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Analysing M. avium-macrophage interactions and establishing a long-term infection in a novel and realistic model of lung alveolar macrophages, Max Planck Institute Cells

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Analysing *M. avium*-macrophage interactions and establishing a long-term infection in a novel and realistic model of lung alveolar macrophages, Max Planck Institute Cells

By

Sanduni Avishka Thantri Hewage Peiris

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

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Author's Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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Abstract

Analysing *M. avium* complex-macrophage interactions and establishing a chronic infection model in novel lung alveolar macrophages, Max Planck Institute Cells.

By

Sanduni Avishka Thantri Hewage Peiris

Mycobacterium avium Complex (MAC) are species of the non-tuberculous branch of the Mycobacteria family that is responsible for the development of pulmonary lung disease in immunocompromised individuals. One of the first lines of defence from the host involve a specialised group of tissue macrophages that reside in the lung alveolar space called alveolar macrophages (AM). They have specialised functions that are suitable for recognition of foreign invaders and the ability to tackle these threats though means of a robust immune response. Alternatively, they also feature as key components in a fairly destructive uncontrolled inflammatory response which is the root cause of MAC lung disease.

As a facultative pathogen that thus far, almost exclusively infiltrates the macrophage intracellularly, MAC has evolved several tools to establish its foothold in the host. MAC persists intracellularly in the host by evading host immune responses via mechanisms such as preventing phagolysosome fusion and acidification which allows it to proliferate intracellularly thus assisting MAC in the spread of infection. It is vital that these mechanisms are investigated so that the disease process is better understood, and therapeutic strides are made.

There is a need for developments in the therapeutic pipeline due to the influx of multi-drug resistant strains of MAC that are ever emerging globally. Current treatments are quickly proving to be inadequate in the face of MAC evasive manoeuvres, and existing literature uses experimental infection models that only cover a short-term infection timeframe. As MAC infection

rarely gets resolved in a short duration (i.e., less than a week), the current literature would benefit from the establishment of a long-term infection model as done here in this project. Using MPI cells, this project focused on the modelling of MAC infection *in vitro* over the span of 20 days. Data from the *modified* gentamicin protection assays shows a marked difference in number of colony forming units (CFUs) from Day 0 to Day 20 infection period, with numbers declining over this time period in antibiotic treated cultures. Two strains with different phenotypes rough and smooth, were able to induce a pro-inflammatory response in this model which aligns with the previously established knowledge of the over exaggerated immune response associated with severe forms of MAC disease. Methods have been indicated to show the model could successfully sustain a *Mycobacterium avium* infection for up to 18 days without extracellular contamination and minimal cell death. The persistence of 2 phenotypically different *M. avium* and *M. intracellularae* were demonstrated in vitro where the strains continued to replicate until day 18 without elimination. The smooth strain was observed to decline intracellularly at a more rapid rate than the rough. In the analysis of cytokines, both the rough and the smooth strain appeared to induce IL-6 levels that were comparable to non-infected cells which could indicate a mechanism of survival.

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List of Abbreviations

ACDP	Advisory Committee on Dangerous Pathogens
AMR	Antimicrobial resistance
AMs	Alveolar macrophages
BALB-C	Inbred mouse cell strain
BMMs	Bone marrow-derived macrophages
CF	Cystic fibrosis
CFUs	Colony-forming units
diH_2O	Deionised water
DPBS	1X Dulbecco's phosphate-buffered saline
ELISA	Enzyme-linked immunosorbent assay
K/G	Kanamycin/Gentamycin – antibiotic combination
g	Relative centrifugal force - units of gravity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPLs	Glycopeptidolipids
D	Days
HRP	Horseradish peroxidase
IFNy	Interferon-y
IL-6	Interleukin-6

iRPMI	RPMI infection media
L	Litres
MAC	*Mycobacterium avium* complex
MAC-PD	*Mycobacterium avium* complex-pulmonary disease
MAPK	Mitogen-activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MDMs	Monocyte-derived macrophages
mg	Milligrams
mL	Millilitres
MLEs	Mouse lung epithelial cells
mM	Millimolar
MOI	Multiplicity of infection
MPIs	Max Planck Institute macrophages
MTB	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation factor 88
Nat	Natural culture type (wells washed without antibiotics added)
NCTC	National Collection of Type Cultures
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTM	Non-tuberculotic mycobacteria
OD	Optical density
p.i	Post-inoculation
PAMPs	Pathogen-associated molecular patterns
pg	Picograms

PRRs	Pattern recognition receptors
R-MAC	Rough *Mycobacterium avium complex*
rpm	Revolutions per minute – units of rotational speed
RPMI	Roswell Park Memorial Institute 1640 (culture medium)
S-MAC	Smooth *Mycobacterium avium complex*
TLR	Toll-like receptor
TLR2	Toll-like receptor 2
TNFα	Tumour necrosis factor-α
µg	Micrograms
µL	Microliters
µm	Micrometres

1. Chapter One: Introduction

1.1 Macrophages

The immune system is comprised of hematopoietic cells that perform protective functions when the host is under threat from foreign invasive agents that seek to cause disease (Marshall et al., 2018). The external environment consists of allergens, toxins and microorganisms that could destabilise homeostasis of the body. Through invasion and replication, microorganisms can thrive and persist in the body via route of excessive host-damage that may be direct (i.e bacterial toxins) or indirect excessive host inflammation (Turvery et al., 2010; Marshall et al., 2018). Defences of the host rely on detecting structural components of the microorganism, thereby allowing the host to mould a relevant immune response upon interaction. These interactions are coordinated by two branches of the immune system, the innate and the adaptive. Innate immunity is the first line of defence from a foreign microorganism and consists of both physical and cellular barriers. This response is delivered rapidly and without specificity (Turvey et al., 2010). The cellular element is spearheaded by antigen presenting cells such as macrophages that recognise pathogen-associated molecular patterns (PAMPS) expressed by foreign microorganisms (Aderam, 2001; Seong and Matzinger, 2004).

Macrophages respond to inflammation and two primary subsets are responsible for this process, the innately recruited and tissue-resident populations (Naito, 2008). Macrophage response is acute, and they differentiate into desired subsets given the correct stimulus (Fejer et al., 2015). As such, macrophages have varied phenotypes and are seen to differentiate from immature precursor cells into a variety of subsets (Murray et al., 2014). Studies in mice have shown that the primitive ectoderm of the yolk sac give rise to macrophages lacking monocytic progenitors at embryonic day 8 (Cline and Moore, 1972). Similar studies prove that macrophages are amongst the very first cells to be embedded in tissues during embryogenesis (Epelman et al., 2014). Therefore, macrophages acquire adaptations that are tissue specific and dependant on their local environment (Das et al., 2015). Another school of thought implies that the process of haematopoiesis occurring within the foetal liver, gives way to circulating monocytes that are fated to be tissue resident macrophages,

noted for their plasticity and ability to self-renew and also be replenished by bone-marrow derived blood monocytes (Cline and Moore, 1972; Shapouri Moghaddam et al., 2018).

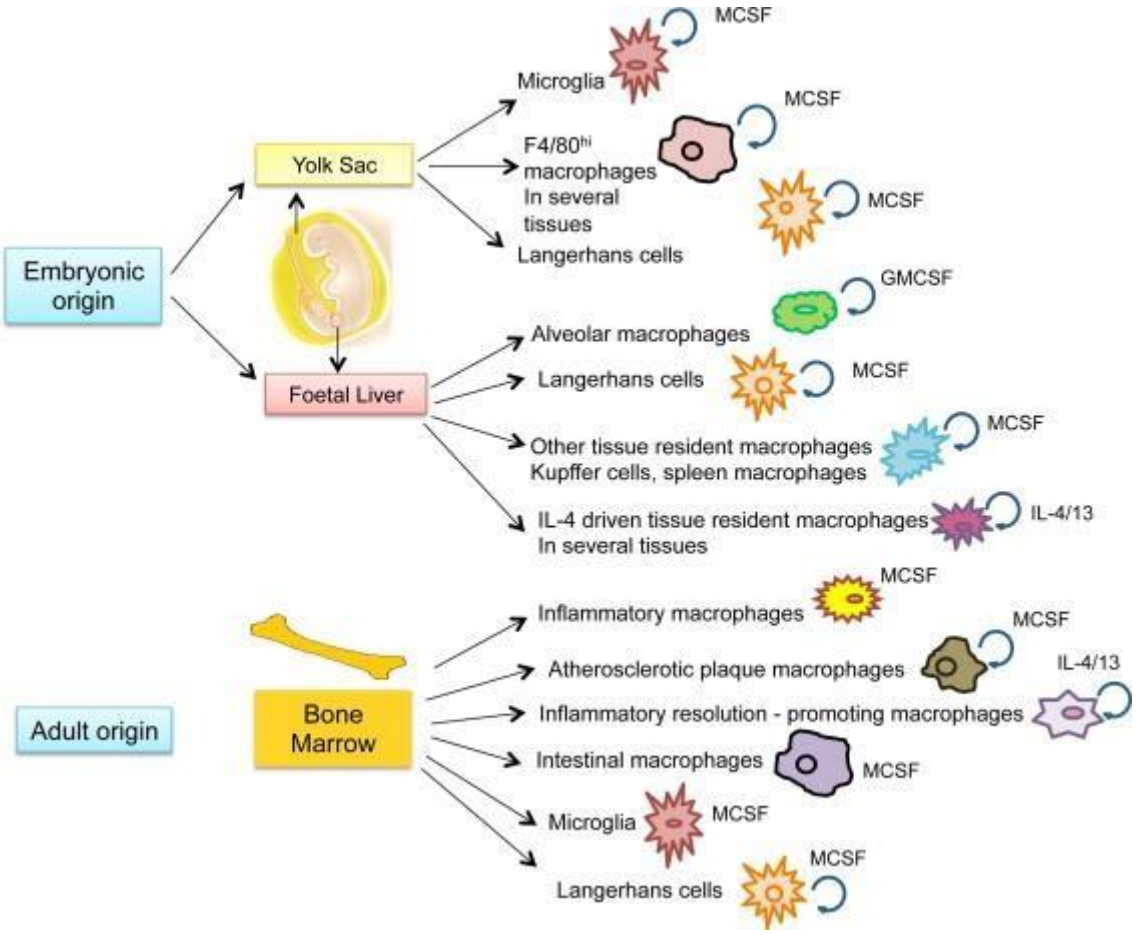


Figure 1.1 Lineages of macrophage development.

