damage in the lungs during EOD. This is due to degradation of the barrier formed by the epithelial and endothelial cells. By doing so, GBS severely compromise the barrier and gain entry to the blood, allowing dissemination from the lung (Maisey *et al*., 2008). This destructive and pro-inflammatory activity is orchestrated by the β-haemolysin/cytolysin (β-H/C), an extensively studied virulence factor of GBS (Pritzlaff *et al*., 2001). β-H/C not only has cytotoxic properties, but is also able to manipulate the host response (Liu *et al*., 2004).

At sub-cytotoxic levels, β-H/C dependent invasion still occurs, but β-H/C now also induces the release of IL-8. The chemoattractant properties of IL-8 towards neutrophils results in neutrophil invasion and further local tissue damage (Sagar *et al*., 2013). Perhaps surprisingly, β-H/C is also able to stimulate the production of nitric oxide (NO) from macrophages. As a result of β-H/C, IFN-γ and the engagement of CR3 by GBS, inducable nitric oxide synthase (iNOS) is activated (Goodrum *et al*., 1994).

Further cytotoxic activity can be achieved with the use of the secreted CAMP factor of GBS (Lang and Palmer, 2003). Although, CAMP factor is not essential for virulence in a mouse model of systemic infection (Hensler *et al*., 2008), it is currently thought that CAMP acts as a 'back up' toxin, available to use when β-H/C would not be appropriate or beneficial. This is supported by the way in which these two toxins are regulated at the gene level. Both the β-H/C gene (*cylE*) and the CAMP factor gene (*cfb*) are regulated by the two-component system CovR/S (Liu *et al*., 2004). The sensor kinase CovS communicates with CovR by phosphorylation of the D53 aspartate of CovR (Lamy *et al*., 2004). CovR then binds and alters transcription at various operon and gene promoters. More specifically, CovR has apposing actions at the *cylE* and *cfb* genes; repressing *cylE* transcription whilst enhancing transcription at the *cfb* gene (Jiang *et al*., 2005; Lamy *et al*., 2004). Once phosphorylated by CovS, these activities of CovR are enhanced (Jiang *et al*., 2005; Jiang *et al*., 2008). However, CovR can also be phosphorylated by the GBS serine/threonine kinase Stk1 (Lin *et al*., 2009). By phosphorylating CovR at a threonine residue (T65), CovR no longer induces CAMP factor expression and instead activates β-H/C expression (Lin *et al*., 2009; Rajagopal *et al*., 2006). Thus, GBS is able to finely regulate the levels of both β-H/C and CAMP by using a combination of CovR/S and Stk1.

This balancing act could allow for GBS to preferentially activate the toxin of choice, depending on the stage of disease progression.

The dissemination of GBS is likely to be further aided by hyaluronate lyase (HylB) (Li and Jedrzejas, 2001). By degrading hyaluron, a particularly abundant ECM component of the placental and lung tissue, HylB aids dissemination of GBS at these particular host sites (Laurent and Fraser, 1992; Rajagopal, 2009).

Group B Streptococcus contributing factors: intracellular survival

It has been known for some time that GBS are able to survive intracellularly for prolonged periods of time (24-48 h) within macrophages (Cornacchione *et al*., 1998; Valenti-Weigand *et al*., 1996). This is an impressive time to be able to persist as most microbes are destroyed after only a few hours in these cells (Cumley *et al*., 2012; Flannagan *et al*., 2012; Kinchen and Ravichandran, 2008). Persistence within macrophages could provide GBS with a 'Trojan horse' strategy of dissemination. By surviving in macrophages, GBS could be transported to distal parts of the body (such as the BBB) without alerting the host immune system. Whether or not GBS manipulates the host phagosome for this survival is not known. The tools that GBS uses to aid persistence are extensive and varied in mechanism (Figure 23).

One such virulence factor is the GBS superoxide dismutase (SodA). By catalysing the conversion of oxygen radicals into oxygen and hydrogen peroxide, products of the macrophage oxidative burst can be fully metabolised by peroxidases (Poyart *et al*., 2001). This action of SodA protects GBS from oxidative damage (Poyart *et al*., 2001). Further protection is provided by large quantities of glutathione. GBS are able to use

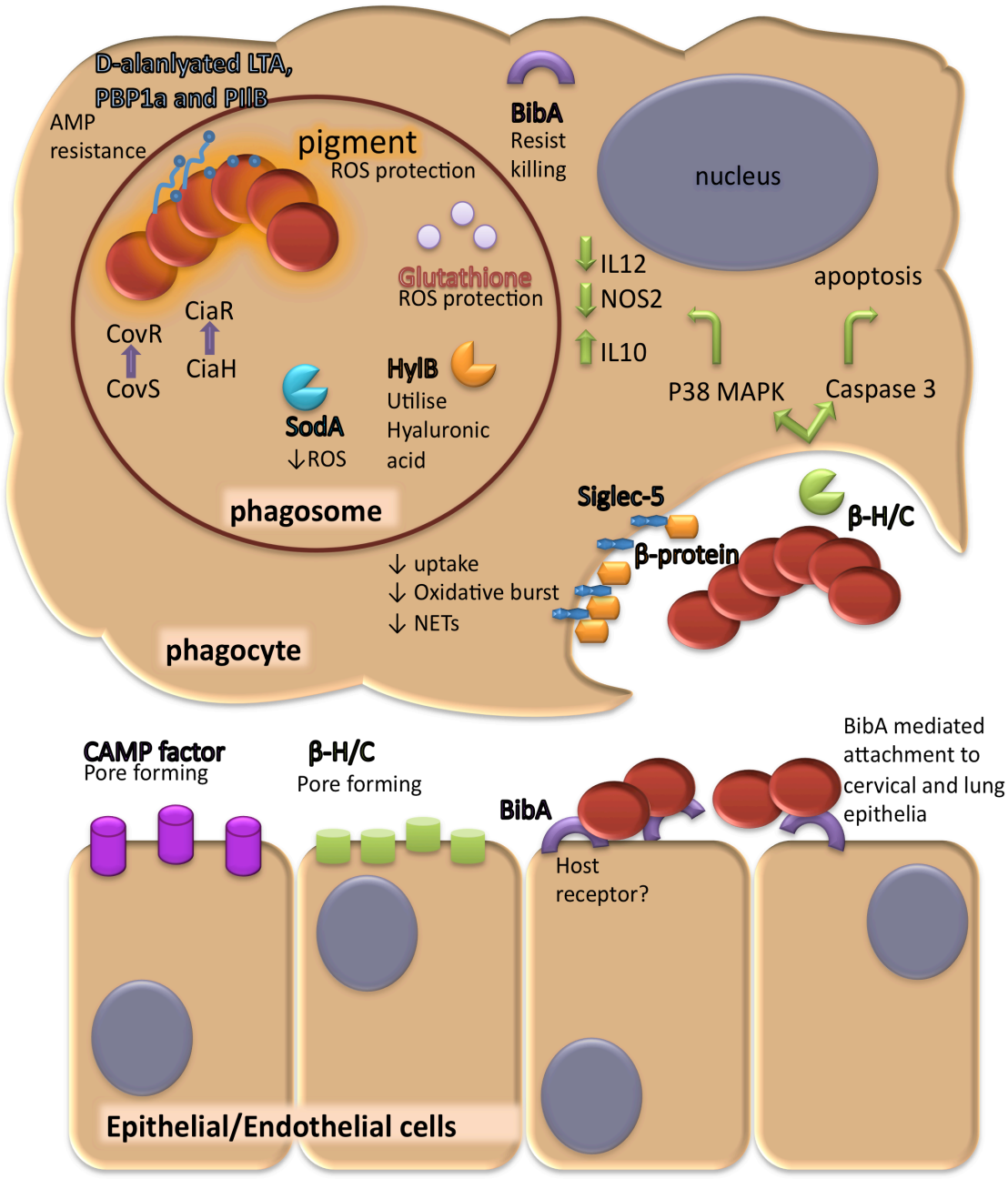

Figure 23. Group B *Streptococcus* factors aiding intracellular survival
The schematic summarises the main mechanisms used by GBS to aid intracellular survival. These range from factors that work extracellularly to those that work intracellularly. LTA, lipoteichoic acid. PBP1a, penicillin binding protein 1 a. PilB, pilus component B. AMP, antimicrobial peptides. BibA, Group B *Streptococcus* immunogenic bacterial adhesion. ROS, reactive oxygen species. β-H/C, β-haemolysin/cytolysin. HylB, hyaluronidase B. SodA, superoxide dismutase A. Siglec-5, Sia-recognising immunoglobulin super family lectin 5. NETs, neutrophil extracellular traps. Cov, control of virulence. Cia, competence induction and altered cefotaxime susceptibility. CAMP, Christie Atkins Munch-Petersen.

glutathione as an oxygen-metabolite scavenger (Wilson and Weaver, 1985). The streptococcal caratenoid pigment is also able to offer protection form oxidative damage by free radical scavenging (Liu *et al*., 2004). Interestingly, neonatal phagocytes are less able to mount a severe oxidative burst (Wilson and Weaver, 1985). This is most likely to further aid the intracellular survival of invading GBS.

The β-H/C of GBS is considered a key virulence factor. This reputation is mainly due to the cytolytic pore forming ability of β-H/C, as discussed previously in this chapter. In addition to its pore forming activity, β-H/C also contributes to survival within macrophages (Liu *et al*., 2004). However, how much of this survival is actually due to the linked caratenoid pigment is unknown, as the *cylE* gene is also responsible for the production of the caratenoid pigment via a currently unknown regulatory mechanism (Spellerberg *et al*., 1999). Interestingly, at sub-lytic concentrations, β-H/C activates macrophage p38 MAPK signaling. This signaling promotes anti-inflammatory IL-10 and suppresses IL-12 and NOS2 (Bebien *et al*., 2012). Targeting of MAPK activation in murine macrophages provides resistance to invasive GBS infection (Bebien *et al*., 2012). Although, how this modulation of host cytokines impacts on the maturation or antimicrobial activity of GBS-containing phagosomes is currently unknown. Despite this, a recent study found that a serotype Ia non-haemolytic mutant (Δ*cylA*) was able to survive within macrophages better than the isogenic wild type (Sagar *et al*., 2013). A possible explanation could lie in the specific genes manipulated in the studies, *cylA* is the ATP binding component of the transporter, where as *cylE* encodes the β-H/C itself. By removing the ability of the transporter to bind ATP, this offers an alternative mechanism for blocking secretion of the toxin, un-linked to the expression levels of the toxin gene

(*cylE*). Thus, the Δ*cylA* mutant can be used to better attribute phenotypes associated specifically with the secretion of β-H/C from GBS.

To aid nutrient acquisition, GBS extracellular hyaluronidase hylB is thought to allow the use of host hyaluronic acid as a sole carbon source. A clinical strain of GBS with the *hylB* gene inactivated displayed attenuated intracellular survival in macrophages and in mouse and zebrafish infection models (Wang *et al*., 2014).

An important host defense relies upon the actions of cationic anti-microbial peptides (AMPs). AMPs are attracted to the electronegative bacterial surface where they are able to bind and form pores (Peschel, 2002). GBS is able to reduce the attraction of AMPs by D-alanylation of its lipoteichoic acid (LTA) to reduce the surface negative charge. Protection from the activities of defensin and cathelicidin AMPs is also achieved by the use of surface associated penicillin binding protein (PBP1a) (Hamilton *et al*., 2006). The mechanism of PBP1a interference of AMPs is still unclear. GBS pilus component PilB, has also been shown to provide increased protection from the AMPs. Additionally, PilB is required for GBS survival when challenged with macrophages and neutrophils (Maisey *et al*., 2008).

Causing host cell apoptosis can also be considered as a GBS defense against phagocytes. β-H/C induces apoptosis, allowing GBS to avoid phagocytosis altogether. The apoptosis induced by GBS appears to require the activation of caspase-3 and modification of the Bcl-2 family of proteins in location and regulation (Ulett *et al*., 2005). An alternative form of GBS induced cell death is also possible via caplains within host cells (Fettucciari *et al*., 2011).

The two-component response regulator CiaR has been found to affect intracellular survival of GBS strain COH1 (Quach *et al*., 2009). This regulator was found to increase resistance to phagocytic killing via resistance to the antimicrobial actions of AMPs, lysozyme and ROS (Quach *et al*., 2009). The function of CiaR is currently unknown although it appears to act as part of the two-component system CiaR/H to sense and respond to the phagosome niche.

β-protein is a cell wall anchored protein of GBS, recently found to have a role in modulating the antimicrobial response of macrophages (Carlin *et al*., 2009). β-protein binds hSiglec-5, a member of the human CD33 related Sia-recognising immunoglobulin super family lectins (hCD33rSiglecs) leukocyte receptor family. Engagement of hSiglec-5 results in the suppression of phagocytic oxidative burst and neutrophil extracellular trap production (Carlin *et al*., 2009). GBS is therefore able to evade uptake by phagocytes and reduce their antimicrobial capacity.

GBS is potentially able to manipulate protein kinase C (PKC) signaling to enhance intracellular survival. PKC is a key component of the macrophage activation pathway (Eason and Martin, 1995; Paul *et al*., 1995). Macrophages infected with GBS become unresponsive to activators requiring PKC, such as PMA and LPS (Cornacchione *et al*., 1998). Depletion of PKC was also seen to enhance GBS intracellular survival, perhaps unsurprisingly. These observations led to the hypothesis that GBS might manipulate PKC to aid persistence within the phagosome, however, the mechanism of PKC manipulation by GBS was not elucidated in the study.

Phagocytosis of opsonised GBS by professional phagocytes provides a crucial clearance mechanism for invasive GBS. Non-opsonised bacteria are poorly killed following uptake

(Valenti-Weigand *et al*., 1996). As seen with other pathogens, the route of entry GBS takes into a macrophage has consequences on its intracellular survival. GBS opsonised with human serum before exposure to J774 macrophages are less able to persist intracellularly compared to non-opsonised bacteria (Valenti-Weigand *et al*., 1996). Furthermore, GBS phagocytosed via the non-opsonic route are able to survive in macrophages for over 24 h (Valenti-Weigand *et al*., 1996).

It is believed that non-opsonised bacteria are able to be recognised by macrophages in a C3 independent but complement receptor 3 (CR3)-dependent manner (Antal *et al*., 1992). CR3 is known to aid the C3-independent uptake of a multitude of microbes (Bullock and Wright, 1987; Mosser and Edelson, 1985; Ross *et al*., 1985). These non-opsonic interactions may be crucial in determining disease progression in neonates, a host with few active opsonic molecules against GBS. The impaired expression and function of neonatal CR3 could contribute to the weakened ability of neonatal macrophages to clear GBS (Anderson *et al*., 1987; Antal *et al*., 1992).

Analysis of the maturation of GBS-containing phagosomes has thus far been limited. GBS are able to survive within macrophages for a prolonged period of time, despite the fact that they appear to reside within a mature, LAMP1 positive phagosome (Cumley *et al*., 2012; Teixeira *et al*., 2001). Interestingly, phagosomal acidification has been hypothesised to be necessary for the survival of internalised GBS, due to a marked drop in the survival of GBS in macrophages treated with the vATPase inhibitor Concanamycin A. Additionally, the two-component system CovRS is required for intracellular survival of GBS (Cumley *et al*., 2012). Although, the precise phagosomal pH required to induce

survival has not been elucidated, the acidic environment likely acts as a stress signal to induce survival genes.

Despite this ability to survive intracellularly, replication within the phagosome has not been documented for GBS, suggesting that GBS are able to persist rather than multiply within phagocytic cells. A novel vaginal colonisation model in mice might be able to provide key answers in the ability of *S. agalactiae* to persist within this vaginal niche and also maintain virulence determinants for opportunistic infection (Carey *et al*., 2014).

Microscopic recordings of GBS within phagocytes has so far concluded that GBS intracellular replication is unlikely (Liu *et al*., 2004; Teixeira *et al*., 2001). The mechanisms employed by GBS to avoid killing and allow intracellular persistence have not been fully elucidated. A combination of known factors and factors yet to be discovered are likely to control the behavior.

In this chapter the molecular basis for GBS survival within macrophages is investigated. The ability of phagocytes to clear GBS is thought to be critical in determining disease outcome. For EOD, phagocytes, particularly the alveolar macrophages in the neonatal lung, are likely to be the first immune cells that GBS encounter. Factors contributing to the outcome of this interaction are therefore of particular interest for the study of GBS pathogenicity. To investigate how GBS variability affects survival within macrophages, a selection of GBS clinical isolates were tested for their intracellular survival. To do this, a collection of 50 clinical isolates including isolates of many serotypes, sequence types and invasive origins (colonising, EOD and LOD) were used. J774 cells are a macrophage-like murine cell line that has been used in the study of many host-pathogen interactions, including studies of GBS (Goodrum *et al*., 1994; Liu *et al*., 2004; Schulert *et al*., 2009;

Valenti-Weigand *et al*., 1996). This cell line was therefore chosen to screen the GBS isolates for their ability to survive intracellularly. The aim of these experiments was to find associations between isolates of GBS from particular clonal lineages (using provided MLST data) and the ability to persist within macrophages, an attribute likely to be critical in disease progression.

To date, the GBS-containing phagosome has not been fully characterised. The persistence of GBS within phagosomes is likely to have a substantial affect on disease outcome. However, the mechanisms used by GBS to allow its survival in the phagosome are not fully elucidated. GBS have many virulence factors thought to aid intracellular survival, as discussed in this introduction. Whether or not GBS are also able to manipulate the phagosome is an under researched area of GBS pathogenicity. If GBS are altering the maturation of the phagosome, then GBS clearance could be achieved via treatment targeted at phagocyte function. Thus, phagosome maturation of the GBS-containing phagosome was also explored.

Experimental procedures

Streptococcal strains and growth conditions

All reagents were purchased from Sigma, unless otherwise stated. Details for the collection of *Streptococcus agalactiae* clinical isolates used in this study, and the strain of *Lactococcus lactis* can be found in Table 1. Group B *Streptococcus* isolates were grown in Todd-Hewitt Broth (THY) (Fluka Analytical) with 5% yeast extract (MP Biomedicals). *L. lactis* MG1363 was grown in M17 broth (OXOID) with 5 % glucose and incubated at 25°C overnight. For survival assays, 1.5 ml of the broth was inoculated with a single colony of bacteria in 2.2 ml 96 well culture plates (Thermo scientific). GBS clinical isolates were tested in groups of 20. Additionally, each repeat survival assay included NEM316, a GBS strain frequently used and known to survive well intracellularly, *L. lactis* and a bacteria-free control.

Macrophage cell line culture

The murine macrophage-like cell line J774A.1 were incubated in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 100 U/ml added (complete DMEM) at 37°C humidified with 5% CO_2.

Table 1. GBS strains and isolates used in this study

Strain	Serotype	MLST	Invasive origin	Source
NEM316	III	23	LOD	MA
2603v/r	V	110	LOD	MA
COH1	III	17	LOD	MA
A909	Ia	7	LOD	MA
COH1Δ*cylE*	III	17		MCL
NEM2089 (NEM316 Δ*CovRS*)	III	23		MCL
NEM2456 (NEM316 Δ*cylE*)	III	23		MA
k1	III	17	LOD	MA
M781	III			MA
H7783				MA
H732	III			MA
COH31	III	17		MA
GBS6313	III			MA
Z50	III	19	C	MA
j76	III		LOD	MA
j95	III	17		MA
m1	III			MA
mk2	III	17	LOD	MA
b9	III	17	EOD	MA
mk3	NT		EOD	MA
b11	III		LOD	MA
j99	Ia		EOD	MA
b3	V		EOD	MA
z77a	II	19	C	MA
z34a	III	17	C	MA
z37	III	17	C	MA
z18a	Ia	24	C	MA
z84a	V	1	C	MA
j87	II		LOD	MA
h11	III	17	EOD	MA
j100	III	17	EOD	MA
j88	III	17	EOD	MA
j96	Ib		EOD	MA
wc3	III	17	EOD	MA
z111	Ib	8	C	MA
z95	V	1	C	MA
z81a	Ia	23	C	MA
z117	III	19	C	MA
z101a	III	19	C	MA
z12a	V	1	C	MA
z41	NT	10	C	MA
z72a	Ib	8	C	MA
j90	III		LOD	MA
z78a	Ib	6	C	MA
j61				MA
j81	III	17	EOD	MA
z87a	V	23	C	MA
r1	III	17	EOD	MA
k9	III		LOD	MA
z69a	Ib	12	C	MA
z73	Ib	10	C	MA
Lactococcus lactis MG1363				HJ

EOD, Early onset disease. LOD, late onset disease. C, colonising. Sources for the collection include; (MA) Dr Mark Anthony, John Radcliffe Hospital, Oxford, UK, (MCL) Dr Marie-Cécile Lamy, Institut Pasteur, Paris. France and (HJ) Professor Howard Jenkinson, University of Bristol, UK.

GBS Intracellular survival assay

At 18 h before infection J774 cells were seeded into duplicate 24 well plates at 2 x 10^5 cells per well in complete DMEM and incubated at 37 °C with 5% CO_2. Just before infection, bacterial cultures were centrifuged at 1967 x g for 10 min and washed with 1 X phosphate buffered saline solution (1XPBS). The PBS suspensions were added to DMEM with L-glutamine 2 mM, at a 100-fold dilution. PBS suspensions were then serially diluted and plated onto THY agar to allow for quantification of CFU in each inoculum.

After triplicate washes with 1XPBS, J774 cells were infected with bacteria in DMEM. Infection of J774 cells continued for 30 min at 37°C and 5% CO_2. After infection, cells were washed in triplicate with 1XPBS and media replaced with DMEM containing 2mM L-glutamine, 100 μg/ml gentamicin and 5 μg/ml penicillin and incubated for 30 min to to kill non-phagocytosed bacteria (Figure 24). One of the plates was then used for time zero (T0) lysis. Media was replaced with 0.02 % triton X-100 and incubated for 15 min to lyse the cells. The resulting suspensions were then serially diluted and plated on THY agar for incubation overnight. Meanwhile, the replicate plate was returned to incubation for a further 6 h. After 6 h the cells in the second plate were lysed, diluted and plated on agar, for time 6 (T6) CFU. After incubation of agar plates overnight the multiplicity of infection (MOI), CFU for T0, CFU for T6 and survival ratio (T6/T0) was calculated for each isolate.