Fejer et al., 2015, outlines a summary of differentiated cell subsets that originate from either the yolk sac or foetal liver i.e., embryonic origin or alternatively, the bone marrow i.e., adult origin. Cells from embryonic origins are tissue-embedded and can replenish and self-renew upon depletion as opposed to the adult originated macrophages derived from differentiated monocytes in the blood stream.

When challenged by infections, there is a constant proliferation of mature macrophages irrespective of function. As a result, macrophages have the most expression of pattern recognition receptors (PRR) on their cell surface which has variation dependant on macrophage tissue location (Aderam, 2001).

Aforementioned, macrophages have the ability to change their functional properties, known as "polarization", in response to the type of infection or stimulus they encounter. Two main types of macrophage polarization have been described: M1 and M2 macrophages (Das et al., 2015).

M1 macrophages, also known as "classically activated" macrophages, are activated by pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) (Shapouri-Moghaddam et al., 2018). They produce high levels of reactive oxygen and nitrogen species and secrete pro-inflammatory cytokines such as TNF-α, Interleukin 1β (IL-1β), and Interleukin-6 (IL-6) (Shapouri-Moghaddam et al., 2018). Intracellular pathogens such as *Mycobacterium avium* are thought to induce the M1 macrophage phenotype which promote the killing of the bacteria, stimulate the formation of granulomas, and activate the adaptive immune system to produce antibodies (Xu et al., 1994).

On the other hand, M2 macrophages, also known as "alternatively activated" macrophages, are activated by anti-inflammatory cytokines such as IL-4 and IL-13 (Murray et al., 2014). They produce high levels of anti-inflammatory cytokines such as IL-10 and TGF- β and promote tissue repair and remodelling. M2 macrophages can also promote the resolution of inflammation and the healing of tissue damage caused by the infection (Murray et al., 2014).

1.1.1 Alveolar Macrophages

Alveolar macrophages (AMs) are an example of a macrophage of embryonic origin and derived from the foetal liver. They are specifically found in the alveolar space and are responsible for protection against respiratory pathogens that infiltrate that specific location (Fejer et al., 2015) (Figure 1).

In the respiratory cavity, AM is the resident macrophage of the lung, continuously exposed to airflow in the alveolar lumen. As a consequence, it is one of the first cells to encounter pathogens that are not cleared by primary epithelial defences such as mucus and cilia (Rubins, 2003). Phenotypically, AMs are round and fairly adherent cells that have a variety of specialised markers on its surface, including high levels of CD206 that recognise microbial carbohydrates and scavenger receptors SR-A and MARCO for particle clearance. Scavenger receptors allow alveolar macrophages to bind to non-opsonized targets and phagocytose them (Byrne et al., 2015; Allard

et al., 2018). Studies investigating mice lacking SR-A and MARCO, observed a high susceptibility to infection from respiratory pathogens (Jozefowski et al., 2005) . Macrophages populate the lung at stages of embryogenesis, and give rise to self-renewing AM populations, although it is theorised that circulating monocytes from haematopoietic origins also contribute to this later in life (Fejer et al., 2013). Recruited monocytes stimulated with granulocyte-macrophage-colony-stimulating factor (GM-CSF), secreted by surrounding alveolar cells, are transformed into AMs (Trapnell and Whitsett, 2002; Fejer et al., 2015). Studies have demonstrated, where there is a lack of GM-CSF, it leads to poor regulation of surfactant clearance and the prevention of AM maturation (Shi et al., 2006). GM-CSF is believed to be the factor that leads to AM specialisation from monocyte precursors and contributes to the maintenance of mature AM function (Trapnell and Whitsett, 2002).

AMs are more specialised than other subsets of macrophages in order to deal with invading respiratory pathogens (Byrne et al., 2015). AMs highly express TLR-2 receptors that recognise respiratory pathogens such as *Mycobacteria* by their ligands that include lipoproteins, lipomannan and several heat shock proteins (Glassroth, 2008; Byrne et al., 2015).

1.1.2 Current cell models of respiratory infection

In the study of respiratory infections, primary alveolar macrophages are the model of choice. However, the collection and *in vitro* use of these AMs has both advantages and limitations. Primary AMs from donors retain their morphological and functional characteristics from tissue origin (Wnorowski et al., 2019). This means all markers and surface proteins are preserved and the cells, when challenged, would mimic a natural infection as close as possible (Sweich et al. 2012). Additionally, animal models can be discontinued in the presence of primary AMs, thus avoiding immune response variation due to inter-species differences (Sweich et al., 2012). Furthermore, results using primary AMs can be more translatable to humans than cell lines such as human THP-1 and U937, as well as BMDM and peritoneal macrophages derived from primarily BALB/c or

C57B/6 mice, which lack key morphological and functional aspects (Parish, 2020; Andrejak et al., 2015; Gaidt et al., 2018; Bosshart and Heinzelmann, 2016). However, the limitations outweigh the advantages and highlight the urgent need to keep developing an optimal cell line for respiratory infections. Primary cells are not immortal and limited in their self-replenishment (Wnorowski et al., 2019). Viability of primary cells are not sustained through passages, and they are known to change cell characteristics and should be used as early as possible (Fejer et al., 2013; Fejer et al., 2015). Thus, maintaining them in culture for long periods of time is ill-advised and the limited number of primary AM's obtained, means that larger scale experimentation is problematic. There are physiological donor factors such as age and genetics that could produce variations in primary cell response even under the same conditions (Wnorowski et al., 2019). Additionally, primary cells may not originate from the same donor, so their source is also limited. Due to their sensitivity, special culture conditions are required such as extra growth factors and additives in the media to maintain healthy populations (Wnorowski et al., 2019).

Murine bone marrow derived macrophages (BMDM) were the preferred model of study over primary AMs. It overcame aforementioned limitations and were sourced from bone marrow cells and stimulated in the presence of MCSF. They can be acquired in high yields; freezer stored and can be obtained from genetically differentiated mice (Zajd et al., 2020). They are highly proliferative and lean towards the M1 polarity and are widely used in studies that investigate inflammatory signalling pathways stimulated by pathogens (Zajd et al., 2020; Fejer et al., 2015). BMDM were also favoured for producing a responsive and highly homogenous population of macrophages that could be activated in-vitro in the presence of M-CSF (Fejer et al., 2015). However, these might not be the most suitable representative of recruited or self-renewing macrophage behaviour *in situ*. BMDMs tend to only represent a particular subset of macrophages that are M-CSF driven as opposed to AM's which thrive in the presence of GM-CSF (Figure 1) therefore cell surface marker variation might exist as demonstrated in a study that showed BMDM had a highly expressed F4/80 and CD11b markers compared to alveolar macrophages which expressed them in

relatively low amounts (Warren and Vogul, 1985; Rampacci et ak., 2020). Researchers attempt to also isolate peritoneal macrophages which are easily harvested from the peritoneal mouse cavity and are non-manipulated macrophages. However, these are not available in sufficient yields which is rectified by the injection of differentiation agents such as Brewer's thioglycolate broth or proteose peptone, which increase migration of monocytes to the peritoneum and are able to increase harvested yield (Zajd et al., 2020). However, along with the high yield obtained brings doubt whether the physiology of the macrophages have undergone alteration due to the presence of the growth agents. Additionally, peritoneal macrophages also appeared to have highly expressed F4/80 and CD11b markers compared to alveolar macrophages (Warren and Vogul, 1985).

1.1.3 Max Planck Institute cells

Importantly, a GM-CSF dependant and self-renewing murine alveolar macrophage cell line called Max Planck Institute cells were created from embryos and were found to replicate tissue-resident macrophage characteristics of alveolar macrophages (Fejer et al., 2013). MPIs are obtained from foetal mouse liver and cultured in the presence of GM-CSF for approximately 6-8 weeks. At this time point, rounded cells form, phenotypically representative of AMs. MPIs are entirely dependent on supplement by GM-CSF and thrive up to 100 weekly passages in its presence (Fejer et al., 2013). Lack of GM-CSF or substitution with MCSF results in reduction of viable cell growth and slow cell growth without morphological changes respectively. Functionally, MPIs were more sensitive than BMDMs to stimulation with TLR-4 dependant LPS and TLR-2 dependant fibro-blast stimulated lipopeptide-1 (FSL-1). This was shown with higher levels of IL-6 and TNF-α produced as a result (Fejer et al., 2015). Fejer et al. (2013), demonstrated the variation in cytokine profiles produced between the widely used BMDM model compared to MPIs, thus highlighting that they are functionally distinct macrophage types. MPIs can be acquired in unlimited amounts and used to study behaviour of pathogens and drug sensitivity. This is especially true in the case of respiratory bacteria such as *Mycobacterium tuberculosis* and more recently, *Mycobacterium abscessus* (MAB)

(Kelly et al., 2022; Woo et al., 2018). In contrast to immortalised cell lines that originate from tumours, and their genetic instability can cause possible phenotypic variations, MPIs offer an inclusive and continuous alveolar lung macrophage model to study host-pathogen interactions without phenotypic changes, as they are non-transformed. MPIs have shown a sensitivity to selected microbial agents that have not previously been seen in other mononuclear phagocyte responses (Fejer et al., 2015). Aforementioned, there was a weak expression of F4/80 on alveolar macrophages in a previous study that were also seen in MPI cells in the described study (Zajd et al., 2020; Fejer et al., 2015). As mentioned previously, scavenger receptors such as MARCO are seen as functionally crucial for human alveolar macrophages to phagocytose unopsonized bacterial targets and as such, a genetic indicator of MARCO is the presence of chitinase 3-like 3 (Sulahian et al., 2008; Fejer et al., 2015). Under mRNA analysis, high levels of chitinase 3-like 3 which is a marker of alternatively activated macrophages was only expressed in MPI cells and not in BMDMs. Additionally, the presence of several other surface markers such as CD11c and low CD14 in MPIs further highlighted the functional and phenotypic similarity between MPIs and lung AMs (Fejer et al., 2015). In summary, although studies have investigated receptor and cytokine pathways in isolated primary cells, they are limited in number, often tainted and difficult to purify. Additionally, large numbers of donors are required to obtain cells in sufficient quantities. MPIs overcome these major limitations especially, the ease of maintaining culture for up to 2 years.

MPIs might be especially advantageous over other cell lines to study the behaviour of airborne respiratory pathogens as demonstrated by their sensitivity to *M. tuberculosis* (Mtb), adenovirus and mycobacterial TDM. Infection and stimulation with these pathogens induce a significantly prominent pro-inflammatory cytokine profile that lacks IL-10 response which is similar to human alveolar macrophage response as demonstrated by Fejer et al. (2015). Woo et al. (2018) demonstrated that MPIs in the presence of live Mtb engulfed the bacteria and supported its replication within the cell as seen in other primary murine macrophages and cell lines. Additionally, drug activity towards Mtb was demonstrable in MPIs, specifically, cytokines such as TNFα, IL-6, IL-1α, and IL-1β were strongly induced in comparison to heat-killed stimulation. Compounding

evidence highlights the similarity between MPIs and lung alveolar macrophages, and thus MPIs are the chosen cell line for the project experiments described in this thesis. *Mycobacterium avium* responses can be reliably investigated in MPI culture as they have been done with other members of the *Mycobacterium* genus.

1.2 Nontuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are the less renowned species of *Mycobacterium* that populate the environment in bodies of water, soil, and vegetation (McCarthy et al., 2012). These bacteria are free-living and over 170 different species have been identified so far with regular new discoveries being made annually (Falkinham et al., 2003). NTM are characteristically known to grow in relatively low oxygen environments and have cell walls that have a spectrum of morphotypes, giving rise to rough, smooth, and mixed colonies (McCarthy et al., 2012; Glassroth, 2008). Unlike a lot of other bacteria, NTMs are slow growers and have a doubling time of up to 3 days. Culturing them in media is a fairly laborious process and does not guarantee its growth. NTM are hardy and resistant to antibiotics and disinfectant. As a result, infections are often very difficult to detect, diagnose and treat (Griffith et al., 2007). NTMs are capable of forming biofilms due to their lipid rich cell walls which means they can be aerosolised in water droplets from shower heads (Griffith et al., 2007). Studies across the globe in continents such as North America, Europe and Asia have seen a drastic increase in NTM associated pulmonary disease (Cassidy et al., 2009). Over the last 20 years the incidence of disease has increased 7-fold since the 1980s in the United States. Similar trends were observed in the United Kingdom which has seen a 3-fold increase in cases since 1995 (Ratnatunga etl., 2020). Generally, this was mimicked by countries such as Denmark and Germany. The primary dominator of NTM disease appeared to be MAC (Levy et al., 2008). In particular, sputum collected from patients with cystic fibrosis were quoted to have ~10,000-fold higher NTM colonisation in comparison to the general population and MAC was the dominantly present species in this colonisation (Levy et al., 2008; Simons et al., 2011).

1.2.1 Mycobacterium avium complex (MAC)

Out of the species formally identified, *Mycobacterium avium* complex (MAC) is noted to be the primary disease-causing agent in non-tuberculous pulmonary infections (Simons et al., 2011; To et al., 2020). NTM do not cause tuberculosis or disease in healthy people as is the case of Mtb infection, rather, it causes chronic lung disease and disseminated infections in individuals with compromised immune systems (Sexton and Harrison et al., 2008; Doucette and Fishman, 2004; Chen et al., 2012; Johnson and Odell., 2014). This is especially the case in immunocompromised individuals that have pre-existing risk factors such as cystic fibrosis, chronic obstructive pulmonary disease, generally weak immune systems, transplant patients and patients undergoing corticosteroid treatment (Griffith et al., 2007; Cassidy et al., 2009; Hojo et al., 2012) . MAC lung disease is known to present itself in two main forms which is the nodular bronchiectasis type and the fibro cavitary type (Simons et al., 2011; Prevots et al., 2017;Ratnatunga et al., 2020). Along with a number of other species, MAC is identified in 80% of global clinical settings. These bacteria are opportunistic in the way they infect as they essentially cohabitate with humans in the water systems and colonise shower heads as well as potting soil and carbon rich land (Falkinham et al., 2020).

MAC was primarily isolated as two individual species, *M. avium* and *M. intracellularae,* rough and smooth respectively (Claeys and Robinson et al., 2018). However, as the number of species discovered grew, it was defined as a complex with *M. avium*, *M. intracellularae* and *M. chimaera*, which were a substantial part of human infection (Boyle et al., 2015). *M. avium* have been successfully isolated from water, whereas *M. intracellularae* was more often found in soil sources. They were found to be resistant to chlorine, chloramines, and ozone with up to 100-fold more resistance to chlorine than other bacteria such as Escherichia coli (Falkinham et al., 2020; Taylor et al., 2000). These factors all contribute to its unparalleled growth in distribution systems. In a

study of 20,182 patients, spanning 30 countries and six continents, MAC accounted for 71% of isolates from Australia and 31% from South America (Hoefsloot., 2013).

1.2.2 Macrophages and NTM interaction

The pathophysiology of non-tuberculous mycobacteria (NTM) lung disease is not fully understood, but it is thought to involve a complex interplay between the bacterium and the host's immune response (Wu et al., 2018). In the case of *Mycobacterium avium* infection, the balance between the M1 and M2 macrophages seems to be important for the outcome of the disease (Wang et al., 2020). Studies have shown that in patients with *Mycobacterium avium* lung disease, there is an imbalance in the ratio of M1 and M2 macrophages, with a shift towards M2 macrophages (Wang et al., 2020). This is thought to contribute to the persistence of the infection, as M2 macrophages may not be as effective at killing the bacteria and promoting the formation of granulomas (Kim et al., 2022).

Similar to tuberculosis, pulmonary complications are associated with NTM (Stout et al., 2016). Both innate and adaptive branches of the immune response are recruited to combat NTM associated disease (Shin et al., 2008). Ciliary function from the epithelial cells lining the airways control the colonization of NTM. However, when the pathogen is able to surpass these physical host barriers, innate cellular defences are alerted and cells such as macrophages are instructed to phagocytose and carry out bacterial killing, as well as present antigens to activate adaptive immune responses (Sampaio., 2008). Multiple TLRs are involved in the response and TLR's 2, 4 and 9 have significant roles in the contribution towards an efficient innate immune response. TLR2 recognizes the cell wall components of *Mycobacterium*, such as lipoproteins, lipoglycans, and peptidoglycans, which

help to initiate the inflammatory response to the infection (Weiss and Schaible., 2015; Shin et al., 2008).

Upon interaction between mycobacterial components and macrophages, the mitogen-activated protein kinase pathway is kick-started which drives pro-inflammatory response and promotes anti-mycobacterial activity (Prasla et al., 2020). NF-κB is a key regulator downstream of these signalling pathways and is translocated to the nucleus in this process and ensures cell survival, cell proliferation, and upregulates transcription of key pro-inflammatory cytokines such as IL-6 (Prasla et al., 2020; Sampaio et al., 2008).

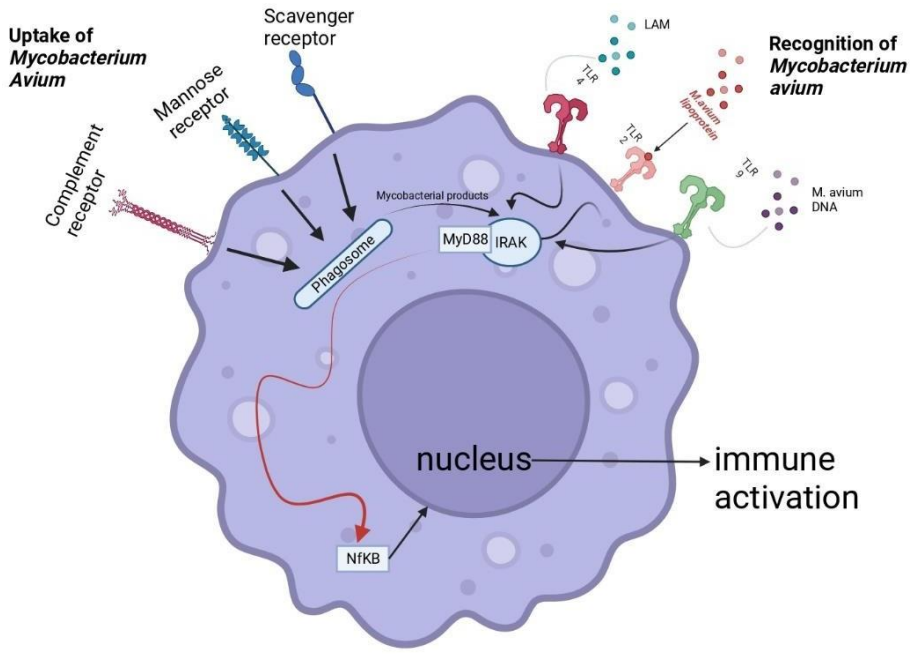
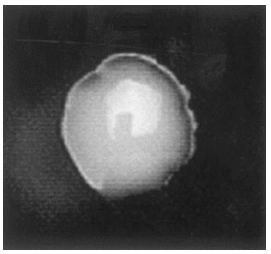
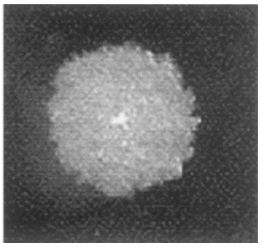


Figure 1.2 *Mycobacteria* Immune recognition and uptake

Overview of immune response from alveolar macrophage towards *Mycobacteria* shows the uptake of mycobacteria from complement receptors, mannose receptors, and scavenger receptors with TLR-4, 2 and 9 which recognise PAMPs from MAC such as LAM, lipoprotein and DNA. The downstream signalling pathways of MyD88 and NfkB are activated with subsequently result in immune activation (Biorender., 2023).