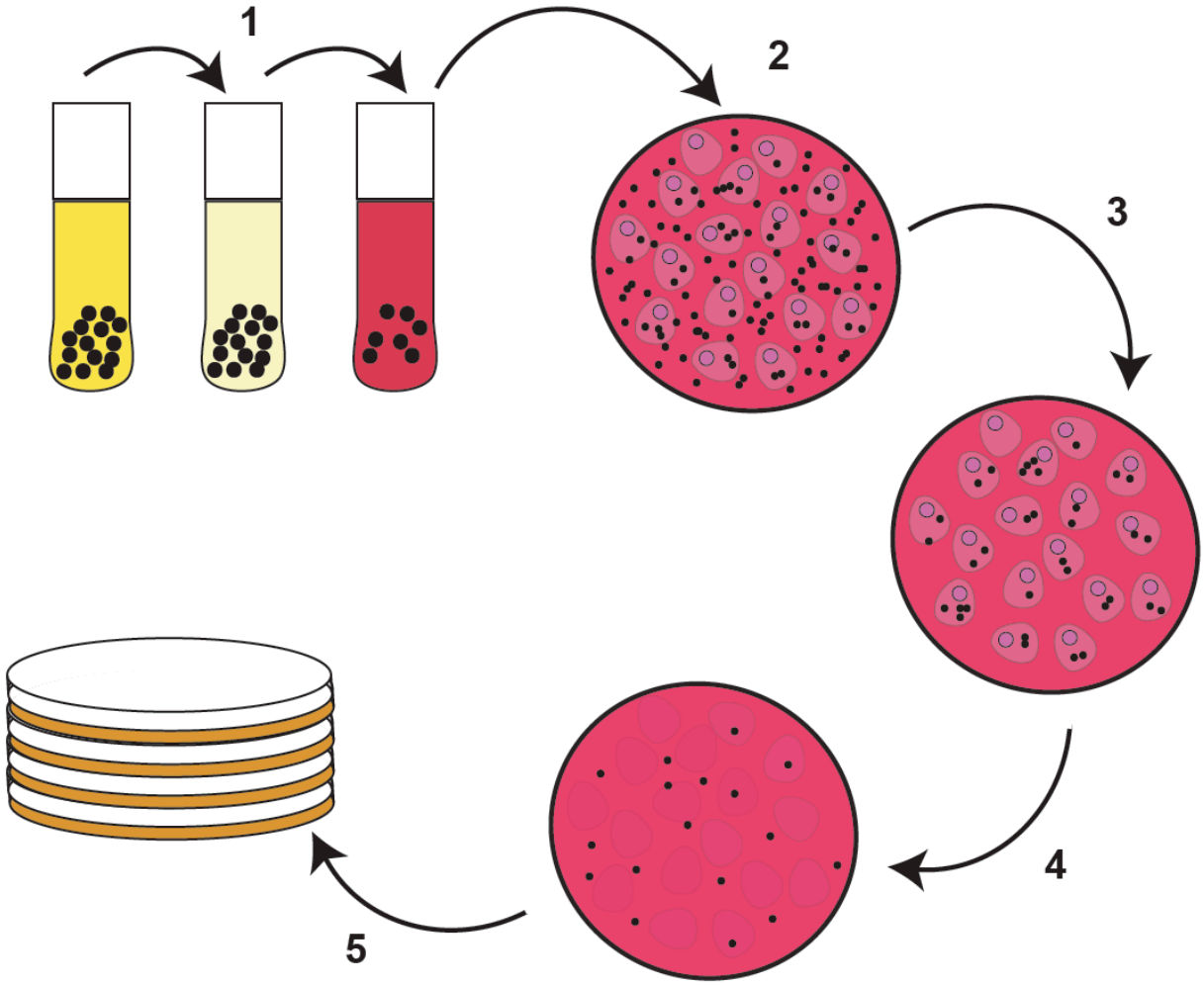

Figure 24. Intracellular survival assay for Group B *Streptococcus*.
1, bacterial cultures isolates were washed in PBS and diluted into DMEM. **2**, J774 macrophages were then infected for 30 min. **3**, media was replaced with antibacterial DMEM to kill extracellular bacteria. **4**, macrophages were then with 0.02 % triton-X100 at T0 or T6. **5**, lysates were then diluted in PBS and plated on agar for incubation.

For GBS initial uptake experiments the following adjustments were made; macrophages were initially seeded onto 13 mm glass coverslips, before infection macrophages were incubated on ice for 10 min, infection DMEM was used at 4°C and after bacteria were added to macrophages they were kept on ice for 15 min to allow extracellular binding of bacteria only. After 15 min media was replaced with warm DMEM + L-glutamine to initiate uptake. Slides were then removed at 15, 30, 60 and 90 min washed three times in 1XPBS and fixed with 4 % paraformaldehyde (PFA) for 10 min.

GBS LysoTracker experiments were conducted in a similar way to initial uptake experiments. However, before ice incubation of macrophages they were first treated with 50 nM LysoTracker Red DND-99 (Sigma) in complete DMEM and incubated for 2 h. The GBS strains COH1 and NEM316 were heat killed by incubation at 70°C for 30 min before infection, when required. Coverslips were then removed at 15 min, 30 min, 90 min and 6 h. At 30 min media on 90 min and 6 h coverslips was replaced with DMEM containing 2mM L-glutamine, 100 μg/ml gentamicin and 5 μg/ml penicillin.

Labeling and imaging of fixed cells

PFA fixed coverslips were first treated with 50 mM ammonium chloride for 10 min. Antibodies were diluted in blocking serum: PBS containing 3% goat serum (invitrogen). For uptake experiments only, coverslips were labeled before permeabilistation, with monoclonal mouse IgG anti-GBS 1.B501 (Santa Cruz Biotechnology) at 2 μg/ml for 20 min and then for 20 min with anti-mouse IgG - FITC (Sigma) at 0.25 μg/ml (to label extracellular bacteria). After treatment with 0.1% triton-X 100 for 4 min intracellular bacteria were labeled with anti-mouse IgG - TRITC (Sigma) as the secondary antibody.

For LysoTracker experiments, GBS were labeled with mouse IgG-FITC (Sigma) after permeabilisation with 0.1% saponin for 4 min. Coverslips were then mounted on glass slides with 6 µl mowiol mounting media (Calbiochem). For uptake experiments images were captured using a Nikon eclipse TE2000-U and Digital Sight DS- Qi1MC camera. For LysoTracker experiments a Nikon eclipse Ti-S microscope and QICAM Fast1394 camera (Q imaging were used. All images were obtained using a Plan Apo 60x/1.40 NA oil DIC objective. (Nikon). Images were processed with NIS-elements AR software (Nikon).

Results

Initial uptake of GBS by macrophages

To validate the intracellular survival assay used, the initial phagocytosis of GBS was assessed by microscopy. To synchronise uptake J774 macrophages were infected with GBS whilst on ice, after 15 min to allow extracellular binding, phagocytosis was initiated by replacing cold media for warm DMEM and continuing incubation at 37°C. Immediately after infection, 5, 15, 30 and 60 min post infection coverslips were removed, washed and fixed. Coverslips were labeled firstly for extracellular GBS (FITC) and then intracellular GBS (TRITC) after permeabilisation. Intracellular GBS were scored if they only carried the red TRITC label. The percentage of macrophages associated GBS found to be intracellular reaches a plateau of approximately 17% by 15 min post infection (Figure 25). The infection assay used in the analysis of clinical isolates of GBS allows 30 min for the phagocytosis of GBS by J774s. This period of time is more than sufficient allow maximum uptake, as demonstrated by the result presented here.

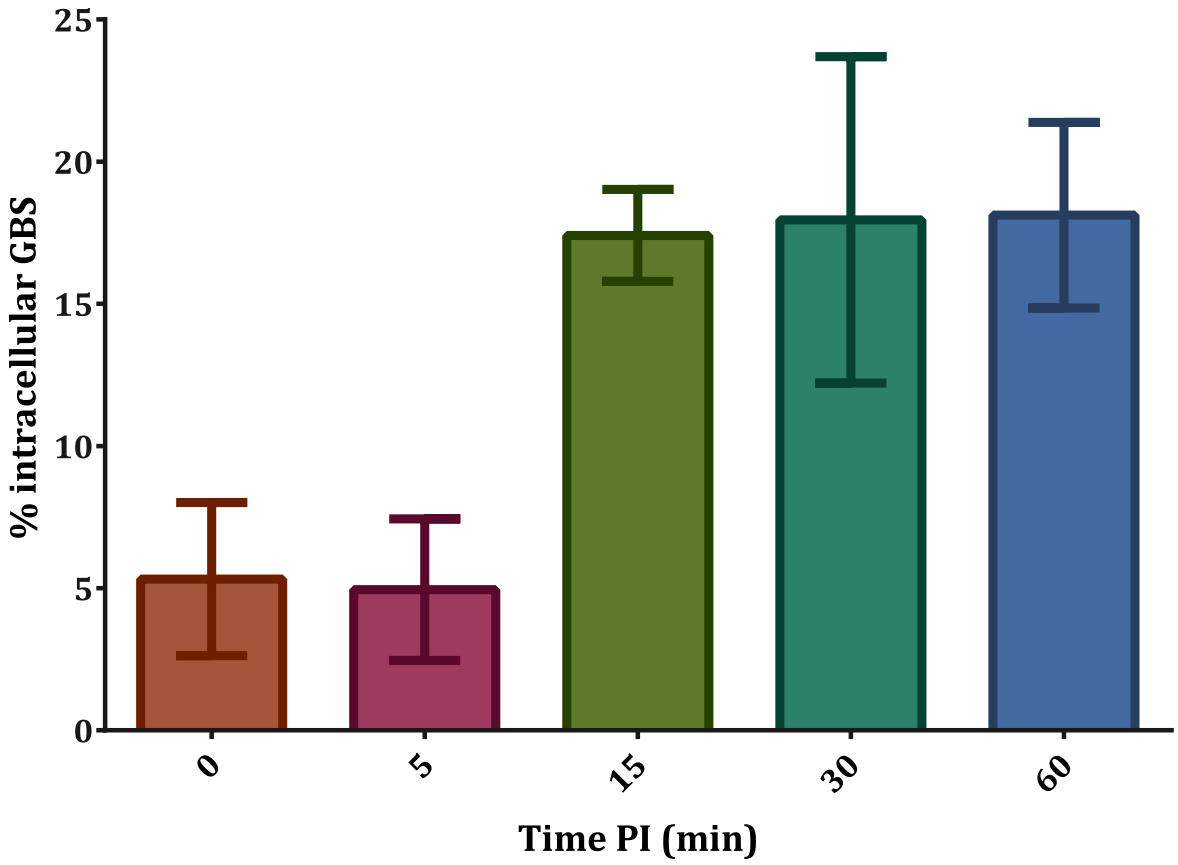

Figure 25. Initial phagocytosis of GBS by J774 macrophages.
J774 macrophages were seeded on to coverslips and infected with GBS strain NEM316. Coverslips were then removed, washed and fixed at 0, 5, 15, 30 and 60 min post infection. Extracellular GBS were immuno-stained with FITC, intracellular GBS were labeled after permeabilisation of J774 cells with TRITC secondary antibody. GBS carrying only red TRITC label were counted as phagocytosed. Data is shown as an average percentage of intracellular GBS, over 1000 GBS scored per time point, n=3, Error bars represent ±SEM.

Clinical isolates of GBS are able to persist within macrophages

The intracellular survival abilities of a collection of GBS clinical isolates was assessed. After 30 min of infection and 30 min of antibiotic treatment J774 cells were lysed immediately (T0) or after 6 h of further incubation (T6) (Figure 26). Viable intracellular bacteria from both time points were used to calculate relative CFU (rCFU = T6CFU/T0CFU). This intracellular survival assay was conducted for GBS clinical isolates and the microbial controls; GBS NEM316, *L. lactis* and a bacteria-free well. *L. lactis* was included in the assay as it is susceptible to phagocyte killing within the time frame of the experiment, it is a commensal of the gastrointestinal tract of humans and is closely related to *Streptococcus*. These results demonstrate that all the clinical isolates of GBS tested vary are able to persist within the J774 macrophage-like cell line (Figure 26). At 6 h post infection all isolates were able to survive intracellularly, at a level significantly greater than the non-pathogenic *L. lactis* ($p<0.01$, Mann-Whitney U test).

Mutant strains of GBS were included in the intracellular survival assays. NEM2089 (NEM316Δ*CovRS*) (Lamy *et al.,* 2004) showed a marked defect in intracellular survival. NEM2456 (NEM316Δ*CylE*) (Forquin *et al.,* 2007) and COH1Δ*CylE* (Pritzlaff *et al.,* 2001), both mutants in the β-H/C virulence factor, showed no defect in intracellular survival.

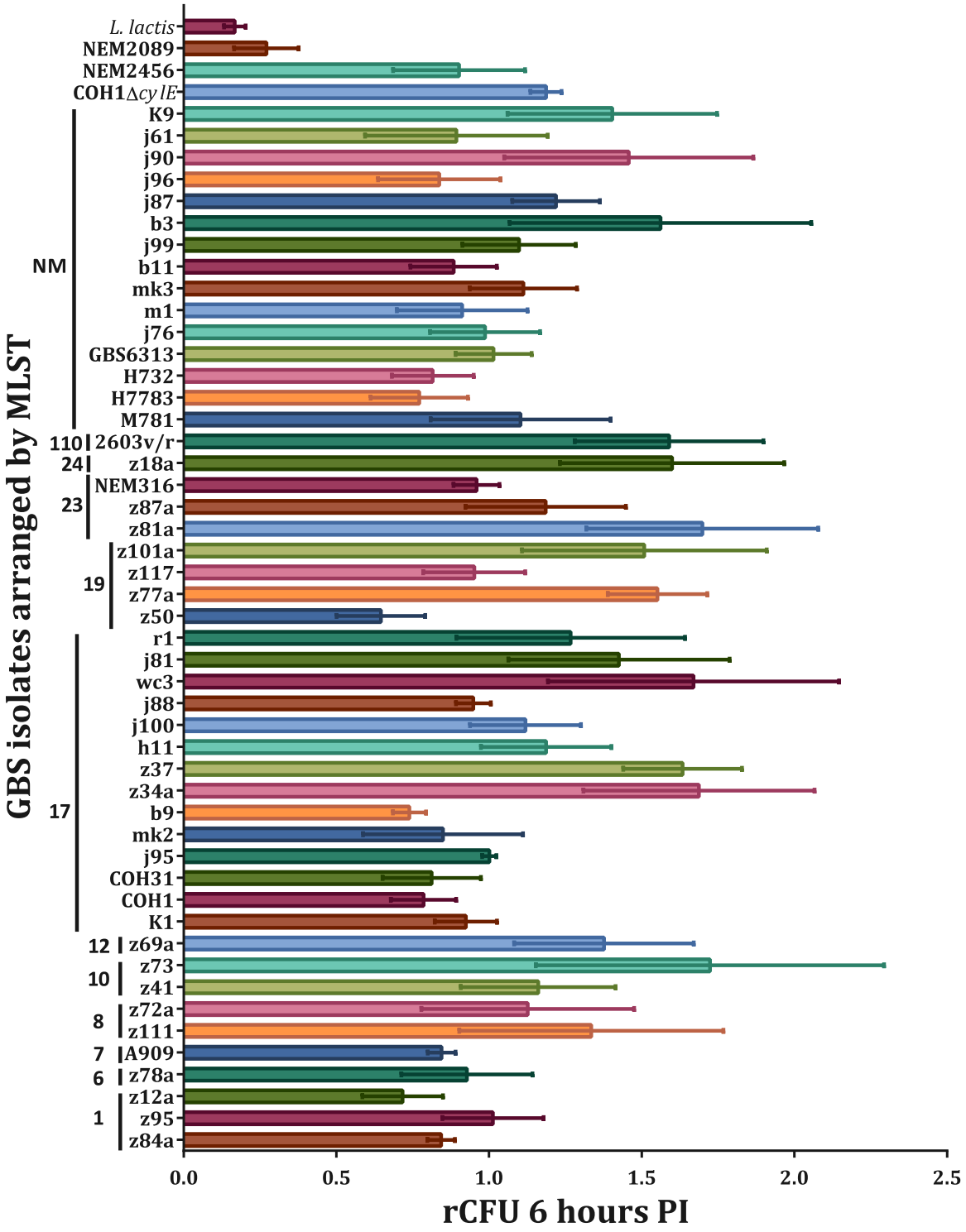

Figure 26. Intracellular survival of GBS clinical isolates in J774 macrophages. Fifty GBS isolates were assessed for intracellular survival in J774 macrophages. Survival is presented as rCFU at 6 h post infection. GBS isolates are arranged by Multilocus Sequence Type (MLST). All GBS isolates show significantly higher survival than *L. lactis* (MG1363) (Mann Whitney U, $p<0.01$ at least three repeats), but do not significantly differ from each other (Kruskal Wallis $p=0.147$). Values displayed are mean ± SE for each isolate. NM, no MLST data available.

For between group variation, survival data was grouped by either MLST, serotype or invasive origin (colonising, EOD or LOD) (Figure 27A-C). No significant differences were found in intracellular survival after values were grouped. No relationship between higher survival ratio and particular origin, serotype or MLST were found. For clarity, the number of isolates in the collection representing each group are also displayed (Figure 27D-F). Similarly to many epidemiological studies, III and 17 are the most abundant serotype and MLST represented in the collection (Figure 27D and E).

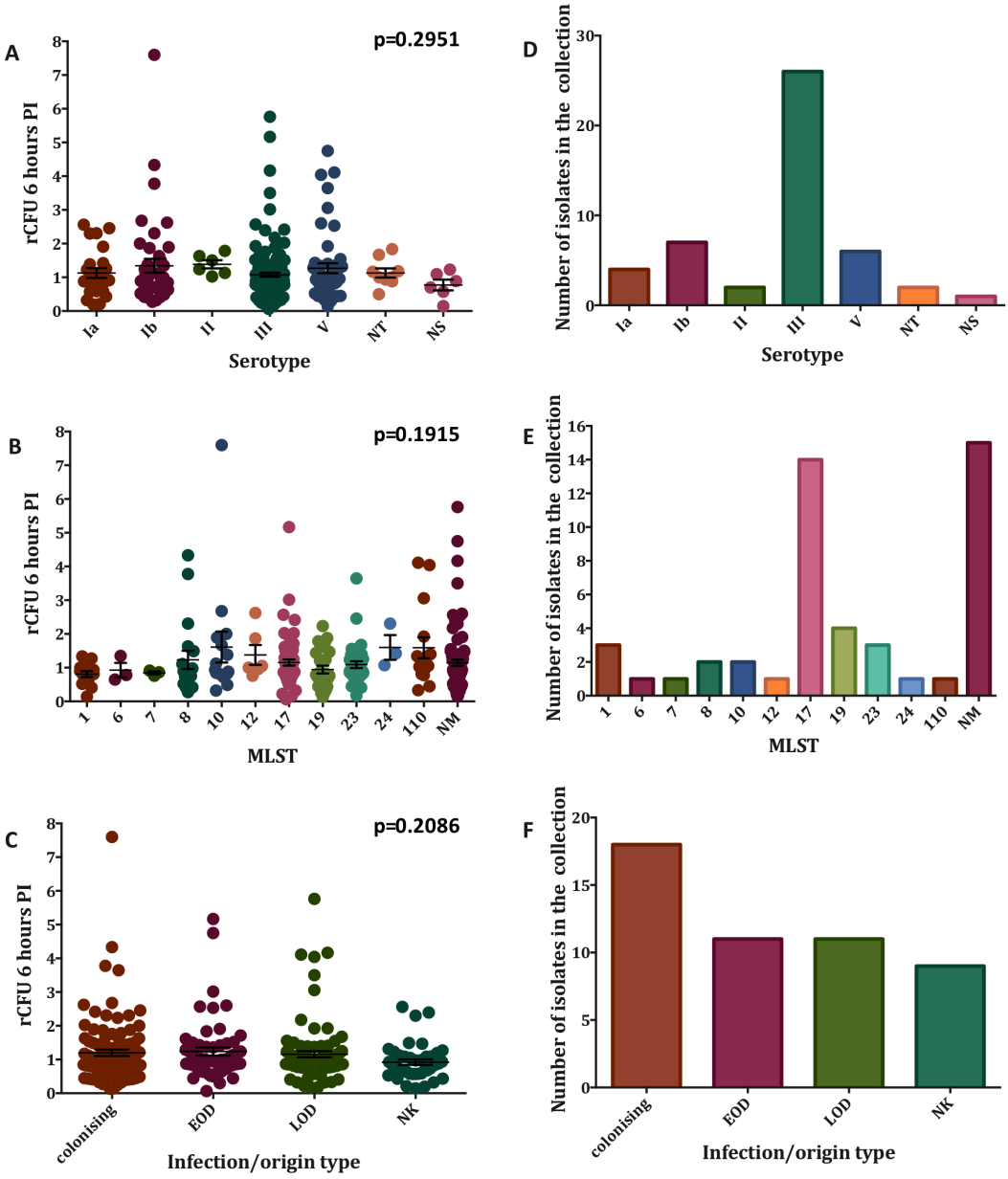

Figure 27. GBS Intracellular survival grouped by MLST, invasive origin and serotype.

A-C display each intracellular survival assay value for each clinical isolate, allowing visualisation of the spread of the raw data. A, GBS grouped serotype (NT, non-typable. NS, no serotype information available). B, GBS grouped by multi-locus sequence type (MLST)(NM, no MLST data available). C, GBS grouped by infection/origin (EOD, Early Onset Disease. LOD, Late Onset Disease. NK, Not Known). E-F display the number of isolates in the collection of tested GBS strains belonging to groups used in panels A-C. No significant difference was found in the rCFU between groups for data in panels A-C, P values displayed are from Kruskal-Wallis test. No significance between groups compared individually with Dunn's multiple comparison test for panels A-C, lines and error bars represent mean ± SEM. Relative CFU (rCFU). Post infection (PI).

GBS phagosome maturation – acidification

Maturation of macrophage phagosomes culminates with an increase in acidity of the phagolysosome lumen as V-ATPases pump hydrogen ions in. Many bacterial pathogens are able to survive intracellularly by modifying or delaying this acidification process (Smith and May, 2013). To determine if GBS resides within acidic phagocytic compartments in macrophages, LysoTracker Red DND-99 was used to label acidic cellular organelles. J774 macrophages were seeded on to coverslips in 24-well plates and treated with LysoTracker for 2 h before infection. Coverslips were then removed, washed and fixed at 15, 30, 90 min and 6 h post infection. Coverslips were then imaged and scored for GBS phagosomes positive for LysoTracker Red (Figure 29). The GBS strains NEM316 and COH1 were used, as well as each in a heat-killed form. Heat-killed GBS co-localised with LysoTracker over three fold more than live GBS (Figure 28). Suggesting heat killed GBS reside in an acidified phagosome. Both the GBS strains NEM316 and COH1, when tested live, were rarely found in acidified phagosomes. These results indicate that live GBS are able to modify phagosome acidification.

In addition, the *CovRS* mutant of NEM316 (NEM2089), only displayed a partial defect in its ability to modify phagosomal pH (Figure 29). Only at 15 and 30 min post infection, was a larger proportion of NEM2089 found in LysoTracker positive phagosomes compared to NEM316. The slight increase in acidic phagosomes was not comparable to that seen for heat killed NEM316.

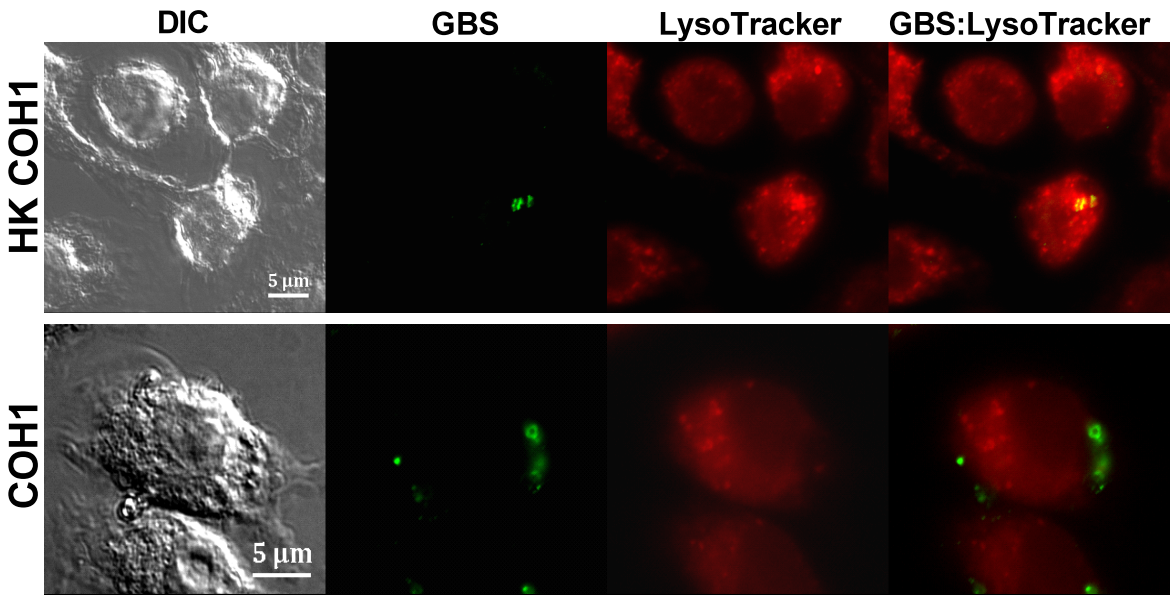

Figure 28. Co-localisation of GBS with LysoTracker Red labelled acidic compartments.

Immunofluorescence and DIC images of J774 macrophages pre-treated with LysoTracker Red after 90 min infection with heat killed GBS strain COH1(HK COH1) and live COH1 (COH1). DIC, image of J774 cells infected. GBS, corresponding fluorescent image of GBS labeled with mouse anti-GBS and anti-mouse IgG-FITC. LysoTracker, fluorescent image for LysoTracker Red. GBS:LysoTracker, merged image of panels GBS and LysoTracker.

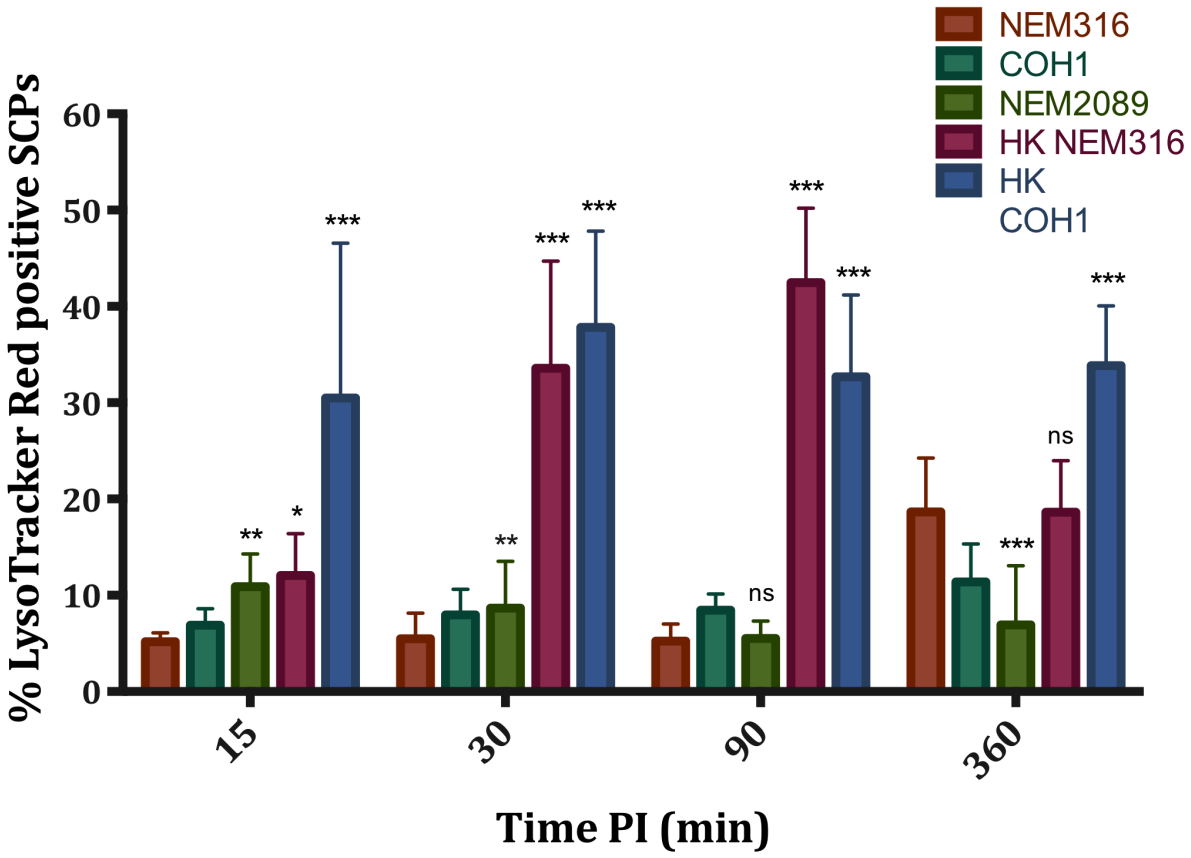

Figure 29. Acidification of GBS containing phagosomes.
J774 macrophages, pre-treated with LysoTracker Red DND-99, were infected with either live *Streptococcus agalactiae* (NEM316), heat killed NEM316 (HK NEM316), NEM316 ΔCovRS (NEM2089), COH1 or HK COH1. After 15, 30, 90 or 360 min of infection, coverslips were fixed and stained with mouse anti-GBS antibody and anti-mouse FITC secondary antibody. Internal streptococci were then scored for LysoTracker Red co-localisation. Error bars represent mean ± SE of at least 5 repeats. Statistical analysis conducted on categorical data with Fisher's Exact Test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Comparisons were performed between heat killed or mutant strains and the corresponding wild type strain.

Discussion

Group B *Streptococcus* (GBS) is known to survive for prolonged periods of time inside macrophages (Cornacchione *et al*., 1998; Teixeira *et al*., 2001; Valenti-Weigand *et al*., 1996). This ability is thought to impact on the pathogenicity of this *Streptococcus* and play a role in disease outcome, as macrophages are such a key cell in the innate immune system. For neonates the innate immune system is their only defence, with many arms of the adaptive immune system requiring time to mature (Nussbaum and Sperandio, 2011; Remington *et al*., 2011). Isolates of GBS that are found colonising pregnant women consist mostly of serotypes Ia, V, and III (Remington *et al*., 2011). A very similar distribution is seen for GBS isolates causing EOD. However, the proportion of serotype III changes dramatically when we look at isolates causing LOD, with the majority of cases caused by a serotype III GBS strains (Figure 20) (Lamagni *et al*., 2013; Remington *et al*., 2011; Teatero *et al*., 2014). These serotype III isolates are often of the MLST 17 genotype (Brochet *et al*., 2008; Springman *et al*., 2009). This association of serotype III, MLST 17, isolates and LOD has led to these isolates being deemed hypervirulent (Harrison *et al*., 1998; Lamy *et al*., 2006; Madzivhandila *et al*., 2011). One explanation for this apparent hypervirulence has come from the discovery of the MLST 17 specific hypervirulent GBS adhesin HvgA (Tazi *et al*., 2010). This cell wall anchored protein increases GBS adherence to intestinal and blood brain barrier constituting cells (Tazi *et al*., 2010). Although this one molecule is unlikely to be solely responsible for the hypervirulence of these isolates.

In this study data is presented that demonstrates GBS isolates of many different serotypes and MLSTs were able to persist with macrophages. These results suggest

there is no obvious virulence gain to the serotype III MLST 17 isolates, at least not in macrophage phagosomes. It is interesting, however, that all isolates appear to survive intracellulary to a level significantly greater than a non-pathogenic organism (*L. lactis*). This finding supports a conclusion that all isolates are potentially capable of intracellular pathogenesis. This perhaps explains the variation of serotypes seen causing invasive GBS infection in adults (Lamagni *et al*., 2013). The restricted serotype distribution seen causing neonatal infection might be due to a neonatal specific selection as yet undetermined. The hypervirulent isolates of GBS are likely to be "accidental" by increasing their chances of becoming a neonatal infection by different mechanisms, such as colonisation and adherence. For instance, if colonising isolates of GBS are less adherent to the vaginal epithelium (usually a niche disadvantage) they are more likely to be freely moving in the vaginal fluids, thus more likely to ascend the reproductive tract and infect the amniotic fluids and cause infection. Moreover, host factors play a role in the level of GBS adherence to vaginal epithelia, such as hormone level changes over the menstrual cycle (Zawaneh *et al*., 1981).

Mutant strains of GBS were included in the intracellular survival assays. NEM2089 (NEM316Δ*CovRS*) showed a marked defect in intracellular survival. This two-component system regulates a large number of GBS genes, equating to 7% of the genome (Jiang *et al*., 2008; Lamy *et al*., 2004). Mutants in this regulatory system have previously been shown to have attenuated virulence in rodent models (Lamy *et al*., 2004; Lembo *et al*., 2010). CovRS is thought to regulate much of the GBS transcriptional response to changes in pH (Santi *et al*., 2009a). Although, interestingly, mutants do not appear to be less tolerant to *in vitro* oxidative stress or acid treatment (Cumley *et al*., 2012). NEM2456 (NEM316Δ*CylE*) and COH1Δ*CylE* both mutants of the β- H/C virulence

factor, showed no defect in intracellular survival. Suggesting this pore-forming toxin plays no role in enhancing GBS intracellular survival in macrophages.

The phagosome of many intracellular pathogens has been extensively characterised. Pathogens have evolved a myriad of mechanisms to escape phagosome death, from delaying lysosomal fusion and acidification to mimicking the endoplasmic reticulum (Smith and May, 2013). However, such a complete characterisation has not yet been achieved for GBS. To begin to answer if the GBS phagosome matures, experiments were conducted to determine the acidity of GBS phagosomes. The use of an acidotropic probe (LysoTracker Red) that labels acidic organelles within cells has been used in many studies of phagosome acidification and maturation (Hara *et al*., 2008; Radtke *et al*., 2011; Toyooka *et al*., 2005; Via *et al*., 1998; Zhou *et al*., 2011). Here, LysoTracker Red was used to analyse fixed samples of J774 cells infected with GBS. This technique has shown that live GBS reside within non-acidified phagosomes when compared to heat killed streptococci. Interestingly, a strain lacking the functionality of the CovRS two-component system (NEM2089), was also found to reside within non-acidified phagosomes. The CovRS system has been previously described as necessary for initiating a survival program within GBS when sensing environmental cues, such as acidification (Cumley *et al*., 2012). Despite being required for reacting to low pH and subsequent intracellular survival, the CovRS system had no exaggerated bearing on the acidity of the phagosome. The proportion of NEM2089 found within acidified phagosomes differed compared to wild type NEM316 only at select time points and not to the level of heat killed NEM316. Perhaps suggesting that gene products of the CovR regulon are not involved directly in increasing phagosomal pH but more so in orchestrating the GBS response to being intracellular. Thus explaining why less

NEM2089 were found in acidic vesicles at later stages of infection compared to early time points.

The data presented demonstrate that the majority of live GBS-containing phagosomes are not acidic enough for LysoTracker positivity. This does not, however, rule out that the GBS – containing phagosome is able to become slightly acidic. This would agree more with previous data suggesting that an acidic environmental cue is required for GBS intracellular survival (Cumley *et al*., 2012). J774s treated with the vATPase inhibitor concanamycin A, were able to kill many of the GBS they were infected with. Thus, without the phagosomal acidification provided by vATPase, GBS were more vulnerable to intracellular killing. Perhaps suggesting that a mildly acidic stimulus is required for GBS intracellular survival.

It should be taken into account that all of the data presented here were obtained from analysing the outcome of non-opsonised phagocytosis by macrophages. While opsonisation with either serum or GBS specific antibody increases the antimicrobial response of phagocytes to GBS (Valenti-Weigand *et al*., 1996). Similarly, activation of macrophages with either IFN-γ or LPS, seriously reduces GBS intracellular survival (Cornacchione *et al*., 1998). Which of these infection scenarios is most appropriate for finding virulence determinants responsible for neonatal infection is difficult to tell. Neonates that succumb to infection are unlikely to have opsonising antibodies or appropriate inflammatory signalling.

To assess the reliability of the intracellular survival assay starting time (T0) the proportion of GBS phagocytosed by macrophages at early time points was determined. It was demonstrated that by 15 min post infection approximately 17% of non-opsonised

GBS are internalised and this figure does not change from 15 min to 60 min post infection. With no clear indication that bacterial death is occurring before T0 (equivalent to 60 min post infection in uptake experiments), concluding that no significant peak or trough of GBS uptake occurs before the first sample time at T0.

The details of the Group B *Streptococcus* - containing phagosome are far from complete. GBS are able to resist killing within a macrophage for up to 24 h, however, a small persister population is still possible. GBS were thought to reside in a mature phagosome (Cumley *et al*., 2012). It now seems that live GBS can alter phagosome pH, allowing prolonged survival within macrophages.

Chapter IV. Creating a transposon mutant library of *Streptococcus agalactiae*

Introduction

Transposons are small mobile genetic elements that are able to move in and out of DNA with the help of transposases. The transposase enzyme recognises the inverted repeats at the ends of a transposon, makes a double strand break in the target sequence and inserts or 'pastes' the transposon into this new genetic location (van Opijnen and Camilli, 2013). By using transposons to create mutant libraries, in which each mutant has a single transposon insertion site, researchers have been able to link genotype with phenotype for the discovery of virulence genes within many microbial pathogens (Chiang and Mekalanos, 1998; Hensel *et al*., 1995; Jones *et al*., 2000). Signature tagged mutagenesis has been used previously for the generation of 1600 *S. agalactiae* serotype Ia A909 mutants (Jones *et al*., 2000). The library was used to search the GBS genome for genes required in the neonatal rat sepsis model of infection. A total of 120 mutants were found to have defective virulence in the rat. Cloning the regions flanking these insertion sites was not successful for all mutants and for those that were successful, 50% were in genes with unknown function at the time (Jones *et al*., 2000).

Transposon insertion techniques and parallel high-throughput sequencing can now be combined, resulting in techniques such as transposon-directed insertion site sequencing (TraDIS). By combining mutant libraries with sequencing technologies, genes and their putative functions can be discovered simultaneously. The technology also allows for mutant libraries to be kept as a mixed population, rather than selecting individual clones with a single insertion site. The entire population's genetic material can be collected

before and after a particular *in vitro* or *in vivo* challenge and the abundance or loss of sequence reads from particular loci compared (van Opijnen and Camilli, 2013).

TraDIS has been used to generate a huge library of 370.000 mutants in *Salmonella enterica* serovar Typhi (Langridge *et al*., 2009). The method resulted in high density of transposon insertion sites in non-essential genes (averaging every 13 bp). The authors discovered 169 genes that are required for *S. enterica* Typhi bile tolerance.

More recently, TraDIS was used to study *Burkholderia pseudomallei*. Using a library of over 240,000 individual transposon mutants the authors discovered 505 putative essential genes that could potentially be targeted therapeutically (Moule *et al*., 2014). The technology has also been applied to; *Escherichia coli* to discover genes required for resistance to ionising radiation (Byrne *et al*., 2014), *Haemopilus parasuis* (Luan *et al*., 2013) and *Staphylococcus aureus* (Santa Maria *et al*., 2014).

Because of the potential for genome-wide analysis and virulence gene discovery, we aimed to create a transposon library in *S. agalactiae* and analyse the mutants generated with TraDIS. The sequenced serotype III GBS strain COH1 was chosen as the target strain for library generation as it belongs to the hypervirulent MLST-17 lineage of *S. agalactiae* (Tettelin *et al*., 2002). MLST-17 is a genetically homogeneous lineage, therefore, findings with this strain could be applied to others in the clonal complex (Springman *et al*., 2009). We aimed to produce a library of mutants spanning the entire COH1 genome to allow us to dissect out what GBS genes are required for persistence within macrophages. The generated library would also be available for the screening of many other virulence attributes of GBS in the future.

Experimental procedures

Unless otherwise stated all reagents were obtained from Sigma. Group B *Streptococcus* strain COH1 was grown in Todd-Hewitt Broth (THB) (Fluka Analytical) with 5% yeast extract (MP Biomedicals)(THY), at 37°C. After transformation attempts COH1 was incubated on Columbia blood agar base with 5% defibrinated horse blood (OXOID) supplemented with 200 µg/ml kanamycin and *Escherichia coli* on Luria Broth (LB) agar supplemented with kanamycin 50 µg/ml and 100 µg/ml ampicillin. The *E. coli* DH5αpTnTMDH strain was a gift from A.K. Turner, University of Cambridge.

Generation of Competent GBS strain COH1

Competent GBS strain COH1 was achieved by use of an adapted published protocol (Framson *et al*., 1997). Briefly, 10 ml of M9 salts solution with 0.2 % glucose, 0.3 % yeast extract, 1 % Casamino Acids, 2 mM magnesium sulphate heptahydrate and 1 mM calcium chloride was inoculated with GBS strain COH1 and incubated overnight at 37°C. This culture was then diluted 1/500 into warmed 50 ml of the same M9 broth with 0.45 % glycine and incubated at 37°C overnight. This culture was then diluted 1/50 into warmed 50 ml of the same broth with 0.45 % glycine and incubated at 37°C until early exponential phase (OD_{600} = 0.13-0.2). Cultures were then rapidly chilled on ice for 10 min before harvesting the bacteria by centrifugation at 4000 rpm for 15 min at 4°C. Cells were then re-suspended in 12.5 ml ice-cold 0.625 M sucrose at pH 4. Bacteria were then centrifuged again at 3000xg for 15 min at 4°C and re-suspended in a total volume of 1 ml ice-cold 0.625 M sucrose pH 4. Competent COH1 cells were then frozen in aliquots and stored at -80°C.

Construction of transposomes

The plasmid pTnTMDH was extracted from *E. coli* DH5α pTnTMDH using a QIAprep® Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. Briefly, *E. coli* were grown overnight in 3 ml Luria Broth supplemented with 5 µg/ml kanamycin at 37°C shaking (200 rpm). Cells were harvested by centrifuging for 10 min at 13,000 rpm. After cell lysis, neutralisation and binding DNA, the plasmid DNA was eluted in 50 µl of EB buffer (10 mM Tris.Cl, pH 8.5) and stored at -20°C. Extracted plasmid DNA was then digested with the restriction enzyme *Msp*A1I (New England Biolabs), 100 units of enzyme was added to 100 ng of plasmid DNA and incubated for 1 h at 37°C in NEB buffer 4 and 100 µg/ml BSA. The transposon was then amplified by TnPCR (Table 3) using the primers TM1 and TM2 (Table 2), PCR reactions in this study used phusion DNA polymerase (New England Biolabs). PCR fragments were then 5'phosphorylated using T4 polynucleotide kinase (New England Biolabs) in accordance with the manufacturers guidelines. Phosphorylated transposon fragments were then used to create transposomes (transposase and DNA complex) with the use of EZ-Tn5 Transposase (Epicentre biotechnologies) following the manufacturer's instructions. Transposomes were then stored at -20°C.

Electroporation of GBS strain COH1

Competent GBS strain COH1cells were thawed on ice before 60 µl of cells were added to pre-chilled 2mm cuvettes with 0.2 µl of transposomes. Using a BIORAD gene pulser, cells were electroporated with 1.4 kV (7 kV/cm), 200 Ω and 25 µF. This suspension was

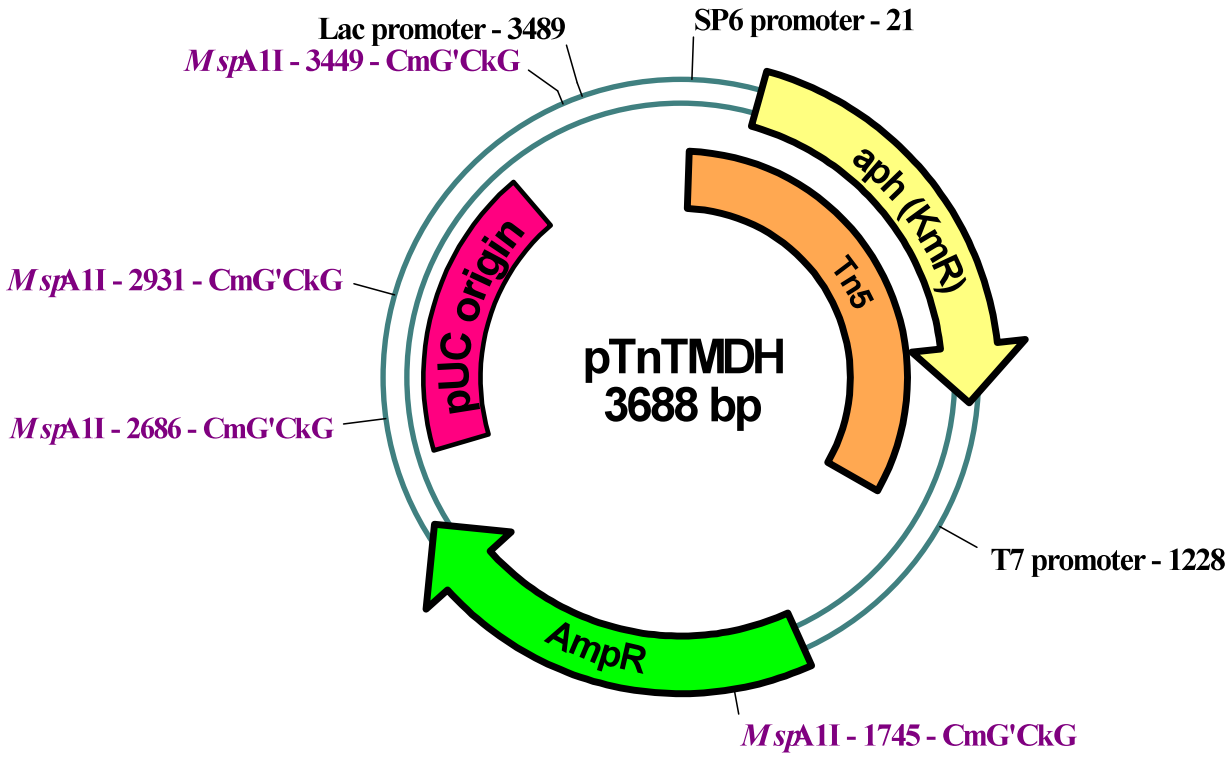

Figure 30. Plasmid map of pTnTMDH

Plasmid map of pTnTMDH with the major features annotated. Aph, aminoglycoside phophotransferase. KmR, Kanamycin resistance selective gene. Tn5, Tn5 transposon. pUC, plasmid University of California. AmpR, Ampicillin resistance selective gene.

immediately diluted in 1 ml Todd-Hewitt broth with 0.25 M sucrose and incubated for 2 h at 37°C. Cells were then harvested by centrifugation at 8000 rpm for 1 min and re-suspended in 100 µl of the same broth. To recover transformants, suspensions were then spread on Todd-Hewitt yeast agar plates supplemented with 50 µg/ml kanamycin and incubated for 2-4 days at 37°C.