1.2.3 NTM colony morphology and host interaction

Toll like receptor 2 has been shown to be over-stimulated in the presence of rough mycobacterium. When plated on agar, MAC colonies can be classed predominantly into three types: smooth opaque, smooth transparent and rough (Sarmento and Appelberg 1995; Kansal et al., 1998). This classification has roots in the variation of Glycopeptidolipids (GPLs) on the surface of cell wall (Reddy et al., 1996; Schorey and Sweet., 2008). Genetic analysis of DNA in various MAC strain identified DNA deletions that would lead to lack of GPL in rough MAC strains (Belisle et al., 1993). In the case of the related NTM *Mycobacterium abscessus*, the lack of GPL was compensated for by the increased number of lipoproteins that resulted in an overactive TLR-2 pathway (Kim et al., 2019; Jonsson et al., 2013; Catherinot., 2007; Rhoades et al., 2009). Therefore, the rough morphotype of MAB was observed to produce a more robust and acute pro-inflammatory response as seen in significantly high intracellular CFU numbers and pro-inflammatory cytokine levels compared to the smooth strain (Kelly et al., 2022; Catherinot., 2007). The present study investigates two observable morphologies of smooth *M. intracellularae* and rough *M. avium*. These variations are thought to exist due to the presence or lack thereof, of GPLs on the surface of MAC strains which could possibly correlate to the morphotypes of some strains being more virulent over others (Nishimura et al., 2020). There exists a spectrum of GPL variation between species that can predetermine the inhibition of intracellular killing of *M. avium* as evidenced by early literature that shows the smooth opaque type to be more virulent and invasive than both the smooth transparent and rough colonies (Reddy et al., 1996; Torrelles et al., 2002; Brambilla et al., 2016; DePas et al.,

Smooth	Rough
Glossy, opaque, domed, convex, scalloped, small 	Dry, opaque, wrinkled, flat, scalloped, small 

2019). **Table 1:** Images of single colonies, grown on 7H9 agar plates, showing contrasting qualitative differences of morphology in rough and smooth mycobacteria avium strains (Torrelles et al., 2002)

Studies have also uncovered that the rough morphotype leads to granulomatous formations due to the clustering effect it causes in immune cells. The rough morphotype has another tendency to clump itself and this is considered to be a virulence factor (Schorey and Sweet ., 2008). In a study that investigated the potential of infection severity between filtered and non-filtered rough colonies of mycobacteria, it was highlighted that the cytotoxicity and bacterial burden of macrophages were high in the unfiltered cohort than the filtered one (Nishimura et al., 2020).

1.2.4 The role of cytokines in MAC infection

Cytokines are produced by immune cells, such as macrophages, T cells and dendritic cells, and they play a crucial role in the body's immune response to infection. In the case of NTM infections, the initial response of the immune system is to produce pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, which activate immune cells and recruit more immune cells to the site of infection (Early et al., 2011; Carpenito et al., 2019).

During an infection with *Mycobacterium avium*, macrophages are activated and produce a pro-inflammatory cytokine reaction that involves the production of the cytokine IL-6, and the resulting inflammatory response involves and activates other immune cells to the site of infection (Cooper et al., 2011; Kim et al., 2014).

However, the relationship between IL-6 and *Mycobacterium avium* infection is not straightforward. Studies have shown that elevated levels of IL-6 in the blood of patients with *Mycobacterium avium* lung infections are associated with more severe disease and a poorer prognosis. This suggests that excessive production of IL-6 may contribute to the chronicity of the infection by promoting the formation of large, necrotic granulomas which are ineffective in containing the infection (Kwon et al., 2007; Orne et al., 2014; Singh and Goyal et al., 2013).

On the other hand, studies also have showed that some patients with *Mycobacterium avium* lung disease have an impaired response to IL-6 and other cytokines. This suggests that the IL-6 and other cytokine response may be important in controlling the infection and that an impaired response may contribute to the persistence of the infection (Beltan et al., 2000; Shiratsuchi et al., 1991; Newman et al., 1991)).

Overall, the role of IL-6 in *Mycobacterium avium* infection is complex, and more research is needed to fully understand how it contributes to the immune response to this type of infection. However, it is clear that IL-6 plays an important role in the host response to Mycobacterium avium and that

understanding the mechanisms that control its production may lead to new therapeutic strategies for treating this infection.

Along with the pro-inflammatory cytokine IL-6, which is studied herein, MAC also induces the production of IL-12 and IFN-γ that regulate intracellular mycobacterial killing (Cooper et al., 2012). However, a ligand called ManLAM which is specific to only select mycobacteria including MAC, interacts with the mannose receptor on the surface of alveolar macrophage cells and dampens the immune response (Shamaei and Mirsaedi., 2021; Sweet et al., 2010; Schorey and Sweet et al., 2008). The binding of the mannose receptor and ManLAM secretes IL-10 that is anti-inflammatory and immune suppressive (Hussain et al., 2016). IL-10 downregulates IL-12 production, thus downregulating intracellular killing, and possibly by extension ,the serum IL-6 levels (Higgins et al., 2009; Hussain et al., 2016). As intracellular killing is downregulated, MAC is able to thrive within the macrophage and sustain a chronic infection within the host which eventually damages lung tissue. These chronic infections can engage with the host for months or years and it is attributed to the persistence of NTM within the macrophage as a mechanism of latency which could cause reinfection in the future.

1.2.5 Review of MAC infection models

There have been several animal models developed to study *Mycobacterium avium* infections. These models have helped researchers understand the pathogenesis of the disease and evaluate new treatments. One model used is the intratracheal instillation of *Mycobacterium avium* into beige mice and ferret lungs , which replicate features of human lung disease like chronic infection, granuloma formation and lung tissue damage (Cynamon and DeStefano., 1999; Buddle et al., 2000). This model is particularly usefμl for studying the host-pathogen interactions and the immunology of the disease. Another model is the use of chickens, which can develop chronic infections with *Mycobacterium avium* that resemble human disease (Long et al., 2000). This model has been used to study the host immune response to the infection, and to evaluate new treatment strategies.

Finally, the subcutaneous needle point injection of *Mycobacterium avium* in the BALB/c mouse footpad has been used to mimic the subcutaneous nodular form of the disease (Gonzalez-Perez et al., 2013). These models are not perfect representations of the human disease as it is multifactorial, but they can be helpfµl in understanding the host-pathogen interactions, evaluating new treatment strategies, and identifying potential targets for drug development.

It is important to note that whilst animal models are used to study the underlying mechanisms of the disease and to test the effectiveness of new treatments before they can be tested in humans, the models can be difficult to manipulate, costly and the reproducibility of in-vivo experiments are questionable (Shi et al., 2019). As *in vivo* models present an overall systemic effect of a *Mycobacterium avium* infection, there is a need for AM-like *in vitro* models to isolate the responses of key cell types such as macrophages and specifically analyse the intracellular behaviour within these macrophages exclusively.

Gentamycin protection assays are used to investigate the impacts of intracellularly residing bacteria and how they persist in *in-vitro* macrophage cell models (Swenson et al., 1990). This assay uses the mechanism of clearing the extracellular environment of bacteria, using gentamycin (or similar) antibiotics, to isolate any reactions and chemical substrates such as cytokines produced to be traced back exclusively to intracellular bacterial behaviour (Subashchandrabose and Mobley et al., 2014; Auster et al., 2019; Kuehnel et al., 2001). This assay has been used effectively with other intracellular species such as *Yersinia pestis*, *E. coli* and *Staphylococcus* species (VanCleave et al., 2017; Hamrick et al., 2003; Flannagan et al., 2016). In previous utilisation with these bacterial species, gentamycin protection assays have led to the discovery of intracellular bacterial invasiveness, by allowing bacterial numbers present within infected cells to be tracked over time.

The literature currently lacks a truly AM-like *in vitro* model that can sustain a MAC infection long enough to provide insight into its described persistent and chronic nature. There is an incomplete picture of MAC infection due to the short length of the current experiments within the literature. Addressed herein is the concern of a previous investigators that claimed, *in vitro* cell cultures could

not be sustained for long periods of time due to an increasing intracellular bacterial burden and proportionally, a decreasing viable cell population (Feng et al., 2020; Kilinc et al., 2022). Notably, the same experiments did not use a particular method of extracellular clearance apart from washing cell monolayers, to control growth of MAC which could have extended the life of the cells in culture. Herein, we adapt the principle of the gentamicin protection assay and produce a novel method to model a long-term *M. avium* infection of two different phenotypes (a) smooth *M. intracellularae* and (b) rough *M. avium avium*. The use of MPI cells within this *in vitro* infection system offers the advantage of an easily obtainable and reproducible murine cell line that delivers post-infection responses that are highly representative of primary alveolar macrophages. To maintain viable cells over a period of 18 days, concentration of GM-CSF administered to the cells in culture were optimal to prevent both culture overgrowth as well as maintain healthy cells.

1.3 Rationale and Aims

Within the lung, intracellular invading species such as MAC have the capability to survive inside host immune cells such as lung alveolar macrophages. This mechanism of survival is also the reason why numerous host and therapeutic attempts to eradicate NTM fail. The immune responses orchestrated by AMs in response to MAC, have been studied *in vitro* using cellular models BMMs, peritoneal and THP-1 which provide only short-term insight into MAC intracellular behaviour due to the length of experimentation. Therefore, existing models would be insufficient to study the persistence and long-term infection that is characteristic of MAC. These specific macrophage subsets represent the inflammatory sector of macrophages and are unrepresentative of the widespread immune sequence that involves other alveolar macrophage interactions with mycobacteria. However, the MPI macrophage provides a cellular model that is more representative of the AM phenotype, as previously discussed.

Project aims

i. To establish an *in vitro* chronic infection model of *Mycobacterium avium* in MPI cells.

ii. Investigate the intracellular persistence of 2 different phenotypes, rough and smooth, of *M. avium*

iii. Compare the differences in IL-6 levels and intracellular colony numbers induced by rough and smooth MAC strains

iv. Optimise the infection of MPI macrophages by MAC and investigate whether significant variances in CFUs and cytokine levels exist between antibiotic treated cultures as opposed to washing monolayers alone.

2 Chapter two: Materials and Methods

2.1 MATERIALS

2.1.1 Cells

2.1.1.1 MPI cells

MPI cells are dependent on GM-CSF and are derived from cell lines established from liver tissue of C57BL/6 (BL6) mouse foetuses between the gestation period of 15- to 19- days (Fejer *et al., 2013)*. For the purposes of this project, the cells were passaged for up to 25 times and fresh stocks were retrieved from liquid nitrogen thereafter.

2.1.2 Bacterial Strains

2.1.2.1 *M. avium* strains

All *M. avium* strains were provided by public health England. *M. avium* (NCTC 13034)and *M. intracellularae* (NCTC 10682) are both reference strains from the National Collection of Type Cultures (NCTC). *M. avium* are Advisory Committee on Dangerous Pathogens (ACDP) hazard group 2 organisms and were handled with the appropriate risk assessments in place.

2.1.3 Media and Supplements

2.1.3.1 Cell Culture media

Table 2.1 cell culture media and manufacturers

Product	Supplier
RPMI 1640	Lonza
DMEM high glucose	Lonza

2.1.3.2 Cell culture supplements

Table 2.2 cell culture supplements and manufacturers

Product	Supplier
Fetal Bovine Serum (FBS)	SLS
L-glutamine 200mM	Lonza
Penicillin 10,000U/mL-Streptomycin 10,000µg/mL	Lonza
HEPES Buffer 1M	Lonza

2.1.3.3 Microbiology Media

Table 2.3 Microbiology media and manufacturers

Product	Supplier
Middlebrook 7H9 Broth Base	Sigma-Aldrich
Middlebrook 7H11 Agar Base	Sigma-Aldrich
Tryptic Soy Broth (TSB)	Sigma-Aldrich
Mueller-Hinton Broth 2, Cation-adjusted (MHB II)	Sigma-Aldrich
Agar powder	Sigma-Aldrich

2.1.3.4 M. avium microbiology supplements

Table 2.4 Media supplements and manufacturers

Product	Supplier
OADC/ADC components:	
Oleic acid ≥99%	Sigma-Aldrich
Sodium Chloride	Fisher S
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Dextrose	Sigma-Aldrich
Catalase from bovine liver	Sigma-Aldrich
Glycerol	Alfa Aesar
Tyloxapol	Sigma-Aldrich

2.1.3.5 OADC/ADC recipes

Mycobacterium avium needs OADC/ADC supplementation in solid and liquid media respectively, to grow and achieve log phase for experimental harvesting. Autoclaved deionised water was used to mix the OADC/ADC and the resulting mixture was stored for upto 6 months at 4 degrees.

Table 2.5 OADC/ADC recipes

Product	Amount per 1L
Oleic acid ≥99% (OADC only)	0.6mL
Sodium Chloride	8.50g
Bovine Serum Albumin (BSA)	50.00g
Dextrose	20.00g
Catalase from bovine liver	0.03g

2.1.4 Reagents

Table 2.6: Reagents and Manufacturers

Product	Supplier
Avidin-HRP	Thermo Fisher Scientific
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dulbecco's PBS 0.0095M (PO4) without Ca and Mg	Lonza
MTT reagent (Thiazolyl Blue Tetrazolium Bromide)	Sigma-Aldrich
Paraformaldehyde Solution, 4% in PBS	Thermo Fisher Scientific
Phosphate Buffered Saline (PBS) EDTA	Lonza
Saponin	Sigma-Aldrich
Super AquaBlue ELISA Substrate	Thermo Fisher Scientific
Trypan Blue Solution, 0.4%, Sterile filtered	Sigma-Aldrich
TWEEN® 20	Sigma-Aldrich

2.1.5 Disposables

Table 2.7: Disposables and Manufacturers

Product	Supplier
Syringes: BD Plastipak™ Luer Slip 10mL/20mL/50mL	BD
Microtitre plate: 96-well, round-bottom, polypropylene	Corning
Petri dishes 90mm x 16mm, triple-vented, sterile	Sarstedt
Centrifuge tubes: Conical 50mL	Greiner Bio-One
Syringes: BD Plastipak™ Luer Lock 5mL	BD
Syringe needles 23G	Greiner Bio-One
Syringe Filter 0.45µm	Sarstedt
Syringe Filter 0.2µm	Sarstedt

2.1.6 Equipment

Table 2.8: Equipment and Manufacturers

Product	Supplier
Inverted Microscope AE2000	Motic
FLUOstar Omega Plate Reader	BMG Labtech
Erlenmeyer Flasks: 50mL, narrow-neck	VWR
CO2 Incubator C150	Binder
Class II Biosafety Cabinet MARS 1200	Labogene
Benchtop Centrifuge Multifuge X1R	Thermo Fisher Scientific
End-over-end Rotator	Cole-Parmer
INCU-Line Incubator - Static	VWR
Incubated Shaker INCU-shake MIDI	SciQuip
Pico™ 21 Microcentrifuge	Thermo Fisher Scientific

2.1.7 Recombinant proteins

Table 2.9: Recombinant proteins and Manufacturers

Product	Supplier
Recombinant Murine IL-6	BD Biosciences

2.1.8 Antibodies

Table 2.10: Antibodies and Manufacturers

Product	supplier
Purified rat anti-mouse polyclonal IL-6	Thermo Fisher Scientific
Biotin Conjugated rat anti-mouse monoclonal IL-6	Thermo Fisher Scientific

2.1.9 Antimicrobials

Table 2.11: Antimicrobials and Manufacturers

Product	Supplier
Genatmicin sulfate	Lonza
Kanamycin sulfate, from S. kanamyceticus	Sigma-Aldrich
Amikacin	Sigma-Aldrich

2.2 Methods

2.2.1 Cell culture

Reagents were incubated in water bath to 37 degrees before use, and cells were kept at 37 degrees and 5% CO2 in a humidified atmosphere prior to passage.

2.2.2 MPI cells and BALB/C cells

Max Plank Institute (MPI) cells and BALB/C cells were cultured twice weekly in-vitro in 75cm^2 tissue culture treated flasks with filter in RPMI 1640 with supplementation; 10% v/v FBS, 100U/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 10mM HEPES buffer. During a passage, old media, together were floating cells, were pipetted into a 50ml centrifuge tube. The adherent cells in the flask were washed twice with 7ml of DPBS and the washings were also transferred to the same tube. 5ml PBS-EDTA for detachment was added to the flask and incubated at 37°C for 20 minutes. Afterwards, the flask was gently tapped for absolute detachment and 6ml of DPBS was used to collect the cells. The fluid was pipetted out into the 50ml containing all the washings and centrifuged at 300x*g* for 5 minutes to produce a pellet. Post-centrifugation, the supernatant was discarded, and the pellet was resuspended in 5ml fresh RMPI. Cells are counted and the flask is reseeded at a density of 3x10^5 cells/mL

2.2.2.1 Cell Counting

Cells were counted manually using the hemocytometer. In an Eppendorf, 10µl of cell suspension and 10µl of 0.4% trypan blue were mixed by pipetting thoroughly and 10µl of the mixture was displaced under the coverslip over the glass counting chamber of the hemocytometer. Under the 20x objective lens of the inverted microscope, cells both viable and non-viable were counted. The average of viable cells was taken and multiplied by 2 for the 1:1 dilution with trypan blue. To give a cells/mL figure, the undiluted cell number was multiplied by 10^4. Cell counting was used in both

reseeding flasks for continuous passage and seeding the well-plates with desired cell density for experiments.

2.2.3 Bacterial culture

Both solid and liquid cultures were grown aerobically at 37°C in a static incubator and with continuous shaking at 120 rpm, respectively.

Manufacturer's instructions were followed accordingly and both solid and liquid medium by autoclaving at 121 degrees and 15psi for 15 minutes.

2.2.3.1 *M. avium* complex culture

Required strains were recovered from fridge stocks and grown in 7H9 media supplemented with 10% v/v ADC, 0.5% Glycerol and 0.05% Tyloxapol. Tyloxapol was used to disperses clumps of bacteria which was a phenomenon observed in rough cultures of *Mycobacterium avium* NCTC 13034.

In the making of fridge stocks, desired MAC cultures were recovered from −80°C every 4 weeks. A 1µl loop of bacteria was submerged in a flask of 7H9 broth and left to grow in a shaking incubator. Once the OD reading reached 1.0 the culture was harvested by pipetting it into a screw cap 15ml tube. This tube was then labelled and stored at 4°C for up to 4 weeks. This pre-culture was used to start cultures for future experiments.

Liquid pre-culture was preferred to an agar streak plate in this instance due to the difficulty in emulsifying very dry colonies of rough MAC that could not be disrupted. When Tyloxapol 0.05% was added to liquid pre-cultures, this problem was resolved.