Extraction of GBS genomic DNA

Extraction of streptococcal genomic DNA from transformed COH1 was achieved by use of the DNeasy® Blood and Tissue kit, following instructions for Gram-positive DNA extraction. Briefly, cells were harvested from an overnight culture of 4 COH1 transformants by centrifugation at 5000 x g for 10 min. Each cell pellet was re-suspended in 180 µl of lysis buffer (20 mM Tris.Cl pH8, 2 mM Na_2EDTA, 1.2 % triton X-100 and 20 mg/ml lysozyme). Lysis reactions were then incubated at 37°C for 40 min. After this the manufacturer's instructions for pre-treatment of Gram-positive bacteria and purification of total DNA from animal tissues were followed. To confirm no contamination had occurred over the competency protocol or during electroporation, a 16s RNA PCR for Gram-positive bacteria was conducted using the Gram-positive primers 16sfwdB and 16srevB (Table 2) and conditions 16sPCR (Table 3). PCR products from 4 transformed COH1 strains were then sequenced using the 16sfwdB primer. Sequence was then used in a ribosomal database search to confirm *S. agalactiae* identification.

Table 2. Primers and Plasmids used in this study

Primer	Sequence/features	Source
TM1	5' – CTGTCTCTTATACACATCTCTTC – 3' Forward in transposon Tn*5*	Dr Keith Turner, Cambridge University, U.K.
TM2	5' – CTGTCTCTTATACACATCTCCCT – 3' Reverse in transposon Tn*5*	Dr Keith Turner, Cambridge University, U.K.
TM3	5' – GGGTATTATGGGTAATACGAC – 3' forward out of transposon	This study
TM4	5' – GTTATCCCTATTTAGGTGACAC – 3' Reverse out of transposon	This study
TM5	5' – AAAGCTCTCATCAACCGTGGC – 3' forward out of transposon	This study
16sfwdB	5' – GCTCAGGAYGAACGCTGG – 3'	This study
16srevB	5'-TACTGCTGCCCTCCCGTA – 3'	This study
pTnTMDH	Plasmid containing a transposon derived from EZ-Tn5 <R6Kγori/KAN-2> (Epicentre biotechnologies) with outward T7 and SP6 promoters and R6Kγori deleted. Kanamycin resistance (*aph*), Ampicillin resistance (*amp*), pUC origin and a *lac* promoter.	Dr Keith Turner, Cambridge University, U.K.

Table 3. Polymerase Chain Reaction conditions used in this study

PCR name	Cycling conditions
TnPCR	1. 30 s 98°C 2. 10 s 98°C 3. 30 s at 52°C 4. 60 s at 72°C Repeat steps 2-4 30 times 5. 10 min at 72°C
16sPCR	1. 30 s at 97°C 2. 10 s at 97°C 3. 30 s at 55°C 4. 60 s at 72°C Repeat steps 2-4 26 times 5. 5 min at 72°C
SP-PCRoutTn	1. 1 min at 94°C 2. 30 s at 94°C 3. 30 s at 50°C 4. 3 min at 72°C Repeat steps 2-4 20 times 5. 30 s at 94°C 6. 30 s at 30°C 7. 2 min at 72°C Repeat steps 5-7 30 times 8. 30 s at 94°C 9. 30 s at 50°C 10. 2 min at 72°C Repeat steps 8-9 30 times 11. 7 min at 72°C
Inverse PCR	1. 5 min at 98°C 2. 30 s at 98°C 3. 60 s at 52°C 4. 60 s at 72°C Repeat steps 2-4 30 times 5. 5 min at 72°C

Confirming insertion sites

To confirm transformed COH1 GBS harboured the transposon insertion within the genome, PCR and sequencing was done on extracted genomic DNA of the four transformants. Single-Primer PCR (Karlyshev *et al*., 2000) was utilised to amplify genomic DNA from the 3' end of the transposon into the GBS genome using the TM5 primer. Sequencing was then conducted using the primer TM3, that anneals to DNA just downstream of TM5. Achieved sequence was then compared to pTnTMDH DNA sequence using the EMBOSS align needle online tool to confirm sequence no longer matched the plasmid downstream of the transposon. For better quality PCR products for sequencing and identification of insertion sites inverse PCR was used with the second batch of transposon mutants (Fernandes *et al*., 2001). For this approach genomic DNA was first digested with *Pst*I and *Hind*III (New England Biolabs), for frequent cuts within the COH1 genome. The digested DNA was then recircularised and ligated. The ligation products were then amplified by inverse PCR with the TM3 and TM4 primers (Table 3). PCR products were then sequenced to identify the insertion site within the COH1 genome.

All sequencing in this study was accomplished at the Functional Genomics facility at the University of Birmingham on an ABI 3730 capillary sequencer.

Results

Production of competent GBS strain COH1

Various protocols exist in the literature for preparing streptococcal species for transformation, as they are not naturally competent (Biswas *et al*., 1993; Dunny *et al*., 1991; Framson *et al*., 1997; May *et al*., 2004; Ricci *et al*., 1994). A protocol involving the subculture of overnight minimal media cultures into minimal media with glycine has been used in this study. After these dilutions and re-growths, the cells are harvested in early exponential phase and washed with ice-cold sucrose. We have found this protocol (an adaptation of a previously published protocol (Framson *et al*., 1997)) to be successful. Competency of batches of GBS strain COH1 was tested by electroporation with plasmids carrying antibiotic resistance genes as selective markers. Transformation efficiencies were found to be in the range of 3×10^2 - 1×10^3 CFU/μg plasmid DNA. This protocol was then used to produce several batches of competent COH1.

Molecular construction of transposomes

For the generation of a transposon mutant library in GBS strain COH1 the method of transposon directed insertion site sequencing (TraDIS) has been adopted. In preparation for this analysis, a comprehensive mutant library in COH1 was required. To achieve this a plasmid carrying a transposon and cognate transposase gene is not appropriate. Instead, we exploited a transposome, composed of a transposon DNA segment and the transposase enzyme attached to the inverted repeats it recognises at either end of the transposon DNA. This enzyme-DNA complex was then electroporated into competent COH1 to create random insertions through-out the COH1 genome. To generate transposomes the transposon DNA was first extracted from the pTnTMDH plasmid. The pTnTMDH plasmid was extracted from *E. coli* DH5α and digested with the restriction enzyme *Msp*A1I (Figure 31). This resulted in several linear fragments, including a fragment of approximately 1984 bp containing the Tn5 transposon region. This region was then amplified by PCR (TnPCR, Table 3) to create a 1266 bp fragment.

Whole plasmid and PCR products were sequenced using primers TM1-TM4 (Table 2). For whole plasmid miniprep, sequence from all four primers correctly matched the plasmid sequence, confirming starting plasmid identity before the next stages (data not shown). PCR product sequence from primers TM1 and TM2 successfully matched the transposon sequence (data not shown) and sequence failed from TM3 and TM4, as expected. After PCR clean up treatment the 5' ends of these DNA fragments were then phosphorylated with T4 polynucleotide kinase. Finally, transposon fragments were incubated with EZ-Tn5 transposase to form transposomes.

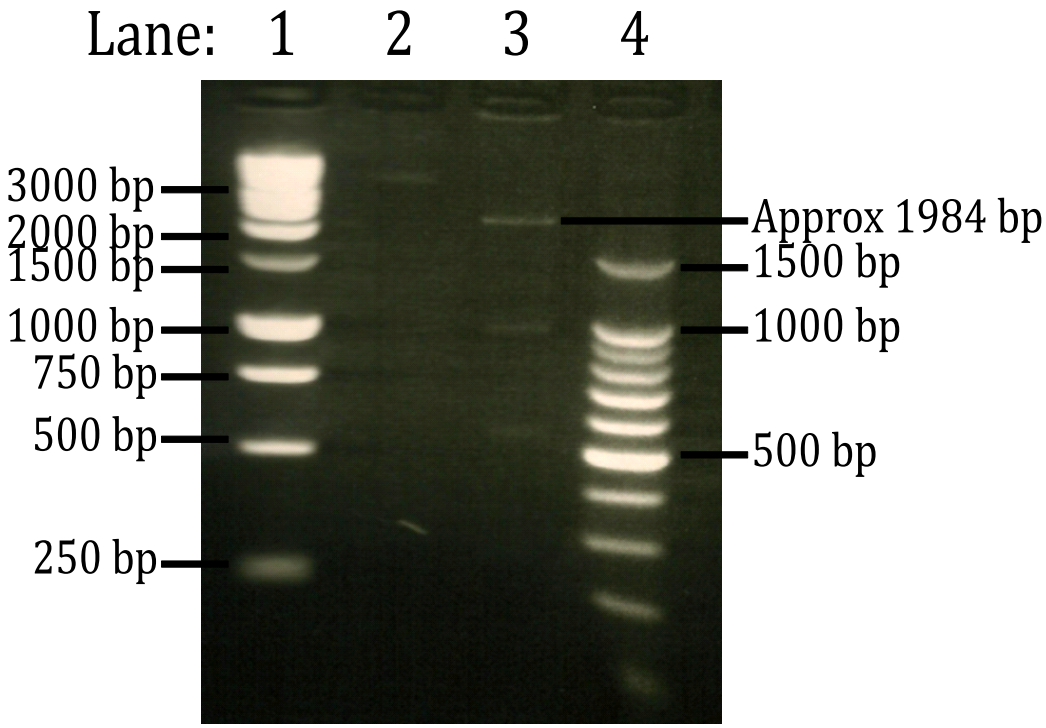

Figure 31. *Msp*A1I digest of pTnTMDH.
After digestion of pTnTMDH with *Msp*A1I for 1 hour at 37°C, a 5 µl sample was loaded into a 2% agarose gel for electrophoresis. Lane 1, 1 Kb promega benchtop DNA Ladder. Lane 2, undigested pTnTMDH miniprep. Lane 3, MspA1I digested pTnTMDH. Lane 4, 100 bp promega benchtop DNA ladder.

Transformation of GBS strain COH1

Constructed transposomes were transformed in to competent COH1 by electroporation. After 2 – 3 h recovery incubation, cell suspensions were plated onto selective kanamycin containing Todd-Hewitt agar. Following four days of incubation, transformant colonies were clearly visible. Four of these transformant colonies were subcultured onto selective plates and subsequent liquid cultures were used or frozen glycerol stocks. To determine if the transposon had been inserted into the chromosome of the transformants, genomic DNA was extracted. Single Primer PCR was then designed and conducted to amplify short regions of DNA from the 3' end of the transposon and into the COH1 genome. These regions were then sequenced, allowing identification of insertion site and concluding success of transformation. Only one of the four transformants returned a sequence read. However, this read matched the end of the transposon *and* the full plasmid. This failed attempt is likely to be due to small amounts of plasmid DNA contamination of the transposon DNA preps. Any remaining plasmid is likely to have been favoured in the transposome generating step. To address this issue, gel purification of the transposon DNA was done at every possible stage, to reduce the risk of plasmid contamination. After another round of transposome generation, competent COH1 Streptococci were transformed again. Several mutant colonies were formed, eleven of which were subcultured and glycerol stocks prepared for freezing. Starting with four of the potential mutants, genomic DNA was extracted and a 16sPCR performed to confirm GBS identity. Next, the transposon DNA was amplified to confirm its presence in the genomic DNA. Both PCR reactions and subsequent sequencing were successful (data not shown). By digestion of the COH1 genome and self-ligation of these fragments inversePCR (iPCR) was completed to determine the transposon insertion

sites. One of the four mutants provided an iPCR product. This product was sequenced and found to match the *glmU* gene in COH1. The colony morphology was also altered in this mutant. It was believed to have a defect in peptidoglycan biosynthesis. With this first successful transposon insertion, the rest of the mutants were recovered from freezer storage and genomic extraction performed. Transposon amplification in all mutants failed at this point, including for the previously identified mutant. How GBS was able to lose the transposon is currently undetermined. It is possible that GBS COH1 harbours an active transposase within the genome which is able to remove this transposon. Other research groups known to be attempting the same transposon library construction technique, in a related *Streptococcus*, appear to have also been unsuccessful and thus it appears that TraDIS may be unfeasible within this lineage of streptococci.

Discussion

Within this chapter we show for the first time that electroporation of transposomes, followed by transposon integration, is feasible with GBS. However, several significant technical hurdles remain that currently prevent widespread adoption of this technique. Firstly, incomplete plasmid digestion leads to carry-over contamination. Such complete plasmids in transposome preparations will have been favoured in late stages (such as transformation). To correct this, larger plasmid preparations were completed (midiprep) and digested for an extended period of time before samples were loaded and separated by electrophoresis. Once visualised (with much higher plasmid concentration than previous digests) it appeared more fragments were present than expected and longer incubation times than suggested were required to lose the complete plasmid. The larger fragments are likely to be explained by incomplete digests and super coiled species of the plasmid. To eliminate the possibility of taking plasmid on to the following stages, a gel extraction and purification of the 1984 bp fragment was conducted. Transposome construction on this new starting material was successful.

Subsequent transformation of competent COH1 provided several transposon mutants. However, between early investigations to determine insertion sites (for which a *glmU* mutant was successfully identified) and later rounds of investigation, the transposon was lost from the COH1 genome. Amplification of the transposon from genomic preps was unsuccessful, even though it had been previously proven to be inserted within the COH1 genome. The reason for this loss of transposon is unknown. However, a strong possibility is that GBS COH1 harbours an active transposase that enabled mobility and removal of the transposon after selection. To make such an approach feasible in the long

term, one would need to generate a GBS strain lacking endogenous transposase activity, which may be feasible if a single transposase locus provides this activity. Future work to generate comprehensive whole genome sequence and transposase activity analyses would thus be very worthwhile.

Thesis Summary

This thesis has concentrated on two human pathogens; the yeast *Cryptococcus neoformans* and the bacterium *Streptococcus agalactiae*. Both of these microbial pathogens are likely to enter the body via the lungs and encounter alveolar macrophages. Thus, the interaction of these microbes with macrophages is likely to play a major part in determining disease progression. This thesis reports findings to suggest that both of these pathogens are able to modify the maturation of the phagocyte phagosome. In the case of *S. agalactiae*, the relevance of these findings to neonatal infection is hard to tell. However, for *C. neoformans*, the intracellular population within macrophages has already been shown by others to be important in determining disease severity. It is hoped that the research presented here will aid further investigation into the macrophage parasitism measures employed by these two pathogens.

List of References

Abadi, J. and Pirofski, L. (1999) Antibodies reactive with the cryptococcal capsular polysaccharide glucuronoxylomannan are present in sera from children with and without human immunodeficiency virus infection. **The journal of infectious diseases,** 180 (3): 915-919.

Adderson, E.E., Takahashi, S., Wang, Y., *et al*. (2003) Subtractive hybridization identifies a novel predicted protein mediating epithelial cell invasion by virulent serotype III group B *Streptococcus agalactiae*. **Infection and immunity,** 71 (12): 6857-6863.

Aga, E., Katschinski, D.M., van Zandbergen, G., *et al*. (2002) Inhibition of the Spontaneous Apoptosis of Neutrophil Granulocytes by the Intracellular Parasite *Leishmania major*. **The journal of immunology,** 169 (2): 898-905.

Aimanianda, V., Bayry, J., Bozza, S., *et al*. (2009) Surface hydrophobin prevents immune recognition of airborne fungal spores**. Nature,** 460 (7259): 1117-1121.

Akhter, S., McDade, H.C., Gorlach, J.M., *et al*. (2003) Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans*. **Infection and immunity,** 71 (10): 5794-5802.

Alanio, A., Desnos-Ollivier, M. and Dromer, F. (2011) Dynamics of *Cryptococcus neoformans*-macrophage interactions reveal that fungal background influences outcome during cryptococcal meningoencephalitis in humans. **mBio,** 2 (4): 10.1128/mBio.00158-11.

Alexander, J. and Russell, D.G. (1992) The interaction of *Leishmania* species with macrophages. **Advances in parasitology,** 31 175-254.

Allaoui, A., Mounier, J., Prevost, M.C., *et al*. (1992) icsB: a *Shigella flexneri* virulence gene necessary for the lysis of protrusions during intercellular spread. **Molecular microbiology,** 6 (12): 1605-1616.

Allen, L.A. and Allgood, J.A. (2002) Atypical protein kinase C-zeta is essential for delayed phagocytosis of *Helicobacter pylori*. **Current biology,** 12 (20): 1762-1766.

Allen, L.A., Schlesinger, L.S. and Kang, B. (2000) Virulent strains of *Helicobacter pylori* demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. **The Journal of experimental medicine,** 191 (1): 115-128.

Altfeld, M., Addo, M.M., Kreuzer, K.A., *et al*. (2000) T(H)1 to T(H)2 shift of cytokines in peripheral blood of HIV-infected patients is detectable by reverse transcriptase polymerase chain reaction but not by enzyme-linked immunosorbent assay under nonstimulated conditions. **Journal of acquired immune deficiency syndromes (1999),** 23 (4): 287-294.

Alvarez, M. and Casadevall, A. (2006) Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. **Current biology,** 16 (21): 2161-2165.

Alvarez-Dominguez, C., Madrazo-Toca, F., Fernandez-Prieto, L., *et al*. (2008) Characterization of a *Listeria monocytogenes* protein interfering with Rab5a. **Traffic,** 9 (3): 325-337.

Alvarez-Dominguez, C., Roberts, R. and Stahl, P.D. (1997) Internalized *Listeria monocytogenes* modulates intracellular trafficking and delays maturation of the phagosome. **Journal of cell science,** 110 (Pt 6): 731-743.

Ampel, N.M., Bejarano, G.C. and Galgiani, J.N. (1992) Killing of *Coccidioides immitis* by human peripheral blood mononuclear cells. **Infection and immunity,** 60 (10): 4200-4204.

Anderson, D.C., Freeman, K.L., Heerdt, B., *et al*. (1987) Abnormal stimulated adherence of neonatal granulocytes: impaired induction of surface Mac-1 by chemotactic factors or secretagogues. **Blood,** 70 (3): 740-750.

Anderson, D.C., Hughes, B.J. and Smith, C.W. (1981) Abnormal mobility of neonatal polymorphonuclear leukocytes. Relationship to impaired redistribution of surface adhesion sites by chemotactic factor or colchicine. **The journal of clinical investigation,** 68 (4): 863-874.

Antal, J.M., Cunningham, J.V. and Goodrum, K.J. (1992) Opsonin-independent phagocytosis of group B streptococci: role of complement receptor type three. **Infection and immunity,** 60 (3): 1114-1121.

Antoine, J.C., Prina, E., Jouanne, C., *et al*. (1990) Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. **Infection and immunity,** 58 (3): 779-787.

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., *et al*. (2005) Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. **Nature immunology,** 6 (6): 618-625.

Arenas, G.N., Grilli, D.J., Samartino, L.E., *et al*. (2010) *Brucella* alters endocytic pathway in J774 macrophages. **Virulence,** 1 (5): 376-385.

Arenas, G.N., Staskevich, A.S., Aballay, A., *et al*. (2000) Intracellular trafficking of *Brucella abortus* in J774 macrophages. **Infection and immunity,** 68 (7): 4255-4263.

Arora, S., Olszewski, M.A., Tsang, T.M., *et al*. (2011) Effect of cytokine interplay on macrophage polarization during chronic pulmonary infection with *Cryptococcus neoformans*. **Infection and immunity,** 79 (5): 1915-1926.

Artavanis-Tsakonas, K., Love, J.C., Ploegh, H.L., *et al*. (2006) Recruitment of CD63 to *Cryptococcus neoformans* phagosomes requires acidification. **Proceedings of the national academy of sciences of the United States of America,** 103 (43): 15945-15950.

Bain, J.M., Lewis, L.E., Okai, B., *et al*. (2012) Non-lytic expulsion/exocytosis of *Candida albicans* from macrophages. **Fungal genetics and biology,** 49 (9): 677-678.

Baker, C.J. and Kasper, D.L. (1976) Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. **The New England journal of medicine,** 294 (14): 753-756.

Bakowski, M.A., Braun, V., Lam, G.Y., *et al*. (2010) The phosphoinositide phosphatase SopB manipulates membrane surface charge and trafficking of the *Salmonella*-containing vacuole. **Cell host & microbe,** 7 (6): 453-462.

Barr, F.A. (2013) Review series: Rab GTPases and membrane identity: causal or inconsequential? **The journal of cell biology,** 202 (2): 191-199.

Barry, A.O., Boucherit, N., Mottola, G., *et al*. (2012) Impaired stimulation of p38alpha-MAPK/Vps41-HOPS by LPS from pathogenic *Coxiella burnetii* prevents trafficking to microbicidal phagolysosomes. **Cell host & microbe,** 12 (6): 751-763.

Basu, M., Czinn, S.J. and Blanchard, T.G. (2004) Absence of catalase reduces long-term survival of *Helicobacter pylori* in macrophage phagosomes. **Helicobacter,** 9 (3): 211-216.

Bauer, K., Zemlin, M., Hummel, M., *et al*. (2002) Diversification of Ig heavy chain genes in human preterm neonates prematurely exposed to environmental antigens. **The journal of immunology,** 169 (3): 1349-1356.

Beaman, L., Benjamini, E. and Pappagianis, D. (1983) Activation of macrophages by lymphokines: enhancement of phagosome-lysosome fusion and killing of *Coccidioides immitis.* **Infection and immunity,** 39 (3): 1201-1207.

Beaman, L., Benjamini, E. and Pappagianis, D. (1981) Role of lymphocytes in macrophage-induced killing of *Coccidioides immitis in vitro*. **Infection and immunity,** 34 (2): 347-353.

Beaman, L. and Holmberg, C.A. (1980) In vitro response of alveolar macrophages to infection with *Coccidioides immitis.* **Infection and immunity,** 28 (2): 594-600.

Bebien, M., Hensler, M.E., Davanture, S., *et al*. (2012) The pore-forming toxin beta hemolysin/cytolysin triggers p38 MAPK-dependent IL-10 production in macrophages and inhibits innate immunity. **PLoS pathogens,** 8 (7): e1002812.

Becker, I.D., Robinson, O.M., Bazan, T.S., *et al*. (1981) Bactericidal capacity of newborn phagocytes against group B beta-hemolytic streptococci. **Infection and immunity,** 34 (2): 535-539.