2.2.4 Bacterial growth curve analysis

OD600 of MAC pre-cultures were measured after syringing 10 times and being vigorously vortexed for 1 minute to disrupt any clumps. Complete 7H9 was used to dilute the broth The OD600 was adjusted to 0.005 (± 0.0005). From this culture, 1ml was taken at 24, 48, 72, 96, 120 and 144 hours to determine ODs that corresponded to this time point and then 10-fold microdilutions were made in 96 well plates and CFUs were plated according to the Miles-Misra method. Droplets of 10µl were plated on agar an allowed to incubate for up to 2 weeks. Counts were taken from colonies that had distinct separation for comfortable counting between 10-100 colonies.

Once colonies were grown, OD counts and corresponding CFU counts were plotted on a graph against time to determine exponential phase of growth for bacteria. A linear equation was created which could be applied to future experiments that required a specific OD for a particular CFU/mL.

2.2.5 Bacterial Challenge experiments

MPI media RPMI 1640 media supplemented with: 10% v/vFBS, 2mM L-glutamine, 10mM HEPES buffer and bacterial culture media was pre-warmed to 37 degrees in a water bath before use. The RPMI media used for infection lacked antibiotics which would have otherwise hindered the infection progression.

2.2.5.1 Live *M. avium* challenge of chronic infection and extracellular antibiotic killing assays in MPI and BALB-c cells.

In 96-well culture plates, MPIs were seeded $3.75x10^4$ cells/200µl iRPMI per well and allowed to attach for 1 hour in the plate before bacterial challenge. Once the exponential growth phase was reached, MAC strains were suspended in solution for the desired multiplicity of infection (MOI) which was calculated using the linear equation of enumerated curves. For experiments an MOI range of 5-112 were used. After the bacteria were added, the 96-well plate was incubated for 1 hour for phagocytosis to take place. To remove any extracellular bacteria that had not been phagocytosed, all wells were gently washed by pipetting 100µl RPMI infection media to displace any extra cellular bacteria.

The aforementioned, would be the procedure to observe intracellular MAC behaviour in a natural MAC infection (named 'natural culture' within the thesis). For the antibiotic treated cultures, wells had 200µl of fresh antibiotic supplemented media with amikacin 200 µg/ml ratio in preliminary experiments and kanamycin/gentamycin in a 400/200 µg/ml ratio in following experiments. Post antibiotic treatment, 6 hours, the supernatant was collected in storage plates for further cytokine analysis via ELISA. The empty wells were washed to remove any residual bacteria and antibiotic. The remaining attached cells were lysed with 0.2% saponin solution. From these lysates, 10-fold microdilutions were made and CFU were calculated as previously described. Media was replaced

in other wells and put back into incubation for timepoints desried. Cells were washed, and media replaced at each timepoint.

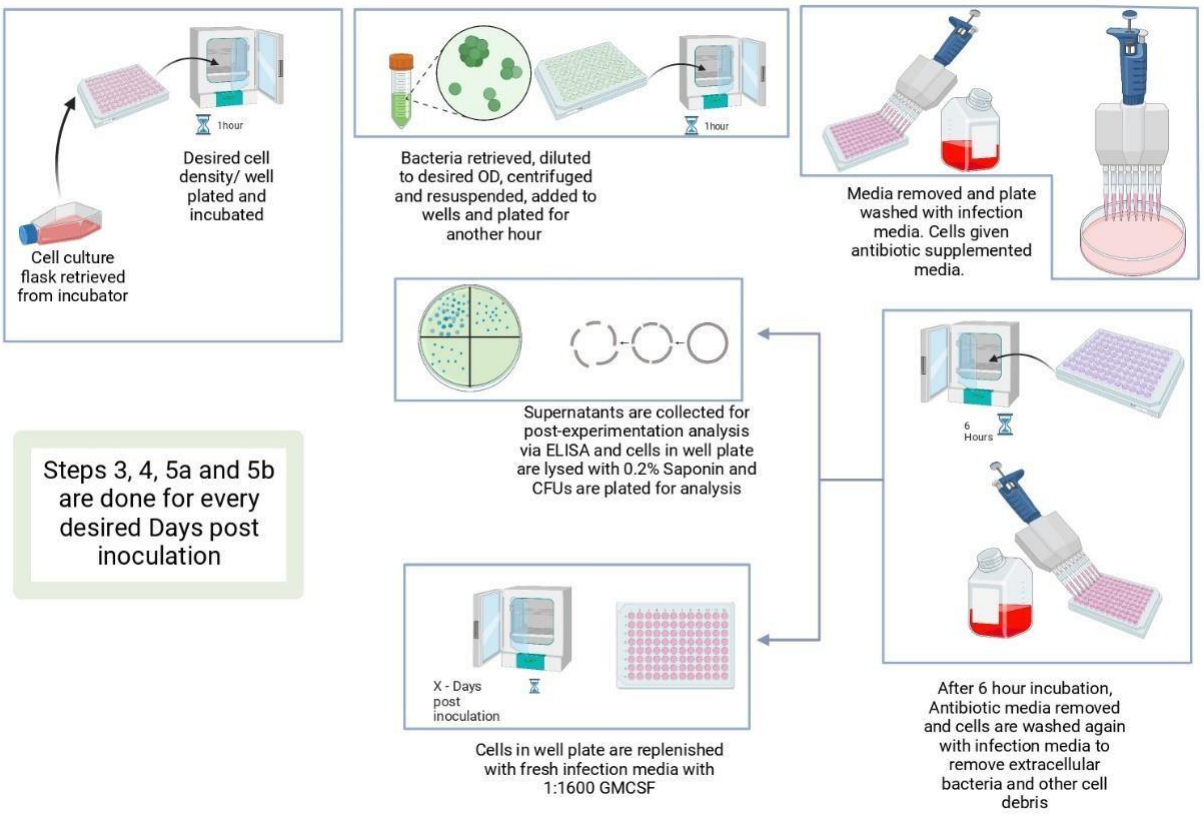


Figure 2.1 Visual representation of live bacterial killing assay

2.2.6 Titration of GM-CSF for modelling chronic infection.

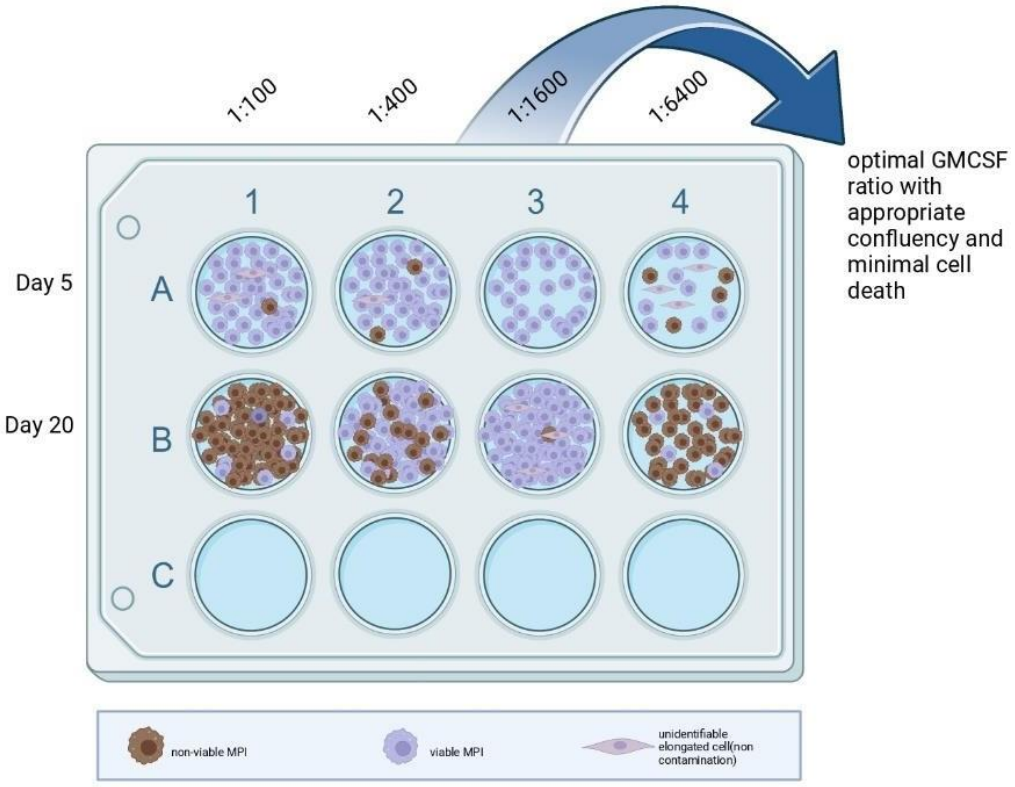


Figure 2.2 Visual representation of the effects of GM-CSF ratios on MPI cells from day 5 to day 20

A GM-CSF titration was done to determine the minimum ratio of GM-CSF to media to keep cells thriving over the duration of 20 days. $3.75x10^4$ cells were seeded into a 96-well plate and observed for the duration of 20 days. Every three days, cells were washed and supplemented with GM-CSF at concentrations of 1:100, 1:400, 1:1600 and 1:6400. A lower concentration of GM-CSF was deemed necessary to slow the growth of MPI's whilst maintaining their viability. The 1:1600 ratio was the concentration at which MPI monolayers were both healthy and appropriately confluent in the well plate.