Ben-Abdallah, M., Sturny-Leclere, A., Ave, P., *et al*. (2012) Fungal-induced cell cycle impairment, chromosome instability and apoptosis via differential activation of NF-kappaB. **PLoS pathogens,** 8 (3): e1002555.

Beron, W., Gutierrez, M.G., Rabinovitch, M., *et al*. (2002) *Coxiella burnetii* localizes in a Rab7-labeled compartment with autophagic characteristics. **Infection and immunity,** 70 (10): 5816-5821.

Beuzon, C.R., Meresse, S., Unsworth, K.E., *et al*. (2000) *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. **EMBO,** 19 (13): 3235-3249.

Bhagwat, A.A., Mithofer, A., Pfeffer, P.E., *et al*. (1999) Further studies of the role of cyclic beta-glucans in symbiosis. An NdvC mutant of *Bradyrhizobium japonicum* synthesizes cyclodecakis-(1-->3)-beta-glucosyl. **Plant physiology,** 119 (3): 1057-1064.

Binker, M.G., Cosen-Binker, L.I., Terebiznik, M.R., *et al*. (2007) Arrested maturation of *Neisseria*-containing phagosomes in the absence of the lysosome-associated membrane proteins, LAMP-1 and LAMP-2. **Cellular microbiology,** 9 (9): 2153-2166.

Birmingham, C.L. and Brumell, J.H. (2006) Autophagy recognizes intracellular *Salmonella enterica* serovar Typhimurium in damaged vacuoles. **Autophagy,** 2 (3): 156-158.

Biswas, I., Gruss, A., Ehrlich, S.D., *et al*. (1993) High-efficiency gene inactivation and replacement system for gram-positive bacteria. **Journal of bacteriology,** 175 (11): 3628-3635.

Blackstock, R. and Murphy, J.W. (2004) Role of interleukin-4 in resistance to *Cryptococcus neoformans* infection. **American journal of respiratory cell and molecular biology,** 30 (1): 109-117.

Blumberg, H.M., Stephens, D.S., Modansky, M., *et al*. (1996) Invasive group B streptococcal disease: the emergence of serotype V. **The journal of infectious diseases,** 173 (2): 365-373.

Bohnsack, J.F., Mollison, K.W., Buko, A.M., *et al*. (1991) Group B streptococci inactivate complement component C5a by enzymic cleavage at the C-terminus. **The biochemical journal,** 273 (Pt 3) (Pt 3): 635-640.

Bohnsack, J.F., Widjaja, K., Ghazizadeh, S., *et al*. (1997) A role for C5 and C5a-ase in the acute neutrophil response to group B streptococcal infections. **The journal of infectious diseases,** 175 (4): 847-855.

Bolduc, G.R., Baron, M.J., Gravekamp, C., *et al*. (2002) The alpha C protein mediates internalization of group B *Streptococcus* within human cervical epithelial cells. **Cellular microbiology,** 4 (11): 751-758.

Borchers, A.T. and Gershwin, M.E. (2010) The immune response in Coccidioidomycosis. **Autoimmunity reviews,** 10 (2): 94-102.

Borlace, G.N., Jones, H.F., Keep, S.J., *et al*. (2011) *Helicobacter pylori* phagosome maturation in primary human macrophages. **Gut pathogens,** 3 (1): 3.

Borlace, G.N., Keep, S.J., Prodoehl, M.J., *et al*. (2012) A role for altered phagosome maturation in the long-term persistence of *Helicobacter pylori* infection. **American journal of physiology. Gastrointestinal and liver physiology,** 303 (2): G169-79.

Bose, I., Reese, A.J., Ory, J.J., *et al*. (2003) A yeast under cover: the capsule of *Cryptococcus neoformans*. **Eukaryotic cell,** 2 (4): 655-663.

Brochet, M., Couve, E., Zouine, M., *et al*. (2006) Genomic diversity and evolution within the species *Streptococcus agalactiae*. **Microbes and infection / Institut Pasteur,** 8 (5): 1227-1243.

Brochet, M., Rusniok, C., Couve, E., *et al*. (2008) Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of *Streptococcus agalactiae.* **Proceedings of the national academy of sciences of the United States of America,** 105 (41): 15961-15966.

Brothers, K.M., Gratacap, R.L., Barker, S.E., *et al*. (2013) NADPH Oxidase-Driven Phagocyte Recruitment Controls *Candida albicans* Filamentous Growth and Prevents Mortality. **PLoS pathogens,** 9 (10): e1003634.

Brothers, K.M., Newman, Z.R. and Wheeler, R.T. (2011) Live Imaging of Disseminated Candidiasis in Zebrafish Reveals Role of Phagocyte Oxidase in Limiting Filamentous Growth. **Eukaryotic Cell,** 10 (7): 932-944.

Brown, S.M., Campbell, L.T. and Lodge, J.K. (2007) *Cryptococcus neoformans*, a fungus under stress. **Current opinion in microbiology,** 10 (4): 320-325.

Brumell, J.H., Tang, P., Zaharik, M.L., *et al*. (2002) Disruption of the *Salmonella*-containing vacuole leads to increased replication of *Salmonella enterica* serovar typhimurium in the cytosol of epithelial cells. **Infection and immunity,** 70 (6): 3264-3270.

Bryk, R., Griffin, P. and Nathan, C. (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. **Nature,** 407 (6801): 211-215.

Bullock, W.E. and Wright, S.D. (1987) Role of the adherence-promoting receptors, CR3, LFA-1, and p150,95, in binding of *Histoplasma capsulatum* by human macrophages. **Journal of experimental medicine,** 165 (1): 195-210.

Burnham, C.A., Shokoples, S.E. and Tyrrell, G.J. (2007) Invasion of HeLa cells by group B *Streptococcus* requires the phosphoinositide-3-kinase signalling pathway and modulates phosphorylation of host-cell Akt and glycogen synthase kinase-3. **Microbiology,** 153 (Pt 12): 4240-4252.

Butchar, J.P., Cremer, T.J., Clay, C.D., *et al*. (2008) Microarray analysis of human monocytes infected with *Francisella tularensis* identifies new targets of host response subversion. **PloS one,** 3 (8): e2924.

Byrne, B. and Swanson, M.S. (1998) Expression of *Legionella pneumophila* Virulence Traits in Response to Growth Conditions. **Infection and immunity,** 66 (7): 3029-3034.

Byrne, R.T., Chen, S.H., Wood, E.A., *et al*. (2014) *Escherichia coli* Genes and Pathways Involved in Surviving Extreme Exposure to Ionizing Radiation. **Journal of bacteriology,** 196 (20): 3534-3545.

Carey, A.J., Tan, C.K., Mirza, S., *et al*. (2014) Infection and cellular defense dynamics in a novel 17beta-estradiol murine model of chronic human group B *Streptococcus* genital tract colonization reveal a role for hemolysin in persistence and neutrophil accumulation. **Journal of immunology,** 192 (4): 1718-1731.

Carlin, A.F., Chang, Y.C., Areschoug, T., *et al*. (2009) Group B *Streptococcus* suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. **The journal of experimental medicine,** 206 (8): 1691-1699.

Carnell, M., Zech, T., Calaminus, S.D., *et al*. (2011) Actin polymerization driven by WASH causes V-ATPase retrieval and vesicle neutralization before exocytosis. **Journal of cell biology,** 193 (5): 831-839.

Carr, R. (2000) Neutrophil production and function in newborn infants. **British journal of haematology,** 110 (1): 18-28.

Carr, R. and Huizinga, T.W. (2000) Low soluble FcRIII receptor demonstrates reduced neutrophil reserves in preterm neonates. **Archives of disease in childhood. Fetal and neonatal edition,** 83 (2): F160.

Carruthers, V.B. (2002) Host cell invasion by the opportunistic pathogen *Toxoplasma gondii*. **Acta tropica,** 81 (2): 111-122.

Casadevall, A. (2012) Amoeba provide insight into the origin of virulence in pathogenic fungi. **Advances in experimental medicine and biology,** 710 1-10.

Casadevall, A., Cassone, A., Bistoni, F., *et al*. (1998) Antibody and/or cell-mediated immunity, protective mechanisms in fungal disease: an ongoing dilemma or an unnecessary dispute? **Medical mycology: official publication of the international society for human and animal mycology,** 36 Suppl 1 95-105.

Castaneda-Ramirez, A., Puente, J.L., Gonzalez-Noriega, A., *et al*. (2012) Silencing of VAMP3 expression does not affect *Brucella melitensis* infection in mouse macrophages. **Virulence,** 3 (5): 434-439.

Celli, J., de Chastellier, C., Franchini, D.M., *et al*. (2003) *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. **The journal of experimental medicine,** 198 (4): 545-556.

Chang, Y.C. and Kwon-Chung, K.J. (1994) Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. **Molecular and cellular biology,** 14 (7): 4912-4919.

Chang, Y.C., Stins, M.F., McCaffery, M.J., *et al*. (2004) Cryptococcal yeast cells invade the central nervous system via transcellular penetration of the blood-brain barrier. **Infection and immunity,** 72 (9): 4985-4995.

Charlier, C., Chretien, F., Baudrimont, M., *et al*. (2005) Capsule structure changes associated with *Cryptococcus neoformans* crossing of the blood-brain barrier. **American journal of pathology,** 166 (2): 421-432.

Charlier, C., Nielsen, K., Daou, S., *et al*. (2009) Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans.* **Infection and immunity,** 77 (1): 120-127.

Chayakulkeeree, M., Johnston, S.A., Oei, J.B., *et al*. (2011) SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*. **Molecular microbiology,** 80 (4): 1088-1101.

Checroun, C., Wehrly, T.D., Fischer, E.R., *et al*. (2006) Autophagy-mediated re-entry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. **Proceedings of the national academy of sciences of the United States of America,** 103 (39): 14578-14583.

Chen, C.S. (2002) Phorbol ester induces elevated oxidative activity and alkalization in a subset of lysosomes. **BMC cell biology,** 3 21.

Chen, H., Langer, R. and Edwards, D.A. (1997) A Film Tension Theory of Phagocytosis. **Journal of colloid and interface science,** 190 (1): 118-133.

Chen, J., Varma, A., Diaz, M.R., *et al*. (2008) *Cryptococcus neoformans* strains and infection in apparently immunocompetent patients, China. **Emerging infectious diseases,** 14 (5): 755-762.

Chen, S.C., Wright, L.C., Golding, J.C., *et al*. (2000) Purification and characterization of secretory phospholipase B, lysophospholipase and lysophospholipase/transacylase from a virulent strain of the pathogenic fungus *Cryptococcus neoformans*. **The biochemical journal,** 347 (Pt 2): 431-439.

Chen, S.H., Stins, M.F., Huang, S.H., *et al*. (2003) *Cryptococcus neoformans* induces alterations in the cytoskeleton of human brain microvascular endothelial cells. **Journal of medical microbiology,** 52 (Pt 11): 961-970.

Chen, Y., Toffaletti, D.L., Tenor, J.L., *et al*. (2014) The *Cryptococcus neoformans* transcriptome at the site of human meningitis. **mBio,** 5 (1): e01087-13.

Cheng, C., Chen, J., Shan, Y., *et al*. (2013) *Listeria monocytogenes* ArcA contributes to acid tolerance. **Journal of medical microbiology,** 62 (Pt 6): 813-821.

Cheng, Q., Stafslien, D., Purushothaman, S.S., *et al.* (2002) The group B streptococcal C5a peptidase is both a specific protease and an invasin. **Infection and immunity.** 70 (5): 2408-2413.

Chiang, S.L. and Mekalanos, J.J. (1998) Use of signature-tagged transposon mutagenesis to identify *Vibrio cholerae* genes critical for colonization. **Molecular microbiology,** 27 (4): 797-805.

Chong, A., Wehrly, T.D., Nair, V., *et al*. (2008) The early phagosomal stage of *Francisella tularensis* determines optimal phagosomal escape and *Francisella* pathogenicity island protein expression. **Infection and immunity,** 76 (12): 5488-5499.

Chrisman, C.J., Albuquerque, P., Guimaraes, A.J., *et al*. (2011) Phospholipids trigger *Cryptococcus neoformans* capsular enlargement during interactions with amoebae and macrophages. **PLoS pathogens,** 7 (5): e1002047.

Chun, C.D., Brown, J.C. and Madhani, H.D. (2011) A major role for capsule-independent phagocytosis-inhibitory mechanisms in mammalian infection by *Cryptococcus neoformans*. **Cell host & microbe,** 9 (3): 243-251.

Clarke, M., Kohler, J., Arana, Q., *et al*. (2002) Dynamics of the vacuolar H(+)-ATPase in the contractile vacuole complex and the endosomal pathway of Dictyostelium cells. **Journal of cell science,** 115 (Pt 14): 2893-2905.

Claus, V., Jahraus, A., Tjelle, T., *et al*. (1998) Lysosomal enzyme trafficking between phagosomes, endosomes, and lysosomes in J774 macrophages. Enrichment of cathepsin H in early endosomes. **Journal of biological chemistry,** 273 (16): 9842-9851.

Clemens, D.L. and Horwitz, M.A. (1996) The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. **Journal of experimental medicine,** 184 (4): 1349-1355.

Clemens, D.L., Lee, B.Y. and Horwitz, M.A. (2004) Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. **Infection and immunity,** 72 (6): 3204-3217.

Clemens, D.L., Lee, B.Y. and Horwitz, M.A. (2005) *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. **Infection and immunity,** 73 (9): 5892-5902.

Coers, J., Monahan, C. and Roy, C.R. (1999) Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. **Nature cell biology,** 1 (7): 451-453.

Coleman, B.I. and Gubbels, M.J. (2012) A genetic screen to isolate *Toxoplasma gondii* host-cell egress mutants. **Journal of visualized experiments : JoVE,** (60). pii: 3807. doi (60): 10.3791/3807.

Cornacchione, P., Scaringi, L., Fettucciari, K., *et al*. (1998) Group B streptococci persist inside macrophages. **Immunology,** 93 (1): 86-95.

Cossart, P., Vicente, M.F., Mengaud, J., *et al*. (1989) Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. **Infection and immunity,** 57 (11): 3629-3636.

Cox, G.M., Harrison, T.S., McDade, H.C., *et al*. (2003) Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. **Infection and immunity,** 71 (1): 173-180.

Cox, G.M., McDade, H.C., Chen, S.C., *et al*. (2001) Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. **Molecular microbiology,** 39 (1): 166-175.

Cox, G.M., Mukherjee, J., Cole, G.T., *et al*. (2000) Urease as a virulence factor in experimental cryptococcosis. **Infection and immunity,** 68 (2): 443-448.

Crabtree, J.N., Okagaki, L.H., Wiesner, D.L., *et al*. (2012) Titan cell production enhances the virulence of *Cryptococcus neoformans*. **Infection and immunity,** 80 (11): 3776-3785.

Cross, C.E. and Bancroft, G.J. (1995) Ingestion of acapsular *Cryptococcus neoformans* occurs via mannose and beta-glucan receptors, resulting in cytokine production and increased phagocytosis of the encapsulated form. **Infection and immunity,** 63 (7): 2604-2611.

Cumley, N.J., Smith, L.M., Anthony, M., *et al*. (2012) The CovS/CovR Acid Response Regulator Is Required for Intracellular Survival of Group B *Streptococcus* in Macrophages. **Infection and immunity,** 80 (5): 1650-1661.

Currie, A.J., Curtis, S., Strunk, T., *et al.* (2011) Preterm infants have deficient monocyte and lymphocyte cytokine responses to group B *Streptococcus*. **Infection and Immunity,** 79 (4): 1588-1596.

Da Cunha, V., Davies, M.R., Douarre, P., *et al*. (2014) *Streptococcus agalactiae* clones infecting humans were selected and fixed through the extensive use of tetracycline. **Nat communications,** 5.

Decken, K., Kohler, G., Palmer-Lehmann, K., *et al*. (1998) Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. **Infection and immunity,** 66 (10): 4994-5000.

Depani, S.J., Ladhani, S., Heath, P.T., *et al*. (2011) The contribution of infections to neonatal deaths in England and Wales. **The pediatric infectious disease journal,** 30 (4): 345-347.

Deretic, V. (2012) Autophagy as an innate immunity paradigm: expanding the scope and repertoire of pattern recognition receptors. **Current opinion in immunology,** 24 (1): 21-31.

Descoteaux, A., Matlashewski, G. and Turco, S.J. (1992) Inhibition of macrophage protein kinase C-mediated protein phosphorylation by *Leishmania donovani* lipophosphoglycan. **Journal of immunology,** 149 (9): 3008-3015.

Desjardins, M., Celis, J.E., van Meer, G., *et al*. (1994) Molecular characterization of phagosomes. **Journal of biological chemistry,** 269 (51): 32194-32200.

Diamond, R.D. and Bennett, J.E. (1973) Growth of *Cryptococcus neoformans* within human macrophages in vitro. **Infection and immunity,** 7 (2): 231-236.

Diamond, R.D., Root, R.K. and Bennett, J.E. (1972) Factors influencing killing of *Cryptococcus neoformans* by human leukocytes in vitro. **The Journal of infectious diseases,** 125 (4): 367-376.

Dimitrakopoulos, O., Liopeta, K., Dimitracopoulos, G., *et al*. (2013) Replication of *Brucella melitensis* inside primary human monocytes depends on mitogen activated protein kinase signaling. **Microbes and infection,** 15 (6-7): 450-460.

Doering, T.L. (2009) How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. **Annual review of microbiology,** 63 223-247.

Doering, T.L., Nosanchuk, J.D., Roberts, W.K., *et al*. (1999) Melanin as a potential cryptococcal defence against microbicidal proteins. **Medical mycology: official publication of the international society for human and animal mycology,** 37 (3): 175-181.

Dooley, D.P., Cox, R.A., Hestilow, K.L., *et al*. (1994) Cytokine induction in human coccidioidomycosis. **Infection and immunity,** 62 (9): 3980-3983.

Doran, K.S., Liu, G.Y. and Nizet, V. (2003) Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. **The journal of clinical investigation,** 112 (5): 736-744.

Doran, K.S. and Nizet, V. (2004) Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. **Molecular microbiology,** 54 (1): 23-31.

Dramsi, S., Caliot, E., Bonne, I., *et al.* (2006) Assembly and role of pili in group B streptococci. **Molecular microbiology.** 60 (6): 1401:1413.

Dunny, G.M., Lee, L.N. and LeBlanc, D.J. (1991) Improved electroporation and cloning vector system for gram-positive bacteria. **Applied and environmental microbiology,** 57 (4): 1194-1201.

Eason, S. and Martin, W. (1995) Involvement of tyrosine kinase and protein kinase C in the induction of nitric oxide synthase by lipopolysaccharide and interferon-gamma in J774 macrophages. **Archives internationales de pharmacodynamie et de therapie,** 330 (2): 225-240.

Egile, C., Loisel, T.P., Laurent, V., *et al*. (1999) Activation of the CDC42 effector N-WASP by the *Shigella flexneri* IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. **Journal of cell biology,** 146 (6): 1319-1332.

Eisenman, H.C., Mues, M., Weber, S.E., *et al*. (2007) *Cryptococcus neoformans* laccase catalyses melanin synthesis from both D- and L-DOPA. **Microbiology,** 153 (Pt 12): 3954-3962.

Ellerbroek, P.M., Walenkamp, A.M., Hoepelman, A.I., *et al*. (2004) Effects of the capsular polysaccharides of *Cryptococcus neoformans* on phagocyte migration and inflammatory mediators. **Current medicinal chemistry,** 11 (2): 253-266.

Endo, T., Sethi, K.K. and Piekarski, G. (1982) *Toxoplasma gondii*: calcium ionophore A23187-mediated exit of trophozoites from infected murine macrophages. **Experimental parasitology,** 53 (2): 179-188.

Enjalbert, B., MacCallum, D.M., Odds, F.C., *et al*. (2007) Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. **Infection and immunity,** 75 (5): 2143-2151.

Erdmann, H., Rossnagel, C., Bohme, J., *et al*. (2013) IL-17A promotes macrophage effector mechanisms against *Trypanosoma cruzi* by trapping parasites in the endolysosomal compartment. **Immunobiology,** 218 (6): 910-923.

Evans, D.J.,Jr, Evans, D.G., Takemura, T., *et al*. (1995) Characterization of a *Helicobacter pylori* neutrophil-activating protein. **Infection and immunity,** 63 (6): 2213-2220.

Feldmesser, M., Kress, Y. and Casadevall, A. (2001a) Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. **Microbiology,** 147 (Pt 8): 2355-2365.

Feldmesser, M., Tucker, S. and Casadevall, A. (2001b) Intracellular parasitism of macrophages by *Cryptococcus neoformans*. **Trends in microbiology,** 9 (6): 273-278.

Fernandes, M.C., Cortez, M., Flannery, A.R., *et al*. (2011) *Trypanosoma cruzi* subverts the sphingomyelinase-mediated plasma membrane repair pathway for cell invasion. **Journal of experimental medicine,** 208 (5): 909-921.

Fernandes, P.J., Powell, J.A. and Archer, J.A. (2001) Construction of *Rhodococcus* random mutagenesis libraries using Tn5 transposition complexes. **Microbiology,** 147 (Pt 9): 2529-2536.

Fernandez-Arenas, E., Bleck, C.K., Nombela, C., *et al*. (2009) *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. **Cellular microbiology,** 11 (4): 560-589.

Fernandez-Mora, E., Polidori, M., Luhrmann, A., *et al*. (2005) Maturation of *Rhodococcus equi*-containing vacuoles is arrested after completion of the early endosome stage. **Traffic,** 6 (8): 635-653.

Fettucciari, K., Quotadamo, F., Noce, R., *et al*. (2011) Group B *Streptococcus* (GBS) disrupts by calpain activation the actin and microtubule cytoskeleton of macrophages. **Cellular microbiology,** 13 (6): 859-884.

Flannagan, R.S., Jaumouille, V. and Grinstein, S. (2012) The cell biology of phagocytosis. **Annual review of pathology,** 7: 61-98.

Fleuridor, R., Lyles, R.H. and Pirofski, L. (1999) Quantitative and qualitative differences in the serum antibody profiles of human immunodeficiency virus-infected persons with and without *Cryptococcus neoformans* meningitis. **The journal of infectious diseases,** 180 (5): 1526-1535.

Forquin, M-P., Tazi, A,, Rosa-Fraile, M., Poyart, C*., et al.* (2007) The putative glycosyltransferase-encoding gene cylJ and the group B streptococcus (GBS)-specific gene cylK modulate hemolysin production and virulence of GBS. **Infection and immunity.** 75:2063–2066.

Framson, P.E., Nittayajarn, A., Merry, J., *et al*. (1997) New genetic techniques for group B streptococci: high-efficiency transformation, maintenance of temperature-sensitive pWV01 plasmids, and mutagenesis with Tn917. **Applied and environmental microbiology,** 63 (9): 3539-3547.

Fratti, R.A., Backer, J.M., Gruenberg, J., *et al*. (2001) Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. **The journal of cell biology,** 154 (3): 631-644.

Fukuto, H.S., Svetlanov, A., Palmer, L.E., *et al*. (2010) Global gene expression profiling of *Yersinia pestis* replicating inside macrophages reveals the roles of a putative stress-induced operon in regulating type III secretion and intracellular cell division. **Infection and immunity,** 78 (9): 3700-3715.

Garcia-Rodas, R., Gonzalez-Camacho, F., Rodriguez-Tudela, J.L., *et al*. (2011) The interaction between *Candida krusei* and murine macrophages results in multiple outcomes, including intracellular survival and escape from killing. **Infection and immunity,** 79 (6): 2136-2144.

Garrison, E., Treeck, M., Ehret, E., *et al*. (2012) A forward genetic screen reveals that calcium-dependent protein kinase 3 regulates egress in *Toxoplasma*. **PLoS pathogens,** 8 (11): e1003049.

Geier, H. and Celli, J. (2011) Phagocytic receptors dictate phagosomal escape and intracellular proliferation of *Francisella tularensis*. **Infection and immunity,** 79 (6): 2204-2214.

Gengenbacher, M. and Kaufmann, S.H. (2012) *Mycobacterium tuberculosis*: success through dormancy. **FEMS microbiology reviews,** 36 (3): 514-532.

Geunes-Boyer, S., Beers, M.F., Perfect, J.R., *et al*. (2012) Surfactant protein D facilitates *Cryptococcus neoformans* infection. **Infection and immunity,** 80 (7): 2444-2453.

Geunes-Boyer, S., Oliver, T.N., Janbon, G., *et al*. (2009) Surfactant protein D increases phagocytosis of hypocapsular *Cryptococcus neoformans* by murine macrophages and enhances fungal survival. **Infection and immunity,** 77 (7): 2783-2794.

Ghigo, E., Colombo, M.I. and Heinzen, R.A. (2012) The *Coxiella burnetii* parasitophorous vacuole. **Advances in experimental medicine and biology,** 984 141-169.

Giannoni, E., Guignard, L., Knaup Reymond, M., *et al.* (2011) Estradiol and progesterone strongly inhibit the innate immune response of mononuclear cells in newborns. **Infection and immunity.** 79 (7): 2690-2698.

Gobert, A.P., McGee, D.J., Akhtar, M., *et al*. (2001) *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. **Proceedings of the national academy of sciences of the United States of America,** 98 (24): 13844-13849.

Goldfine, H., Wadsworth, S.J. and Johnston, N.C. (2000) Activation of host phospholipases C and D in macrophages after infection with *Listeria monocytogenes*. **Infection and immunity,** 68 (10): 5735-5741.

Goldman, D.L., Khine, H., Abadi, J., *et al*. (2001) Serologic evidence for *Cryptococcus neoformans* infection in early childhood. **Pediatrics,** 107 (5): E66.

Goodrum, K.J., McCormick, L.L. and Schneider, B. (1994) Group B *Streptococcus*-induced nitric oxide production in murine macrophages is CR3 (CD11b/CD18) dependent. **Infection and immunity,** 62 (8): 3102-3107.

Gonzalez, A., Hung, C.Y. and Cole, G.T. (2011) *Coccidioides* releases a soluble factor that suppresses nitric oxide production by murine primary macrophages. **Microbial pathogenesis,** 50 (2): 100-108.

Gouin, E., Egile, C., Dehoux, P., *et al*. (2004) The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex. **Nature,** 427 (6973): 457-461.

Grabenstein, J.P., Fukuto, H.S., Palmer, L.E., *et al*. (2006) Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. **Infection and immunity,** 74 (7): 3727-3741.

Graham, J.G., Macdonald, L.J., Hussain, S.K., *et al*. (2012) Virulent *Coxiella burnetii* pathotypes productively infect primary human alveolar macrophages. **Cellular microbiology,** .

Hall, B.F., Webster, P., Ma, A.K., *et al*. (1992) Desialylation of lysosomal membrane glycoproteins by *Trypanosoma cruzi*: a role for the surface neuraminidase in facilitating parasite entry into the host cell cytoplasm. **Journal of experimental medicine,** 176 (2): 313-325.

Hamilton, A., Popham, D.L., Carl, D.J., *et al*. (2006) Penicillin-binding protein 1a promotes resistance of group B *Streptococcus* to antimicrobial peptides. **Infection and immunity,** 74 (11): 6179-6187.

Hammer, B.K. and Swanson, M.S. (1999) Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. **Molecular microbiology,** 33 (4): 721-731.

Hansen, S.M., Uldbjerg, N., Kilian, M., *et al*. (2004) Dynamics of *Streptococcus agalactiae* Colonization in Women during and after Pregnancy and in Their Infants. **Journal of clinical microbiology,** 42 (1): 83-89.

Hara, H., Tsuchiya, K., Nomura, T., *et al*. (2008) Dependency of caspase-1 activation induced in macrophages by *Listeria monocytogenes* on cytolysin, listeriolysin O, after evasion from phagosome into the cytoplasm. **The journal of immunology,** 180 (12): 7859-7868.

Harris, T.O., Shelver, D.W., Bohnsack, J.F., *et al*. (2003) A novel streptococcal surface protease promotes virulence, resistance to opsonophagocytosis, and cleavage of human fibrinogen. **The journal of clinical investigation,** 111 (1): 61-70.

Harrison, L.H., Elliott, J.A., Dwyer, D.M., *et al*. (1998) Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. **The journal of infectious diseases,** 177 (4): 998-1002.

Harrison, T.S., Griffin, G.E. and Levitz, S.M. (2000) Conditional lethality of the diprotic weak bases chloroquine and quinacrine against *Cryptococcus neoformans*. **The journal of infectious diseases,** 182 (1): 283-289.

Heiss, C., Skowyra, M.L., Liu, H., *et al*. (2013) Unusual galactofuranose modification of a capsule polysaccharide in the pathogenic yeast *Cryptococcus neoformans*. **Journal of biological chemistry,** 288 (16): 10994-11003.

Henneke, P. and Berner, R. (2006) Interaction of neonatal phagocytes with group B *Streptococcus*: recognition and response. **Infection and immunity,** 74 (6): 3085-3095.

Henry, T. and Monack, D.M. (2007) Activation of the inflammasome upon *Francisella tularensis* infection: interplay of innate immune pathways and virulence factors. **Cellular microbiology,** 9 (11): 2543-2551.

Hensel, M., Shea, J.E., Gleeson, C., *et al*. (1995) Simultaneous identification of bacterial virulence genes by negative selection. **Science,** 269 (5222): 400-403.

Hensler, M.E., Quach, D., Hsieh, C.J., *et al*. (2008) CAMP factor is not essential for systemic virulence of Group B *Streptococcus*. **Microbial pathogenesis,** 44 (1): 84-88.

Hernandez, L.D., Hueffer, K., Wenk, M.R., *et al*. (2004) *Salmonella* modulates vesicular traffic by altering phosphoinositide metabolism. **Science,** 304 (5678): 1805-1807.

Hertzen, E., Johansson, L., Wallin, R., *et al*. (2010) M1 protein-dependent intracellular trafficking promotes persistence and replication of *Streptococcus pyogenes* in macrophages. **Journal of innate immunity,** 2 (6): 534-545.

Holm, A., Tejle, K., Magnusson, K.E., *et al*. (2001) *Leishmania donovani* lipophosphoglycan causes periphagosomal actin accumulation: correlation with impaired translocation of PKCalpha and defective phagosome maturation. **Cellular microbiology,** 3 (7): 439-447.

Horwitz, M.A. (1983a) Formation of a novel phagosome by the Legionnaires' disease bacterium *(Legionella pneumophila*) in human monocytes. **Journal of experimental medicine,** 158 (4): 1319-1331.

Horwitz, M.A. (1983b) The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. **Journal of experimental medicine,** 158 (6): 2108-2126.

Houpt, D.C., Pfrommer, G.S., Young, B.J., *et al*. (1994) Occurrences, immunoglobulin classes, and biological activities of antibodies in normal human serum that are reactive with *Cryptococcus neoformans* glucuronoxylomannan. **Infection and immunity,** 62 (7): 2857-2864.

Hull, C.M. and Heitman, J. (2002) Genetics of *Cryptococcus neoformans*. **Annual review of genetics,** 36 557-615.

Hull, J.R., Tamura, G.S. and Castner, D.G. (2008) Interactions of the streptococcal C5a peptidase with human fibronectin. **Acta biomaterialia,** 4 (3): 504-513.

Huppert, M., Sun, S.H. and Harrison, J.L. (1982) Morphogenesis throughout saprobic and parasitic cycles of *Coccidioides immitis.* **Mycopathologia,** 78 (2): 107-122.

Isaac, D.T., Coady, A., Van Prooyen, N., *et al*. (2013) The 3-hydroxy-methylglutaryl coenzyme A lyase HCL1 is required for macrophage colonization by human fungal pathogen *Histoplasma capsulatum*. **Infection and immunity,** 81 (2): 411-420.

Jacobsen, I.D., Brunke, S., Seider, K., *et al*. (2010) *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. **Infection and immunity,** 78 (3): 1066-1077.

Jacobson, E.S., Ayers, D.J., Harrell, A.C., *et al*. (1982) Genetic and phenotypic characterization of capsule mutants of *Cryptococcus neoformans.* **Journal of bacteriology,** 150 (3): 1292-1296.

Jacobson, E.S., Jenkins, N.D. and Todd, J.M. (1994) Relationship between superoxide dismutase and melanin in a pathogenic fungus. **Infection and immunity,** 62 (9): 4085-4086.

Jarva, H., Hellwage, J., Jokiranta, T.S., *et al*. (2004) The group B streptococcal beta and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion. **The journal of immunology,** 172 (5): 3111-3118.

Jarva, H., Jokiranta, T.S., Wurzner, R., *et al*. (2003) Complement resistance mechanisms of streptococci. **Molecular immunology,** 40 (2-4): 95-107.

Jaumouillé, V. and Grinstein, S. (2011) Receptor mobility, the cytoskeleton, and particle binding during phagocytosis. **Current opinion in cell biology,** 23 (1): 22-29.

Jerlstrom, P.G., Talay, S.R., Valentin-Weigand, P., *et al*. (1996) Identification of an immunoglobulin A binding motif located in the beta-antigen of the c protein complex of group B streptococci. **Infection and immunity,** 64 (7): 2787-2793.

Jha, S.S., Danelishvili, L., Wagner, D., *et al*. (2010) Virulence-related *Mycobacterium avium* subsp hominissuis MAV_2928 gene is associated with vacuole remodeling in macrophages. **BMC microbiology,** 10 100-2180-10-100.

Ji, Y.S., Sun, X.M., Liu, X.Y., *et al*. (2013) *Toxoplasma gondii*: Effects of exogenous nitric oxide on egress of tachyzoites from infected macrophages. **Experimental parasitology,** 133 (1): 70-74.

Jiang, S.M., Cieslewicz, M.J., Kasper, D.L., *et al*. (2005) Regulation of virulence by a two-component system in group B *Streptococcus*. **Journal of bacteriology,** 187 (3): 1105-1113.

Jimenez-Lopez, C., Collette, J.R., Brothers, K.M., *et al*. (2013) *Candida albicans* Induces Arginine Biosynthetic Genes in Response to Host-Derived Reactive Oxygen Species. **Eukaryotic cell,** 12 (1): 91-100.

Jiang, S.M., Ishmael, N., Dunning Hotopp, J., *et al*. (2008) Variation in the group B *Streptococcus* CsrRS regulon and effects on pathogenicity. **Journal of bacteriology,** 190 (6): 1956-1965.

Johnson, M.B. and Criss, A.K. (2013) *Neisseria gonorrhoeae* phagosomes delay fusion with primary granules to enhance bacterial survival inside human neutrophils. **Cellular microbiology,** 15 (8): 1323-1340.

Johnston, S.A. and May, R.C. (2012) *Cryptococcus* interactions with macrophages: evasion and manipulation of the phagosome by a fungal pathogen. **Cellular microbiology,** .

Johnston, S.A. and May, R.C. (2010) The human fungal pathogen *Cryptococcus neoformans* escapes macrophages by a phagosome emptying mechanism that is inhibited by Arp2/3 complex-mediated actin polymerisation. **PLoS pathogens,** 6 (8): e1001041.

Johri, A.K., Paoletti, L.C., Glaser, P., *et al*. (2006) Group B *Streptococcus*: global incidence and vaccine development. **Nature reviews microbiology,** 4 (12): 932-942.

Jones, A.L., Knoll, K.M. and Rubens, C.E. (2000) Identification of *Streptococcus agalactiae* virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. **Molecular microbiology,** 37 (6): 1444-1455.

Jones, B.D., Faron, M., Rasmussen, J.A., *et al*. (2014) Uncovering the components of the *Francisella tularensis* virulence stealth strategy. **Frontiers in cellular and infection microbiology,** 4 32.

Jones, N., Oliver, K., Jones, Y., *et al.* (2006) Carriage of group B *Streptococcus* in pregnant women from Oxford, UK. **Journal of clinical pathology.**

Jong, A., Wu, C.H., Gonzales-Gomez, I., *et al*. (2012) Hyaluronic acid receptor CD44 deficiency is associated with decreased *Cryptococcus neoformans* brain infection. **Journal of biological chemistry,** 287 (19): 15298-15306.

Jong, A., Wu, C.H., Prasadarao, N.V., *et al*. (2008a) Invasion of *Cryptococcus neoformans* into human brain microvascular endothelial cells requires protein kinase C-alpha activation. **Cellular microbiology,** 10 (9): 1854-1865.

Jong, A., Wu, C.H., Shackleford, G.M., *et al*. (2008b) Involvement of human CD44 during *Cryptococcus neoformans* infection of brain microvascular endothelial cells. **Cellular microbiology,** 10 (6): 1313-1326.

Kaambwa. B., Bryan, S., Gray, J., *et al.* (2010) Cost-effectiveness of rapid tests and other existing strategies for screening and management of early-onset group B *Streptococcus* during labour. **British journal of gynaecology.** 117 (13): 1616-1627.

Kafsack, B.F., Pena, J.D., Coppens, I., *et al*. (2009) Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. **Science,** 323 (5913): 530-533.

Kageyama, S., Omori, H., Saitoh, T., *et al*. (2011) The LC3 recruitment mechanism is separate from Atg9L1-dependent membrane formation in the autophagic response against *Salmonella.* **Molecular biology of the cell,** 22 (13): 2290-2300.

Karlyshev, A.V., Pallen, M.J. and Wren, B.W. (2000) Single-primer PCR procedure for rapid identification of transposon insertion sites. **BioTechniques,** 28 (6): 1078, 1080, 1082.

Kasper, L., Seider, K., Gerwien, F., *et al*. (2014) Identification of *Candida glabrata* genes involved in pH modulation and modification of the phagosomal environment in macrophages. **PloS one,** 9 (5): e96015.

Kaur, R., Ma, B. and Cormack, B.P. (2007) A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. **Proceedings of the national academy of sciences of the United States of America,** 104 (18): 7628-7633.

Kawakami, K., Kohno, S., Kadota, J., *et al*. (1995) T cell-dependent activation of macrophages and enhancement of their phagocytic activity in the lungs of mice inoculated with heat-killed *Cryptococcus neoformans*: involvement of IFN-gamma and its protective effect against cryptococcal infection. **Microbiology and immunology,** 39 (2): 135-143.

Kechichian, T.B., Shea, J. and Del Poeta, M. (2007) Depletion of alveolar macrophages decreases the dissemination of a glucosylceramide-deficient mutant of *Cryptococcus neoformans* in immunodeficient mice. **Infection and immunity,** 75 (10): 4792-4798.

Keefe, G.P. (1997) *Streptococcus agalactiae* mastitis: A review. **Canadian veterinary journal,** 38 (7): 429-437.

Kim, J.C., Crary, B., Chang, Y.C., *et al*. (2012) *Cryptococcus neoformans* activates RhoGTPase proteins followed by protein kinase C, focal adhesion kinase, and ezrin to promote traversal across the blood-brain barrier. **Journal of biological chemistry,** 287 (43): 36147-36157.

Kinchen, J.M. and Ravichandran, K.S. (2008) Phagosome maturation: going through the acid test. **Nature reviews. Molecular cell biology,** 9 (10): 781-795.

Klein, K.A., Fukuto, H.S., Pelletier, M., *et al*. (2012) A transposon site hybridization screen identifies galU and wecBC as important for survival of *Yersinia pestis* in murine macrophages. **Journal of bacteriology,** 194 (3): 653-662.

Knodler, L.A., Vallance, B.A., Celli, J., *et al*. (2010) Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. **Proceedings of the national academy of sciences of the United States of America,** 107 (41): 17733-17738.

Kocks, C., Gouin, E., Tabouret, M., *et al*. (1992) *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. **Cell,** 68 (3): 521-531.

Konto-Ghiorghi, Y., Mairey, E., Mallet, A., *et al*. (2009) Dual role for pilus in adherence to epithelial cells and biofilm formation in *Streptococcus agalactiae*. **PLoS pathogens,** 5 (5): e1000422.

Lalioui, L., Pellegrini, E., Dramsi, S., *et al*. (2005) The SrtA Sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. **Infection and immunity,** 73 (6): 3342-3350.

Lamagni, T.L., Keshishian, C., Efstratiou, A., *et al*. (2013) Emerging Trends in the Epidemiology of Invasive Group B Streptococcal Disease in England and Wales, 1991–2010. **Clinical infectious diseases.**

Lambrechts, A., Gevaert, K., Cossart, P., *et al*. (2008) *Listeria* comet tails: the actin-based motility machinery at work. **Trends in cell biology,** 18 (5): 220-227.

Lamy, M.C., Dramsi, S., Billoet, A., *et al*. (2006) Rapid detection of the "highly virulent" group B *Streptococcus* ST-17 clone. **Microbes and infection / Institut Pasteur,** 8 (7): 1714-1722.

Lamy, M.C., Zouine, M., Fert, J., *et al*. (2004) CovS/CovR of group B *Streptococcus*: a two-component global regulatory system involved in virulence. **Molecular microbiology,** 54 (5): 1250-1268.

Lang, S. and Palmer, M. (2003) Characterization of *Streptococcus agalactiae* CAMP factor as a pore-forming toxin. **Journal of biological chemistry,** 278 (40): 38167-38173.

Langridge, G.C., Phan, M.D., Turner, D.J., *et al*. (2009) Simultaneous assay of every *Salmonella Typhi* gene using one million transposon mutants. **Genome research,** 19 (12): 2308-2316.

Laurent, T.C. and Fraser, J.R. (1992) Hyaluronan. **FASEB,** 6 (7): 2397-2404.

Lembo, A., Gurney, M.A., Burnside, K., *et al*. (2010) Regulation of CovR expression in Group B *Streptococcus* impacts blood-brain barrier penetration. **Molecular microbiology,** 77 (2): 431-443.

Lennon-Dumenil, A.M., Bakker, A.H., Maehr, R., *et al*. (2002) Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. **Journal of experimental medicine,** 196 (4): 529-540.

Levent, F., Baker, C.J., Rench, M.A., *et al*. (2010) Early outcomes of group B *Streptococcus* meningitis in the 21st century. **Pediatric infectious diseases journal.** 29 (11): 1009-1012.

Levitz, S.M., Dupont, M.P. and Smail, E.H. (1994) Direct activity of human T lymphocytes and natural killer cells against *Cryptococcus neoformans.* **Infection and immunity,** 62 (1): 194-202.

Levitz, S.M., Farrell, T.P. and Maziarz, R.T. (1991) Killing of *Cryptococcus neoformans* by human peripheral blood mononuclear cells stimulated in culture. **The journal of infectious diseases,** 163 (5): 1108-1113.

Levitz, S.M., Nong, S.H., Seetoo, K.F., *et al*. (1999) *Cryptococcus neoformans* resides in an acidic phagolysosome of human macrophages. **Infection and immunity,** 67 (2): 885-890.

Levitz, S.M. and Tabuni, A. (1991) Binding of *Cryptococcus neoformans* by human cultured macrophages. Requirements for multiple complement receptors and actin. **Journal of clinical investigation,** 87 (2): 528-535.

Li, S. and Jedrzejas, M.J. (2001) Hyaluronan binding and degradation by *Streptococcus agalactiae* hyaluronate lyase. **Journal of biological chemistry,** 276 (44): 41407-41416.

Lin, F.Y., Clemens, J.D., Azimi, P.H., *et al*. (1998) Capsular polysaccharide types of group B streptococcal isolates from neonates with early-onset systemic infection. **The journal of infectious diseases,** 177 (3): 790-792.

Lin, F.Y., Weisman, L.E., Azimi, P.H., *et al.* (2004) Level of maternal IgG anti-group B *Streptococcus* type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen. **The journal of infectious diseases.** 190 (5): 928-934.

Lin, W.J., Walthers, D., Connelly, J.E., *et al*. (2009) Threonine phosphorylation prevents promoter DNA binding of the Group B *Streptococcus* response regulator CovR. **Molecular microbiology,** 71 (6): 1477-1495.

Lindemann, S.R., Peng, K., Long, M.E., *et al*. (2011) *Francisella tularensis* Schu S4 O-antigen and capsule biosynthesis gene mutants induce early cell death in human macrophages. **Infection and immunity,** 79 (2): 581-594.

Liu, G.Y., Doran, K.S., Lawrence, T., *et al*. (2004) Sword and shield: linked group B streptococcal beta-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. **Proceedings of the national academy of sciences of the United States of America,** 101 (40): 14491-14496.

Liu, O.W., Chun, C.D., Chow, E.D., *et al*. (2008) Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. **Cell.** 135 (1): 174-188.

Lopez-Castejon, G., Corbett, D., Goldrick, M., *et al*. (2012) Inhibition of Calpain Blocks the Phagosomal Escape of *Listeria monocytogenes*. **PloS one,** 7 (4): e35936.

Lorenz, M.C., Bender, J.A. and Fink, G.R. (2004) Transcriptional Response of *Candida albicans* upon Internalization by Macrophages. **Eukaryotic cell,** 3 (5): 1076-1087.

Lorenzen, D.R., Gunther, D., Pandit, J., *et al*. (2000) *Neisseria gonorrhoeae* porin modifies the oxidative burst of human professional phagocytes. **Infection and immunity,** 68 (11): 6215-6222.

Lourido, S., Shuman, J., Zhang, C., *et al*. (2010) Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. **Nature,** 465 (7296): 359-362.

Luan, S.L., Chaudhuri, R.R., Peters, S.E., *et al*. (2013) Generation of a Tn5 transposon library in *Haemophilus parasuis* and analysis by transposon-directed insertion-site sequencing (TraDIS). **Veterinary microbiology,** 166 (3-4): 558-566.

Luberto, C., Martinez-Marino, B., Taraskiewicz, D., *et al*. (2003) Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans*. **Journal of clinical investigation,** 112 (7): 1080-1094.

Lupo, P., Chang, Y.C., Kelsall, B.L., *et al*. (2008) The presence of capsule in *Cryptococcus neoformans* influences the gene expression profile in dendritic cells during interaction with the fungus. **Infection and immunity,** 76 (4): 1581-1589.