2.2.7 ELISA

The wells of a high binding 96-well microtiter plate were coated with 50 µl of primary antibody (capture). The plate was then sealed and incubated overnight at 4°C to allow the primary antibody to bind to the well surfaces. The capture antibody was rinsed out by three washes of 0.05% Tween® 20 in DPBS across the wells of interest with a multichannel wash bottle. To prevent non-specific binding of sample components or other antibodies to the well surfaces, the plate was then incubated with a 100µl of 2% BSA in DPBS, for 1h at room temperature. During the incubation period, recombinant protein standards were prepared in 2-fold dilution series from 2000 to 15.625pg/mL and samples were thawed and diluted in iRPMI as necessary. After an hour, the BSA was removed, and the plate was again washed thrice. Then standards, samples and blanks were added in 50µl duplicates of wells. These plates were again incubated overnight for at 4°C. Fluid from wells was again removed and the plate washed three times. 50µl the detection antibody, diluted in PBS was added to all the wells and incubated at room temperature for an hour. Plate was washed thrice and Avidin-HRP was added in 50µl for all the wells for another 30-minute room temperature incubation. After incubation, 50µl of Super Aqua blue substrate was added and plates were left to develop for the necessary time. The plate was read by a Spectrophotometer at OD 405nm and MARS data analysis produced standard curve graphs plus concentration of cytokines in unknown samples.

Table 2.12 ELISA antibody concentrations

Target	Concentration (μg/mL)	
	Capture antibody	Detect antibody
IL-6	2	2

2.2.8 Software

2.2.8.1 Minitab

Data analysis software is used to create graphs and perform Students T-test statistical analysis on technical replicates obtained throughout the duration of the experiment. In addition to this, the software was also used to obtain linear regression equations of Optical Density vs. Colony Forming Units/mL during *Mycobacterium avium complex* growth analysis.

2.2.8.2 MARS Data analysis software v4.01 R2

Data analysis software used to produce standard curves and interpolate unknown values in ELISA.

2.2.8.3 BioRender

Original figure images for this thesis were created with BioRender.com

3.0 Results

3.1 Introduction

There have been many discrepancies with previous macrophage infections involving MAC, one mainly being the methodology which could not guarantee an accurate CFU counts from OD600 measurements which could ensure the desired MOI to be used during challenge experiments were achieved.

MAC has the inclination to form clumps through large aggregations that are not easily disrupted and therefore, the standard methods for enumeration published previously were not suitable. Due to the clumping which is especially noted in rough strains of the species, the method was then adapted to counteract this by vigorous dispersal and addition of compounds such as Tyloxapol to subsidise the tendency to clump.

Aforementioned alveolar macrophages respond to MAC invasion by producing a prolific cytokine response which involves primarily IL-6, known for its pro-inflammatory role. IL-6 is released during the infection cycle and promotes the recruitment of other immune cells which extensively contributes to the adaptive immunity towards MAC.

The tailored enumeration method and the mimicking of chronic infection over a period of 18 days, establishes the behaviour of 2 MAC strains, *M. intracellularae* (M.INT) and *M. avium* (M. AVI) when MPI cells are infected and also highlights MPIs

as a novel model that proves advantageous over currently used in vitro cell models such as BMMs and primary alveolar macrophages.

The data presented in the results section shows the strain differences over a period of 18 days and establishes whether there are any notifiable variations with respect to CFUs and IL-6 production. This data is also likely to be more reflective of the events that occur during live respiratory infection due to the in-vitro use of MPI cells for immune challenge.

3.2 M. avium growth curves

Individual growth curves were needed for all strains as one growth curve could not account for the variable replication rate between them. Growth curves were produced for strains Intracellularae and Avium where the exponential phase was identified between 5 hours and 120 hours so that bacteria were optimally harvested during this window for experimentational purposes. The rate of growth was then calculated using graphically derived linear equation from CFU/mL vs Optical Density plotted graph. The smooth strain, Intracellularae had a faster rate of growth in broth culture than the rough avium strain.

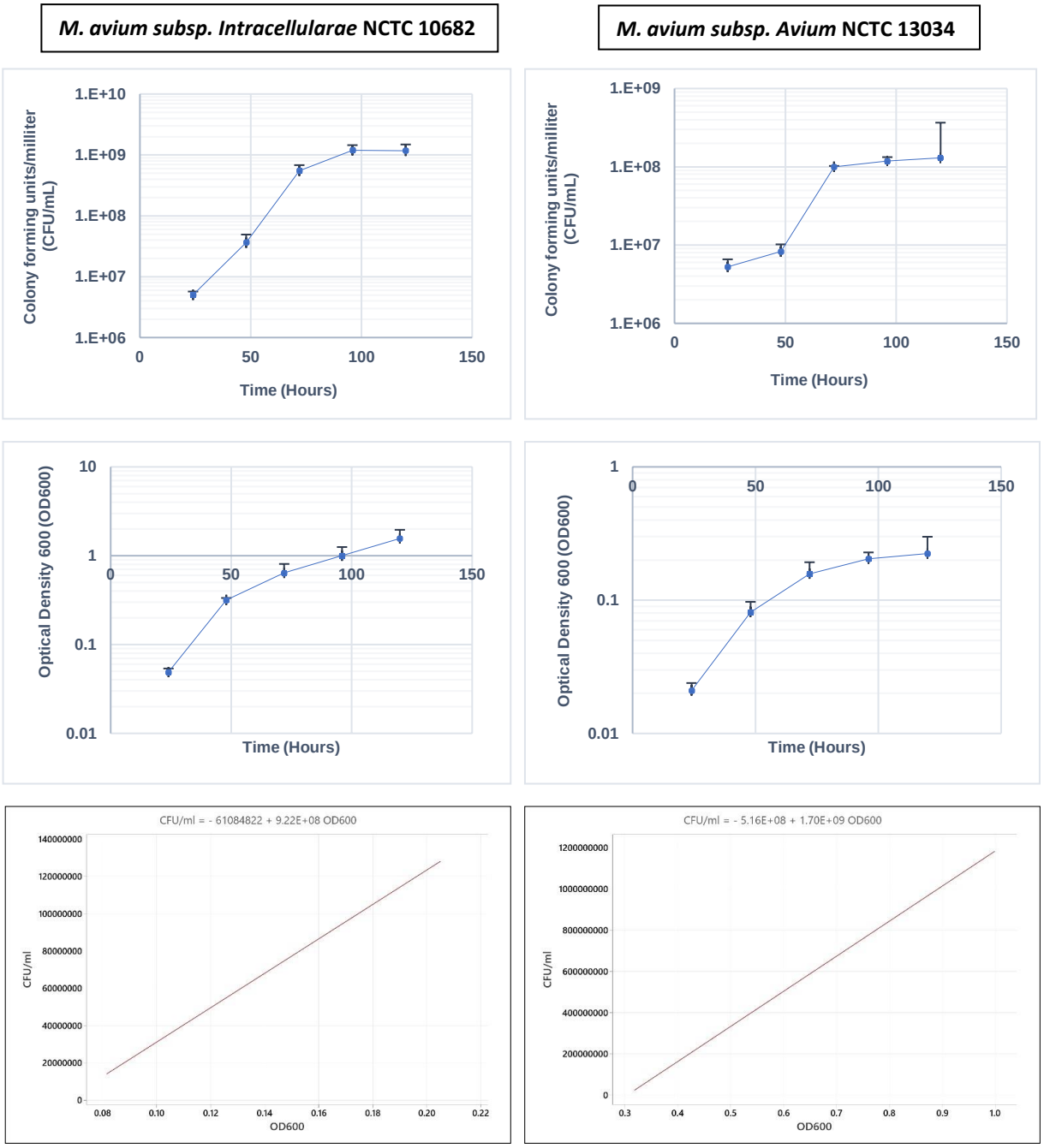


Figure 3.1 Growth curves for 2 Mycobacterium avium strains

Growth curves were produced for MAC pathogens studied: NCTC 10682 (M.INT) and NCTC 13034 (M. AVI). The data were produced by growing cultures from recovered fridge stocks and taking regular interval OD600 measurements with corresponding CFU counts plated and enumerated using the Miles-Misra method mentioned previously. Bacteria were harvested from the exponential phase and then used for any experimentation purposes. Regression analysis performed on growth curve exponential phase to get linear equation to interpolate the required OD600 of bacterial suspension that is needed to achieve the desired MOI for infection experiments.