Ma, H., Croudace, J.E., Lammas, D.A., *et al*. (2007) Direct cell-to-cell spread of a pathogenic yeast. **BMC immunology,** 8 15.

Ma, H., Croudace, J.E., Lammas, D.A., *et al*. (2006) Expulsion of live pathogenic yeast by macrophages. **Current biology,** 16 (21): 2156-2160.

Ma, H. and May, R.C. (2009) Virulence in *Cryptococcus* species. **Advances in applied microbiology,** 67 131-190.

Ma, L.L., Spurrell, J.C., Wang, J.F., *et al*. (2002) CD8 T cell-mediated killing of *Cryptococcus neoformans* requires granulysin and is dependent on CD4 T cells and IL-15. **Journal of immunology,** 169 (10): 5787-5795.

Ma, L.L., Wang, C.L., Neely, G.G., *et al*. (2004) NK cells use perforin rather than granulysin for anticryptococcal activity. **Journal of immunology,** 173 (5): 3357-3365.

MacDonald, L.J., Kurten, R.C. and Voth, D.E. (2012) *Coxiella burnetii* alters cyclic AMP-dependent protein kinase signaling during growth in macrophages. **Infection and immunity,** 80 (6): 1980-1986.

Macher, A.M., Bennett, J.E., Gadek, J.E., *et al*. (1978) Complement depletion in cryptococcal sepsis. **The journal of immunology,** 120 (5): 1686-1690.

Madoff, L.C., Michel, J.L., Gong, E.W., *et al*. (1996) Group B streptococci escape host immunity by deletion of tandem repeat elements of the alpha C protein. **Proceedings of the national academy of sciences of the United States of America,** 93 (9): 4131-4136.

Madzivhandila, M., Adrian, P.V., Cutland, C.L., *et al*. (2011) Serotype distribution and invasive potential of group B *Streptococcus* isolates causing disease in infants and colonizing maternal-newborn dyads. **PloS one,** 6 (3): e17861.

Maganto-Garcia, E., Punzon, C., Terhorst, C., *et al*. (2008) Rab5 activation by Toll-like receptor 2 is required for *Trypanosoma cruzi* internalization and replication in macrophages. **Traffic,** 9 (8): 1299-1315.

Maisey, H.C., Quach, D., Hensler, M.E., *et al*. (2008) A group B streptococcal pilus protein promotes phagocyte resistance and systemic virulence. **FASEB,** 22 (6): 1715-1724.

Malik, Z.A., Thompson, C.R., Hashimi, S., *et al*. (2003) Cutting edge: *Mycobacterium tuberculosis* blocks Ca2+ signaling and phagosome maturation in human macrophages via specific inhibition of sphingosine kinase. **Journal of immunology,** 170 (6): 2811-2815.

Malik-Kale, P., Jolly, C.E., Lathrop, S., *et al*. (2011) *Salmonella* - at home in the host cell. **Frontiers in microbiology,** 2 125.

Mansour, M.K., Vyas, J.M. and Levitz, S.M. (2011) Dynamic virulence: real-time assessment of intracellular pathogenesis links *Cryptococcus neoformans* phenotype with clinical outcome. **mBio,** 2 (5): 10.1128/mBio.00217-11.

Mansour, M.K., Tam, J.M. and Vyas, J.M. (2012) The cell biology of the innate immune response to *Aspergillus fumigatus*. **Annals of the New York academy of sciences,** 1273 (1): 78-84.

Marchlewicz, B.A. and Duncan, J.L. (1980) Properties of a hemolysin produced by group B streptococci. **Infection and immunity,** 30 (3): 805-813.

Marteyn, B., Gazi, A. and Sansonetti, P. (2012) *Shigella*: a model of virulence regulation in vivo. **Gut microbes,** 3 (2): 104-120

Maruvada, R., Prasadarao, N.V. and Rubens, C.E. (2009) Acquisition of factor H by a novel surface protein on group B *Streptococcus* promotes complement degradation. **FASEB,** 23 (11): 3967-3977.

Matheoud, D., Moradin, N., Bellemare-Pelletier, A., *et al*. (2013) *Leishmania* evades host immunity by inhibiting antigen cross-presentation through direct cleavage of the SNARE VAMP8. **Cell host & microbe,** 14 (1): 15-25.

May, J.P., Walker, C.A., Maskell, D.J., *et al*. (2004) Development of an in vivo Himar1 transposon mutagenesis system for use in *Streptococcus equi* subsp. *equi*. **FEMS microbiology letters,** 238 (2): 401-409.

McCoy, J.M., Whitehead, L., van Dooren, G.G., *et al*. (2012) TgCDPK3 Regulates Calcium-Dependent Egress of *Toxoplasma gondii* from Host Cells. **PLoS pathogens,** 8 (12): e1003066.

Melin, P. (2011) Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. **Clinical microbiology and infection: the official publication of the european society of clinical microbiology and infectious diseases,** 17 (9): 1294-1303.

Meyer, W., Aanensen, D.M., Boekhout, T., *et al*. (2009) Consensus multi-locus sequence typing scheme for *Cryptococcus neoformans* and *Cryptococcus gattii*. **Medical mycology,** 47 (6): 561-570.

Milam, J.E., Herring-Palmer, A.C., Pandrangi, R., *et al*. (2007) Modulation of the pulmonary type 2 T-cell response to *Cryptococcus neoformans* by intratracheal delivery of a tumor necrosis factor alpha-expressing adenoviral vector. **Infection and immunity,** 75 (10): 4951-4958.

Ming, M., Ewen, M.E. and Pereira, M.E. (1995) *Trypanosome* invasion of mammalian cells requires activation of the TGF beta signaling pathway. **Cell,** 82 (2): 287-296.

Mitchell, T.G. and Friedman, L. (1972) In vitro phagocytosis and intracellular fate of variously encapsulated strains of *Cryptococcus neoformans*. **Infection and immunity,** 5 (4): 491-498.

Mitchell, T.G. and Perfect, J.R. (1995) Cryptococcosis in the era of AIDS--100 years after the discovery of *Cryptococcus* neoformans. **Clinical microbiology reviews,** 8 (4): 515-548.

Monack, D.M. (2012) *Salmonella* persistence and transmission strategies. **Current opinion in microbiology,** 15 (1): 100-107.

Morton, C.O., Bouzani, M., Loeffler, J., *et al*. (2012) Direct interaction studies between *Aspergillus fumigatus* and human immune cells; what have we learned about pathogenicity and host immunity? **Frontiers in microbiology,** 3 413.

Mosleh, I.M., Huber, L.A., Steinlein, P., *et al*. (1998) *Neisseria gonorrhoeae* porin modulates phagosome maturation. **Journal of biological chemistry,** 273 (52): 35332-35338.

Mosser, D.M. and Edelson, P.J. (1985) The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania promastigotes*. **The journal of immunology,** 135 (4): 2785-2789.

Mottola, G., Boucherit, N., Trouplin, V., *et al*. (2014) *Tropheryma whipplei*, the agent of Whipple's disease, affects the early to late phagosome transition and survives in a Rab5- and Rab7-positive compartment. **PloS one,** 9 (2): e89367.

Moule, M.G., Hemsley, C.M., Seet, Q., *et al*. (2014) Genome-wide saturation mutagenesis of *Burkholderia pseudomallei* K96243 predicts essential genes and novel targets for antimicrobial development. **mBio,** 5 (1): e00926-13.

Mukherjee, J., Scharff, M.D. and Casadevall, A. (1992) Protective murine monoclonal antibodies to *Cryptococcus neoformans*. **Infection and immunity,** 60 (11): 4534-4541.

Mukherjee, S., Feldmesser, M. and Casadevall, A. (1996) J774 murine macrophage-like cell interactions with *Cryptococcus neoformans* in the presence and absence of opsonins. **The journal of infectious diseases,** 173 (5): 1222-1231.

Muller, U., Stenzel, W., Kohler, G., *et al*. (2007) IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. **The journal of immunology,** 179 (8): 5367-5377.

Myrvik, Q.N., Leake, E.S. and Wright, M.J. (1984) Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of Mycobacterium tuberculosis. A correlate of virulence. **The American review of respiratory disease,** 129 (2): 322-328.

Nagai, H., Kagan, J.C., Zhu, X., *et al*. (2002) A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. **Science,** 295 (5555): 679-682.

Nakagawa, Y. (2008) Catalase gene disruptant of the human pathogenic yeast *Candida albicans* is defective in hyphal growth, and a catalase-specific inhibitor can suppress hyphal growth of wild-type cells. **Microbiology and immunology,** 52 (1): 16-24.

Nasution, O., Srinivasa, K., Kim, M., *et al*. (2008) Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. **Eukaryotic cell,** 7 (11): 2008-2011

Newman, S.L., Gootee, L., Bucher, C., *et al*. (1991) Inhibition of intracellular growth of *Histoplasma capsulatum* yeast cells by cytokine-activated human monocytes and macrophages. **Infection and immunity,** 59 (2): 737-741.

Newman, S.L., Gootee, L., Hilty, J., *et al*. (2006) Human macrophages do not require phagosome acidification to mediate fungistatic/fungicidal activity against *Histoplasma capsulatum*. **Journal of immunology,** 176 (3): 1806-1813.

Nicola, A.M., Albuquerque, P., Martinez, L.R., *et al*. (2012) Macrophage autophagy in immunity to *Cryptococcus neoformans* and *Candida albicans*. **Infection and immunity,** 80 (9): 3065-3076.

Nicola, A.M., Robertson, E.J., Albuquerque, P., *et al*. (2011) Nonlytic exocytosis of *Cryptococcus neoformans* from macrophages occurs in vivo and is influenced by phagosomal pH. **mBio,** 2 (4): 10.1128/mBio.00167-11.

Nkadi, P.O., Merritt, T.A. and Pillers, D.A. (2009) An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. **Molecular genetics and metabolism,** 97 (2): 95-101.

Nordenfelt, P., Grinstein, S., Bjorck, L., *et al*. (2012) V-ATPase-mediated phagosomal acidification is impaired by *Streptococcus pyogenes* through Mga-regulated surface proteins. **Microbes and infection,** 14 (14): 1319-1329.

Nosanchuk, J.D. and Casadevall, A. (2006) Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. **Antimicrobial agents and chemotherapy,** 50 (11): 3519-3528.

Nosanchuk, J.D. and Casadevall, A. (2003) Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-dopa. **Microbiology,** 149 (Pt 7): 1945-1951.

Nosanchuk, J.D., Steenbergen, J.N., Shi, L., *et al*. (2003) Antibodies to a cell surface histone-like protein protect against *Histoplasma capsulatum*. **Journal of clinical investigation,** 112 (8): 1164-1175.

Noverr, M.C., Cox, G.M., Perfect, J.R., *et al*. (2003) Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. **Infection and immunity,** 71 (3): 1538-1547.

Nussbaum, C. and Sperandio, M. (2011) Innate immune cell recruitment in the fetus and neonate. **Journal of reproductive immunology,** 90 (1): 74-81.

Nusse, O. (2011) Biochemistry of the phagosome: the challenge to study a transient organelle. **The scientific world journal,** 11 2364-2381.

Oh, Y.K., Alpuche-Aranda, C., Berthiaume, E., *et al*. (1996) Rapid and complete fusion of macrophage lysosomes with phagosomes containing *Salmonella typhimurium.* **Infection and immunity,** 64 (9): 3877-3883.

Okagaki, L.H. and Nielsen, K. (2012) Titan cells confer protection from phagocytosis in *Cryptococcus neoformans* infections. **Eukaryotic cell,** 11 (6): 820-826.

Okagaki, L.H., Strain, A.K., Nielsen, J.N., *et al*. (2010) Cryptococcal cell morphology affects host cell interactions and pathogenicity. **PLoS pathogens,** 6 (6): e1000953.

Okagaki, L.H., Wang, Y., Ballou, E.R., *et al*. (2011) Cryptococcal titan cell formation is regulated by G-protein signaling in response to multiple stimuli. **Eukaryotic cell,** 10 (10): 1306-1316.

Onfelt, B., Nedvetzki, S., Benninger, R.K., *et al*. (2006) Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. **Journal of immunology,** 177 (12): 8476-8483.

Onfelt, B., Nedvetzki, S., Yanagi, K., *et al*. (2004) Cutting edge: Membrane nanotubes connect immune cells. **Journal of immunology,** 173 (3): 1511-1513.

Orsi, C.F., Colombari, B., Ardizzoni, A., *et al*. (2009) The ABC transporter-encoding gene AFR1 affects the resistance of *Cryptococcus neoformans* to microglia-mediated antifungal activity by delaying phagosomal maturation. **FEMS yeast research,** 9 (2): 301-310.

Osterholzer, J.J., Surana, R., Milam, J.E., *et al*. (2009) Cryptococcal urease promotes the accumulation of immature dendritic cells and a non-protective T2 immune response within the lung. **The American journal of pathology,** 174 (3): 932-943.

Page, A.L., Ohayon, H., Sansonetti, P.J., *et al*. (1999) The secreted IpaB and IpaC invasins and their cytoplasmic chaperone IpgC are required for intercellular dissemination of *Shigella flexneri.* **Cellular microbiology,** 1 (2): 183-193.

Park, B.J., Wannemuehler, K.A., Marston, B.J., *et al*. (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. **AIDS,** 23 (4): 525-530.

Paul, A., Pendreigh, R.H. and Plevin, R. (1995) Protein kinase C and tyrosine kinase pathways regulate lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 murine macrophages. **British journal of pharmacology,** 114 (2): 482-488.

Perfect, J.R. (2005) *Cryptococcus neoformans*: a sugar-coated killer with designer genes. **FEMS immunology and medical microbiology,** 45 (3): 395-404.

Pericolini, E., Cenci, E., Monari, C., *et al*. (2006) *Cryptococcus neoformans* capsular polysaccharide component galactoxylomannan induces apoptosis of human T-cells through activation of caspase-8. **Cellular microbiology,** 8 (2): 267-275.

Peschel, A. (2002) How do bacteria resist human antimicrobial peptides? **Trends in microbiology,** 10 (4): 179-186.

Pistor, S., Grobe, L., Sechi, A.S., *et al*. (2000) Mutations of arginine residues within the 146-KKRRK-150 motif of the ActA protein of *Listeria monocytogenes* abolish intracellular motility by interfering with the recruitment of the Arp2/3 complex. **Journal of cell science,** 113 (Pt 18) (Pt 18): 3277-3287.

Pizarro-Cerda, J., Meresse, S., Parton, R.G., *et al*. (1998) *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. **Infection and immunity,** 66 (12): 5711-5724.

Portnoy, D.A., Auerbuch, V. and Glomski, I.J. (2002) The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. **Journal of cell biology,** 158 (3): 409-414.

Polando, R., Dixit, U.G., Carter, C.R. *et al*. (2013) The roles of complement receptor 3 and Fcγ receptors during *Leishmania* phagosome maturation. **Journal of leukocyte biology,** 93 (6): 921-932.

Powderly, W.G. (1993) Cryptococcal meningitis and AIDS. **Clinical infectious diseases,** 17 (5): 837-842.

Poyart, C., Pellegrini, E., Gaillot, O., *et al*. (2001) Contribution of Mn-Cofactored Superoxide Dismutase (SodA) to the Virulence of *Streptococcus agalactiae*. **Infection and immunity,** 69 (8): 5098-5106.

Pritzlaff, C.A., Chang, J.C., Kuo, S.P., *et al*. (2001) Genetic basis for the beta-haemolytic/cytolytic activity of group B *Streptococcus*. **Molecular microbiology,** 39 (2): 236-247.

Pujol, C., Klein, K.A., Romanov, G.A., *et al*. (2009) *Yersinia pestis* can reside in autophagosomes and avoid xenophagy in murine macrophages by preventing vacuole acidification. **Infection and immunity,** 77 (6): 2251-2261

Purdy, G.E., Owens, R.M., Bennett, L., *et al*. (2005) Kinetics of phosphatidylinositol-3-phosphate acquisition differ between IgG bead-containing phagosomes and *Mycobacterium tuberculosis*-containing phagosomes. **Cellular microbiology,** 7 (11): 1627-1634.

Qin, Q.M., Luo, J., Lin, X., *et al*. (2011) Functional analysis of host factors that mediate the intracellular lifestyle of *Cryptococcus neoformans*. **PLoS pathogens,** 7 (6): e1002078.

Quach, D., van Sorge, N.M., Kristian, S.A., *et al*. (2009) The CiaR response regulator in group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. **Journal of bacteriology,** 191 (7): 2023-2032.

Radtke, A.L., Anderson, K.L., Davis, M.J., *et al*. (2011) *Listeria monocytogenes* exploits cystic fibrosis transmembrane conductance regulator (CFTR) to escape the phagosome. **Proceedings of the national academy of sciences of the United States of America,** 108 (4): 1633-1638.

Rai, M.N., Balusu, S., Gorityala, N., *et al*. (2012) Functional genomic analysis of *Candida glabrata*-macrophage interaction: role of chromatin remodeling in virulence. **PLoS pathogens,** 8 (8): e1002863.

Rajagopal, L. (2009) Understanding the regulation of Group B Streptococcal virulence factors. **Future microbiology,** 4 (2): 201-221.

Rajagopal, L., Vo, A., Silvestroni, A., *et al*. (2006) Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in *Streptococcus agalactiae*. **Molecular microbiology,** 62 (4): 941-957.

Ramond, E., Gesbert, G., Rigard, M., *et al*. (2014) Glutamate utilization couples oxidative stress defense and the tricarboxylic acid cycle in *Francisella* phagosomal escape. **PLoS pathogens,** 10 (1): e1003893.

Remington, J.S. Klein, J.O. Wilson, C.B. *et al*. (2011) **Infectious diseases of the fetus and newborn infant.** 7th ed. USA: Elsevier Saunders.

Ricci, M.L., Manganelli, R., Berneri, C., *et al*. (1994) Electrotransformation of *Streptococcus agalactiae* with plasmid DNA. **FEMS microbiology letters,** 119 (1-2): 47-52.

Ricketts, H.T. (1906) Infection, Immunity and Serum Therapy. **American medical association press,** 47 : 358.

Rodriguez, N.E., Gaur Dixit, U., Allen, L.A., *et al*. (2011) Stage-specific pathways of *Leishmania infantum chagasi* entry and phagosome maturation in macrophages. **PloS one,** 6 (4): e19000.

Roehrich, A.D., Guillossou, E., Blocker, A.J., *et al*. (2013) *Shigella* IpaD has a dual role: signal transduction from the type III secretion system needle tip and intracellular secretion regulation. **Molecular microbiology,** 87 (3): 690-706.

Rohatgi, S., Gohil, S., Kuniholm, M.H., *et al*. (2013) Fc gamma receptor 3A polymorphism and risk for HIV-associated cryptococcal disease. **mBio,** 4 (5): e00573-13.

Romano, P.S., Arboit, M.A., Vazquez, C.L., *et al*. (2009) The autophagic pathway is a key component in the lysosomal dependent entry of *Trypanosoma cruzi* into the host cell. **Autophagy,** 5 (1): 6-18.

Rosales-Reyes, R., Perez-Lopez, A., Sanchez-Gomez, C., *et al*. (2012) *Salmonella* infects B cells by macropinocytosis and formation of spacious phagosomes but does not induce pyroptosis in favor of its survival. **Microbial pathogenesis,** 52 (6): 367-374.

Rosas, A.L. and Casadevall, A. (2001) Melanization decreases the susceptibility of *Cryptococcus neoformans* to enzymatic degradation. **Mycopathologia,** 151 (2): 53-56.

Rosas, A.L. and Casadevall, A. (1997) Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. **FEMS microbiology letters,** 153 (2): 265-272.

Ross, G.D., Thompson, R.A., Walport, M.J., *et al*. (1985) Characterization of patients with an increased susceptibility to bacterial infections and a genetic deficiency of leukocyte membrane complement receptor type 3 and the related membrane antigen LFA-1. **Blood,** 66 (4): 882-890.

Roy, C.R., Berger, K.H. and Isberg, R.R. (1998) *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. **Molecular microbiology,** 28 (3): 663-674.

Rubens, C.E., Smith, S., Hulse, M., *et al*. (1992) Respiratory epithelial cell invasion by group B streptococci. **Infection and immunity,** 60 (12): 5157-5163.

Rubens, C.E., Wessels, M.R., Heggen, L.M., *et al*. (1987) Transposon mutagenesis of type III group B *Streptococcus*: correlation of capsule expression with virulence. **Proceedings of the national academy of sciences of the United States of America,** 84 (20): 7208-7212.

Rutherford, J.C. (2014) The emerging role of urease as a general microbial virulence factor. **PLoS pathogens,** 10 (5): e1004062.

Sabiiti, W. and May, R.C. (2012) Capsule independent uptake of the fungal pathogen *Cryptococcus neoformans* into brain microvascular endothelial cells. **PloS one,** 7 (4): e35455.

Sabiiti, W., Robertson, E., Beale, M.A., *et al*. (2014) Efficient phagocytosis and laccase activity affect the outcome of HIV-associated cryptococcosis. **Journal of clinical investigation,** 124 (5): 2000-2008.

Sacks, D. and Sher, A. (2002) Evasion of innate immunity by parasitic protozoa. **Nature immunology,** 3 (11): 1041-1047.

Sagar, A., Klemm, C., Hartjes, L., *et al*. (2013) The beta-hemolysin and intracellular survival of *Streptococcus agalactiae* in human macrophages. **PloS one,** 8 (4): e60160.

Sahni, S.K. and Rydkina, E. (2009) Host-cell interactions with pathogenic *Rickettsia* species. **Future microbiology,** 4 (3): 323-339.

Samen, U., Eikmanns, B.J., Reinscheid, D.J., *et al*. (2007) The surface protein Srr-1 of *Streptococcus agalactiae* binds human keratin 4 and promotes adherence to epithelial HEp-2 cells. **Infection and immunity,** 75 (11): 5405-5414.

Santa Maria, J.P.,Jr, Sadaka, A., Moussa, S.H., *et al*. (2014) Compound-gene interaction mapping reveals distinct roles for *Staphylococcus aureus* teichoic acids. **Proceedings of the national academy of sciences of the United States of America,** 111 (34): 12510-12515.

Santi, I., Grifantini, R., Jiang, S.M., *et al*. (2009a) CsrRS regulates group B *Streptococcus* virulence gene expression in response to environmental pH: a new perspective on vaccine development. **Journal of bacteriology,** 191 (17): 5387-5397.

Santi, I., Maione, D., Galeotti, C.L., *et al*. (2009b) BibA induces opsonizing antibodies conferring in vivo protection against group B *Streptococcus*. **The journal of infectious diseases,** 200 (4): 564-570.

Santi, I., Scarselli, M., Mariani, M., *et al*. (2007) BibA: a novel immunogenic bacterial adhesin contributing to group B *Streptococcus* survival in human blood. **Molecular microbiology,** 63 (3): 754-767.

Santic, M., Molmeret, M., Klose, K.E., *et al*. (2006) *Francisella tularensis* travels a novel, twisted road within macrophages. **Trends in microbiology,** 14 (1): 37-44.

Santic, M., Asare, R., Skrobonja, I., *et al*. (2008) Acquisition of the vacuolar ATPase proton pump and phagosome acidification are essential for escape of *Francisella tularensis* into the macrophage cytosol. **Infection and immunity,** 76 (6): 2671-2677.

Schwartz, J.T. and Allen, L.A. (2006) Role of urease in megasome formation and *Helicobacter pylori* survival in macrophages. **Journal of leukocyte biology,** 79 (6): 1214-1225.

Scott, C.C., Botelho, R.J. and Grinstein, S. (2003) Phagosome maturation: a few bugs in the system. **Journal of membrane biology,** 193 (3): 137-152.

Scott, C.C., Cuellar-Mata, P., Matsuo, T., *et al*. (2002) Role of 3-phosphoinositides in the maturation of *Salmonella*-containing vacuoles within host cells. **Journal of biological chemistry,** 277 (15): 12770-12776.

Seider, K., Brunke, S., Schild, L., *et al*. (2011) The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. **The journal of immunology,** 187 (6): 3072-3086.

Seifert, K.N., Adderson, E.E., Whiting, A.A., *et al*. (2006) A unique serine-rich repeat protein (Srr-2) and novel surface antigen (epsilon) associated with a virulent lineage of serotype III *Streptococcus agalactiae*. **Microbiology,** 152 (Pt 4): 1029-1040.

Shao, X., Mednick, A., Alvarez, M., *et al*. (2005) An innate immune system cell is a major determinant of species-related susceptibility differences to fungal pneumonia. **The journal of immunology,** 175 (5): 3244-3251.

Shapiro, S., Beenhouwer, D.O., Feldmesser, M., *et al*. (2002) Immunoglobulin G monoclonal antibodies to *Cryptococcus neoformans* protect mice deficient in complement component C3. **Infection and immunity,** 70 (5): 2598-2604.

Shea, J.M., Kechichian, T.B., Luberto, C., *et al*. (2006) The cryptococcal enzyme inositol phosphosphingolipid-phospholipase C confers resistance to the antifungal effects

of macrophages and promotes fungal dissemination to the central nervous system. **Infection and immunity,** 74 (10): 5977-5988.

Sheen, T.R., Jimenez, A., Wang, N.Y., *et al*. (2011) Serine Rich Repeat Proteins and Pili Promote *Streptococcus agalactiae* Colonization of the Vaginal Tract. **Journal of bacteriology,** 93 (24): 6834-42.

Sherman, M.P., Johnson, J.T., Rothlein, R., *et al*. (1992) Role of pulmonary phagocytes in host defense against group B streptococci in preterm versus term rabbit lung. **The journal of infectious diseases,** 166 (4): 818-826.

Sherman, M.P. and Lehrer, R.I. (1985) Oxidative metabolism of neonatal and adult rabbit lung macrophages stimulated with opsonized group B streptococci. **Infection and immunity,** 47 (1): 26-30.

Shi, L., Albuquerque, P.C., Lazar-Molnar, E., *et al*. (2008) A monoclonal antibody to *Histoplasma capsulatum* alters the intracellular fate of the fungus in murine macrophages. **Eukaryotic cell,** 7 (7): 1109-1117.

Shi, M., Li, S.S., Zheng, C., *et al*. (2010) Real-time imaging of trapping and urease-dependent transmigration of *Cryptococcus neoformans* in mouse brain. **Journal of clinical investigation,** 120 (5): 1683-1693.

Sibley, L.D., Weidner, E. and Krahenbuhl, J.L. (1985) Phagosome acidification blocked by intracellular *Toxoplasma gondii*. **Nature,** 315 (6018): 416-419.

Siddiqui, A.A., Shattock, R.J. and Harrison, T.S. (2006) Role of capsule and interleukin-6 in long-term immune control of *Cryptococcus neoformans* infection by specifically activated human peripheral blood mononuclear cells. **Infection and immunity,** 74 (9): 5302-5310.

Singh, A., Panting, R.J., Varma, A., *et al*. (2013) Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*. **mBio,** 4 (3): e00220-13.

Singh, R., Jamieson, A. and Cresswell, P. (2008) GILT is a critical host factor for *Listeria monocytogenes* infection. **Nature,** 455 (7217): 1244-1247.

Slesiona, S., Gressler, M., Mihlan, M., *et al*. (2012) Persistence versus escape: *Aspergillus terreus* and *Aspergillus fumigatus* employ different strategies during interactions with macrophages. **PloS one,** 7 (2): e31223.

Smirnov, A., Daily, K.P. and Criss, A.K. (2014) Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression State of *Neisseria gonorrhoeae*. **Infection and immunity,** 82 (3): 1036-1044.

Smith, J., Manoranjan, J., Pan, M., *et al*. (2008) Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in *Mycobacterium marinum* escape from the vacuole. **Infection and immunity,** 76 (12): 5478-5487.

Smith, L.M. and May, R.C. (2013) Mechanisms of microbial escape from phagocyte killing. **Biochemical society transactions,** 41 (2): 475-490.

Soldati, T. and Neyrolles, O. (2012) Mycobacteria and the intraphagosomal environment: take it with a pinch of salt(s)! **Traffic,** 13 (8): 1042-1052.

Spellerberg, B., Pohl, B., Haase, G., *et al*. (1999) Identification of genetic determinants for the hemolytic activity of *Streptococcus agalactiae* by ISS1 transposition. **Journal of bacteriology,** 181 (10): 3212-3219.

Spinner, J.L., Winfree, S., Starr, T., *et al*. (2014) *Yersinia pestis* survival and replication within human neutrophil phagosomes and uptake of infected neutrophils by macrophages. **Journal of leukocyte biology,** 95 (3): 389-398.

Springman, A.C., Lacher, D.W., Wu, G., *et al*. (2009) Selection, recombination, and virulence gene diversity among group B streptococcal genotypes. **Journal of bacteriology,** 191 (17): 5419-5427.

Srikanta, D., Santiago-Tirado, F.H. and Doering, T.L. (2013) *Cryptococcus neoformans*: historical curiosity to modern pathogen. **Yeast,** 31 (2): 47-60

Stalhammar-Carlemalm, M., Stenberg, L., Lindahl, G. (1993) Protein rib: a novel group B streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. **Journal of experimental medicine**. 177 (6): 1593-1603

Stamm, L.M., Morisaki, J.H., Gao, L.Y., *et al*. (2003) *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. **Journal of experimental medicine,** 198 (9): 1361-1368.

Stano, P., Williams, V., Villani, M., *et al*. (2009) App1: an antiphagocytic protein that binds to complement receptors 3 and 2. **The journal of immunology,** 182 (1): 84-91.

Starr, T., Ng, T.W., Wehrly, T.D., *et al*. (2008) *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. **Traffic,** 9 (5): 678-694.

Steenbergen, J.N., Nosanchuk, J.D., Malliaris, S.D., *et al*. (2003) *Cryptococcus neoformans* virulence is enhanced after growth in the genetically malleable host Dictyostelium discoideum. **Infection and immunity,** 71 (9): 4862-4872.

Steenbergen, J.N., Shuman, H.A. and Casadevall, A. (2001) *Cryptococcus* neoformans interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. **Proceedings of the national academy of sciences of the United States of America,** 98 (26): 15245-15250.

Stevens, J.M., Galyov, E.E. and Stevens, M.P. (2006) Actin-dependent movement of bacterial pathogens. **Nature reviews. Microbiology,** 4 (2): 91-101.

Stevens, M.P., Stevens, J.M., Jeng, R.L., *et al*. (2005) Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. **Molecular microbiology,** 56 (1): 40-53.

Stevens, M.P., Wood, M.W., Taylor, L.A., *et al*. (2002) An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. **Molecular microbiology,** 46 (3): 649-659.

Straley, S.C. and Harmon, P.A. (1984) *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. **Infection and immunity,** 45 (3): 655-659.

Strasser, J.E., Newman, S.L., Ciraolo, G.M., *et al*. (1999) Regulation of the macrophage vacuolar ATPase and phagosome-lysosome fusion by *Histoplasma capsulatum.* **Journal of immunology,** 162 (10): 6148-6154.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., *et al*. (1994) Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. **Science,** 263 (5147): 678-681.

Sun, J., Wang, X., Lau, A., *et al*. (2010) Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation in murine RAW 264.7 macrophages. **PloS one,** 5 (1): e8769.

Sunderkötter, C., Kunz, M., Steinbrink, K., *et al*. (1993) Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages in vitro and in vivo. **Journal of Immunology,** 151 (9): 4891-4901.

Suzuki, T., Franchi, L., Toma, C., *et al*. (2007) Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in *Shigella*-infected macrophages. **PLoS pathogens,** 3 (8): e111.

Swanson, M.S. and Sturgill-Koszycki, I. (2000) Exploitation of macrophages as a replication niche by *Legionella pneumophila*. **Trends in microbiology,** 8 (2): 47-49.

Sydor, T., von Bargen, K., Hsu, F.F., *et al*. (2012) Diversion of phagosome trafficking by pathogenic *Rhodococcus equi* depends on mycolic acid chain length. **Cellular microbiology,** 15 (3): 458-473

Syme, R.M., Bruno, T.F., Kozel, T.R., *et al*. (1999) The capsule of *Cryptococcus neoformans* reduces T-lymphocyte proliferation by reducing phagocytosis, which can be restored with anticapsular antibody. **Infection and immunity,** 67 (9): 4620-4627.

Taborda, C.P. and Casadevall, A. (2001) Immunoglobulin M efficacy against *Cryptococcus neoformans*: mechanism, dose dependence, and prozone-like effects in passive protection experiments. **Journal of immunology,** 166 (3): 2100-2107.

Tamura, G.S., Kuypers, J.M., Smith, S., *et al*. (1994) Adherence of group B streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. **Infection and immunity,** 62 (6): 2450-2458.

Tardieux, I., Webster, P., Ravesloot, J., *et al*. (1992) Lysosome recruitment and fusion are early events required for trypanosome invasion of mammalian cells. **Cell,** 71 (7): 1117-1130.

Tazi, A., Disson, O., Bellais, S., *et al*. (2010) The surface protein HvgA mediates group B *Streptococcus* hypervirulence and meningeal tropism in neonates. **Journal of experimental medicine,** 207 (11): 2313-2322.

Teatero, S., McGeer, A., Low, D.E., *et al*. (2014) Characterization of Invasive Group B *Streptococcus* Strains from the Greater Toronto Area, Canada. **Journal of clinical microbiology,** 52 (5): 1441-1447.

Teixeira, C.F., Azevedo, N.L., Carvalho, T.M., *et al*. (2001) Cytochemical study of *Streptococcus agalactiae* and macrophage interaction. **Microscopy research and technique,** 54 (4): 254-259.

Tettelin, H., Masignani, V., Cieslewicz, M.J., *et al*. (2002) Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. **Proceedings of the national academy of sciences of the United States of America,** 99 (19): 12391-12396.

Theriot, J.A., Mitchison, T.J., Tilney, L.G., *et al*. (1992) The rate of actin-based motility of intracellular *Listeria monocytogenes* equals the rate of actin polymerization. **Nature,** 357 (6375): 257-260.

Thywissen, A., Heinekamp, T., Dahse, H.M., *et al*. (2011) Conidial Dihydroxynaphthalene Melanin of the Human Pathogenic Fungus *Aspergillus fumigatus* Interferes with the Host Endocytosis Pathway. **Frontiers in microbiology,** 2 : 96.

Toyooka, K., Takai, S. and Kirikae, T. (2005) *Rhodococcus equi* can survive a phagolysosomal environment in macrophages by suppressing acidification of the phagolysosome. **Journal of medical microbiology,** 54 (Pt 11): 1007-1015.

Truelsen, K., Young, T. and Kozel, T.R. (1992) In vivo complement activation and binding of C3 to encapsulated *Cryptococcus neoformans*. **Infection and immunity,** 60 (9): 3937-3939.

Tucker, S.C. and Casadevall, A. (2002) Replication of *Cryptococcus neoformans* in macrophages is accompanied by phagosomal permeabilization and accumulation of vesicles containing polysaccharide in the cytoplasm. **Proceedings of the national academy of sciences of the United States of America,** 99 (5): 3165-3170.

Tyler, K.M., Luxton, G.W., Applewhite, D.A., *et al*. (2005) Responsive microtubule dynamics promote cell invasion by *Trypanosoma cruzi*. **Cellular microbiology,** 7 (11): 1579-1591.

Ulett, G.C., Maclean, K.H., Nekkalapu, S., *et al*. (2005) Mechanisms of group B streptococcal-induced apoptosis of murine macrophages. **The journal of immunology,** 175 (4): 2555-2562.

Utaisincharoen, P., Arjcharoen, S., Limposuwan, K., *et al*. (2006) *Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7). **Microbial pathogenesis,** 40 (4): 184-189.

Valenti-Weigand, P., Benkel, P., Rohde, M., *et al*. (1996) Entry and intracellular survival of group B streptococci in J774 macrophages. **Infection and immunity,** 64 (7): 2467-2473.

Vanaporn, M., Wand, M., Michell, S.L., *et al*. (2011) Superoxide dismutase C is required for intracellular survival and virulence of *Burkholderia pseudomallei.* **Microbiology,** 157 (Pt 8): 2392-2400.

van Opijnen, T. and Camilli, A. (2013) Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. **Nature reviews. Microbiology,** 11 (7): 435-442.

van Zandbergen, G., Klinger, M., Mueller, A., *et al*. (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. **Journal of immunology,** 173 (11): 6521-6525.

Vecchiarelli, A., Pericolini, E., Gabrielli, E., *et al*. (2013) Elucidating the immunological function of the *Cryptococcus neoformans* capsule. **Future microbiology,** 8 (9): 1107-1116.

Vecchiarelli, A., Pietrella, D., Dottorini, M., *et al*. (1994) Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. **Clinical and experimental immunology,** 98 (2): 217-223.

Vergne, I., Chua, J. and Deretic, V. (2003) Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. **The journal of experimental medicine,** 198 (4): 653-659.

Via, L.E., Deretic, D., Ulmer, R.J., *et al*. (1997) Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. **Journal of biological chemistry,** 272 (20): 13326-13331.

Via, L.E., Fratti, R.A., McFalone, M., *et al*. (1998) Effects of cytokines on mycobacterial phagosome maturation. **Journal of cell science,** 111 ((Pt 7): 897-905.

Voelz, K., Johnston, S.A., Smith, L.M., *et al*. (2014) "Division of labour" in response to host oxidative burst drives a fatal *Cryptococcus gattii* outbreak. **Nature Communications,** In Press.

Voelz, K., Lammas, D.A. and May, R.C. (2009) Cytokine signaling regulates the outcome of intracellular macrophage parasitism by *Cryptococcus neoformans*. **Infection and immunity,** 77 (8): 3450-3457.

Voelz, K. and May, R.C. (2010) Cryptococcal interactions with the host immune system. **Eukaryotic cell,** 9 (6): 835-846.

von Bargen, K., Gorvel, J.P. and Salcedo, S.P. (2012) Internal affairs: investigating the *Brucella* intracellular lifestyle. **FEMS microbiology reviews,** 36 (3): 533-562.

Vylkova, S. and Lorenz, M.C. (2014) Modulation of phagosomal pH by *Candida albicans* promotes hyphal morphogenesis and requires Stp2p, a regulator of amino acid transport. **PLoS pathogens,** 10 (3): e1003995.

Wadhwa, A., Kaur, R. and Bhalla, P. (2008) Profile of central nervous system disease in HIV/AIDS patients with special reference to cryptococcal infections. **The neurologist,** 14 (4): 247-251.

Walker, D.H., Feng, H.M. and Popov, V.L. (2001) Rickettsial phospholipase A2 as a pathogenic mechanism in a model of cell injury by typhus and spotted fever group rickettsiae. **The American journal of tropical medicine and hygiene,** 65 (6): 936-942.

Wang, Y., Aisen, P. and Casadevall, A. (1995) *Cryptococcus neoformans* melanin and virulence: mechanism of action. **Infection and immunity,** 63 (8): 3131-3136.

Wang, Y. and Casadevall, A. (1994) Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. **Infection and immunity,** 62 (7): 3004-3007.

Wang, Z., Guo, C., Xu, Y., *et al*. (2014) Two novel functions of hyaluronidase from *Streptococcus agalactiae* are enhanced intracellular survival and inhibition of proinflammatory cytokine expression. **Infection and immunity,** 82 (6): 2615-2625.

Weber, S., Wagner, M. and Hilbi, H. (2014) Live-cell imaging of phosphoinositide dynamics and membrane architecture during *Legionella* infection. **mBio,** 5 (1): e00839-13.

Weiss, D.S., Brotcke, A., Henry, T., *et al*. (2007) In vivo negative selection screen identifies genes required for *Francisella* virulence. **Proceedings of the national academy of sciences of the United States of America,** 104 (14): 6037-6042.

Welch, M.D., Iwamatsu, A. and Mitchison, T.J. (1997) Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. **Nature,** 385 (6613): 265-269.

Welin, A. and Lerm, M. (2012) Inside or outside the phagosome? The controversy of the intracellular localization of *Mycobacterium tuberculosis.* **Tuberculosis,** 92 (2): 113-120.

Wellington, M., Dolan, K. and Krysan, D.J. (2009) Live *Candida albicans* suppresses production of reactive oxygen species in phagocytes. **Infection and immunity,** 77 (1): 405-413.

Wessels, M.R., Kasper, D.L., Johnson, K.D., *et al*. (1998) Antibody responses in invasive group B streptococcal infection in adults. **The Journal of infectious diseases,** 178 (2): 569-572.

Wilson, D.E., Bennett, J.E. and Bailey, J.W. (1968) Serologic grouping of *Cryptococcus neoformans.* **Proceedings of the society for experimental biology and medicine,** 127 (3): 820-823.

Wilson, C.B. and Weaver, W.M. (1985) Comparative susceptibility of group B streptococci and *Staphylococcus aureus* to killing by oxygen metabolites. **The journal of infectious diseases**, 152 (2): 323-329.

Winberg, M.E., Holm, A., Sarndahl, E., *et al*. (2009) *Leishmania donovani* lipophosphoglycan inhibits phagosomal maturation via action on membrane rafts. **Microbes and infection,** 11 (2): 215-222.

Winchell, C.G., Graham, J.G., Kurten, R.C., *et al*. (2014) *Coxiella burnetii* type IV secretion-dependent recruitment of macrophage autophagosomes. **Infection and immunity,** 82 (6): 2229-2238.

Wiseman, J.C., Ma, L.L., Marr, K.J., *et al*. (2007) Perforin-dependent cryptococcal microbicidal activity in NK cells requires PI3K-dependent ERK1/2 signaling. **Journal of immunology,** 178 (10): 6456-6464.

Wormley, F.L.,Jr, Perfect, J.R., Steele, C., *et al*. (2007) Protection against cryptococcosis by using a murine gamma interferon-producing *Cryptococcus neoformans* strain. **Infection and immunity,** 75 (3): 1453-1462.

Wozniak, K.L. and Levitz, S.M. (2008) *Cryptococcus neoformans* enters the endolysosomal pathway of dendritic cells and is killed by lysosomal components. **Infection and immunity,** 76 (10): 4764-4771.

Wright, L.C., Santangelo, R.M., Ganendren, R., *et al*. (2007) Cryptococcal lipid metabolism: phospholipase B1 is implicated in transcellular metabolism of macrophage-derived lipids. **Eukaryotic cell,** 6 (1): 37-47.

Yauch, L.E., Lam, J.S. and Levitz, S.M. (2006) Direct inhibition of T-cell responses by the *Cryptococcus* capsular polysaccharide glucuronoxylomannan. **PLoS pathogens,** 2 (11): e120.

Zaghouani, H., Hoeman, C.M., Adkins, B. (2009) Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. **Trends in Immunology.** 30 (12): 585-591.

Zaragoza, O., Chrisman, C.J., Castelli, M.V., *et al*. (2008) Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival. **Cellular microbiology,** 10 (10): 2043-2057.

Zaragoza, O., Garcia-Rodas, R., Nosanchuk, J.D., *et al*. (2010) Fungal cell gigantism during mammalian infection. **PLoS pathogens,** 6 (6): e1000945.

Zaragoza, O., Taborda, C.P. and Casadevall, A. (2003) The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. **European journal of immunology,** 33 (7): 1957-1967.

Zawaneh, S.M., Ayoub, E.M., Baer, H., *et al*. (1981) Cyclic variation in the adherence of group B streptococci to human vaginal epithelial cells. **American Journal of obstetrics and gynecology,** 140 (4): 381-386.

Zawaneh, S.M., Ayoub, E.M., Baer, H., *et al*. (1979) Factors influencing adherence of group B streptococci to human vaginal epithelial cells. **Infection and immunity,** 26 (2): 441-447.

Zhao, Y., Marple, A.H., Ferguson, D.J., *et al*. (2014) Avirulent strains of *Toxoplasma gondii* infect macrophages by active invasion from the phagosome. **Proceedings of the national academy of sciences of the United States of America,** 111 (17): 6437-6442.

Zheng, C.F., Ma, L.L., Jones, G.J., *et al*. (2007) Cytotoxic CD4+ T cells use granulysin to kill *Cryptococcus neoformans,* and activation of this pathway is defective in HIV patients. **Blood,** 109 (5): 2049-2057.

Zheng, P.Y. and Jones, N.L. (2003) *Helicobacter pylori* strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. **Cellular microbiology,** 5 (1): 25-40.

Zhou, R., Yazdi, A.S., Menu, P., *et al*. (2011) A role for mitochondria in NLRP3 inflammasome activation. **Nature,** 469 (7329): 221-225.

Appendix

Movie 1. Example lack of acidification of the live *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualisation of infection of J774 macrophages with live *C. neoformans* H99. LysoTracker Red does not accumulate in phagosomes containing live H99. Media contains cell permeable LysoTracker Red, displayed in red in this phase contrast and fluorescent channel merge. Each frame represents 5 min real time.

Movie 2. Example acidification of the heat-killed *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualisation of infection of J774 macrophages with heat-killed *C. neoformans* H99. LysoTracker Red signal accumulates rapidly and intensely in phagosomes containing heat-killed H99. Media contains cell permeable LysoTracker Red, displayed in red in this phase contrast and fluorescent channel merge. Each frame represents 5 min real time.

Movie 3. Example lack of Cathepsin L activity of the live *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualisation of infection of J774 macrophages with live *C. neoformans* H99. Magic Red signal does not accumulate in phagosomes containing live H99, indicating a lack of cathepsin L activity. Media contains cell permeable Magic Red, displayed in red in this phase contrast and fluorescent channel merge. Each frame represents 5 min real time.

Movie 4. Example of rapid acquisition of Cathepsin L activity to the Heat-killed *Cryptococcus*-containing phagosome. Time lapse experiment allowing visualisation of infection of J774 macrophages with heat-killed *C. neoformans* H99. Strong Magic Red signal is seen, indicating significant acquisition of active cathepsin L to phagosomes containing heat-killed H99. Media contains cell permeable Magic Red, displayed in red in this phase contrast and fluorescent channel merge. Each frame represents 5 min real time.