3.3 Assessing Amikacin efficacy in *M. intracellularae*

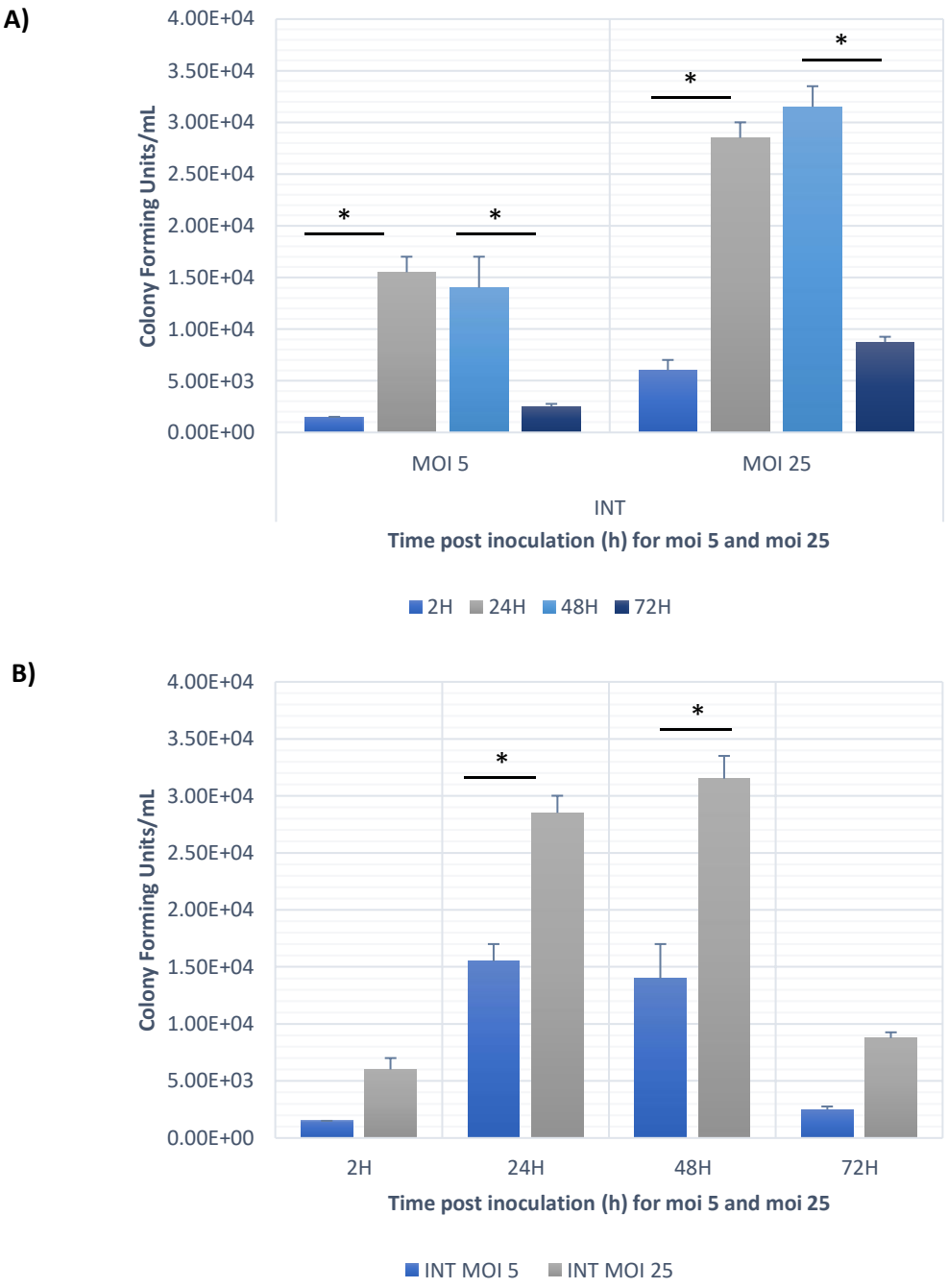


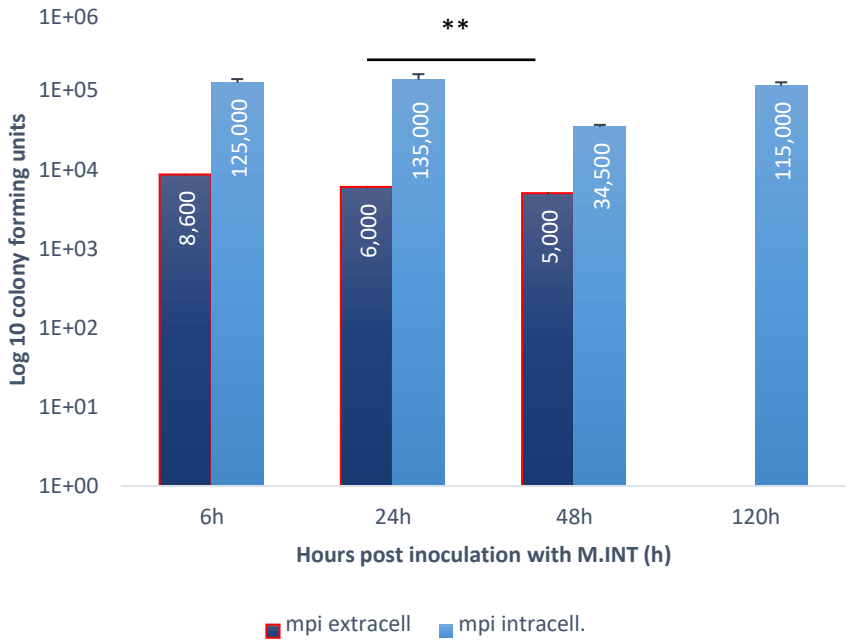
Figure 3.2 Extracellular killing assay using 400µg/ml Amikacin with live NCTCC 10682 and MPI cells, MOI (5) and (25).

MPI macrophages infected with strain *M. intracellularae* (M. INT) NCTC 10682 for MOI (5) and MOI (25). A) highlights comparisons between timepoints within MOI groups and B) portrays timepoint comparisons between MOI groups. 3-day extracellular killing assay with infection spread across 2, 24, 48 and 72 hours. Points represent mean from 2 technical replicates from one experiment specific to the aforementioned MOIs. Two sample Student t-test was used to identify significant differences between strains and sequential timepoints, and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

There is an increase in intracellular M.INT CFUs between timepoints 2H and 24H by a 10-fold increase for MOI 5 and a 5-fold increase for MOI 25. Both increases were significant; MOI 5 (p= 0.047), MOI 25 (p= 0.043). CFU's were maintained at peak levels for 48H and then significantly dropped for both MOI 5 (p= 0.043) and MOI 25 (p=0.042). At 24 hours (p=0.014) and 48 hours (p=0.032), graph B shows significance between MOI 5 and 25 but no significance detected otherwise.

3.4 Comparing *M. intracellularae* and *M. avium* infection in wild type MPI

A)



B)

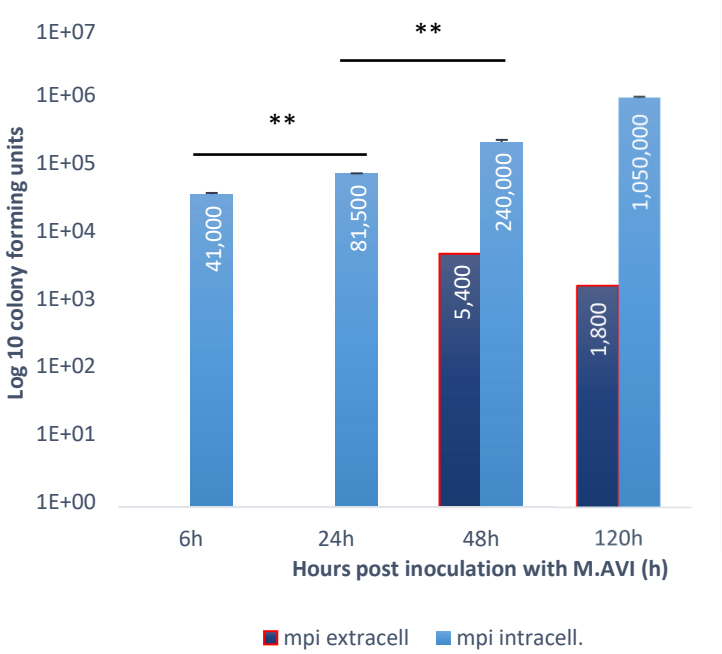


Figure 3.3 Log CFU values for a 5 – day Extracellular killing assay using 400µg/ml kanamycin and 200µg/ml gentamycin (k/g) antibiotic combination, with live M. INT: NCTC 10682 MOI (22) and M. AVI: NCTC 13034 MOI (52) in MPI cells.

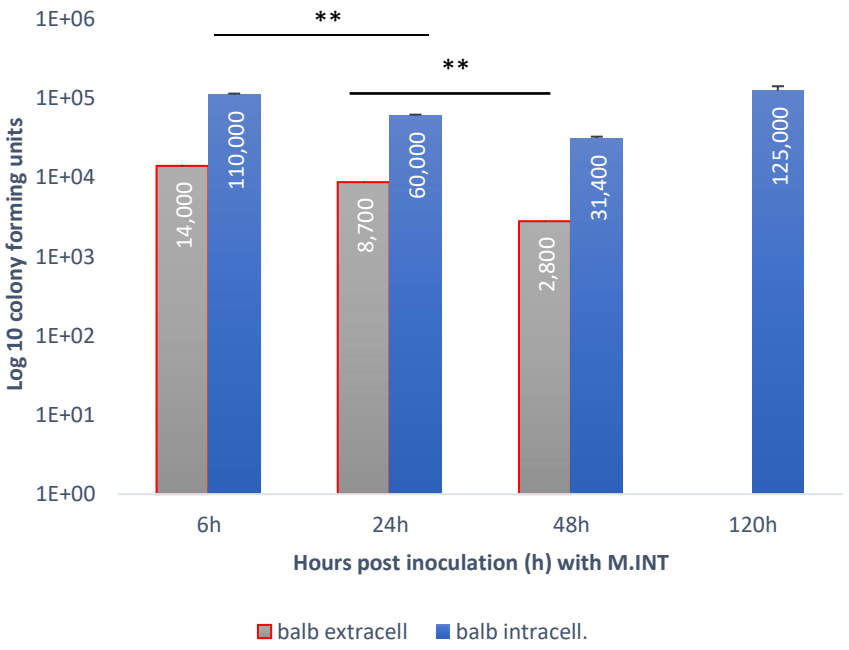
Wild-type MPI macrophages infected with strain A) *M. intracellularae* (M. INT) NCTC 10682 for MOI (22) and B) strain *M. avium* (M.AVI) NCTC 13034 for MOI (52). Graphs highlight comparisons between timepoints within each bacterial group. Data provided for 5-day extracellular killing assay with infection spread across 6, 24, 48 and 120 hours. CFUs were plotted to show the change in bacterial colony number in both the intracellular and extracellular environment post antibiotic treatment for 6-hours. Points represent mean from 3 technical replicates from one experiment specific to the aforementioned MOIs. Two sample Student t-test was used to identify significant differences between sequential timepoints, and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

Whilst both strains had an increase in the uptake of bacteria between 6 and 24 hours, it was only significant for M.AVI (p=0.000). During this time, M.INT had extracellular contamination of bacteria in the supernatant. Between hours 24 and 48, M. INT experienced a 4-fold decrease (p= 0.015) whilst M.AVI had a significant 3-fold increase (p=0.004). The extracellular bacteria for M.INT decreased between the 24 and 48 hours whilst M.AVI experienced a rise. Between 48h and 72h, M.INT and M.AVI experienced a mould growth in the agar growth plate whilst incubation which led to plate disposal and inconclusive results at 72 hours. On the final day of

the experiment, the intracellular numbers of M.INT had increased 3-fold from 48 hours and M.AVI had a 4-fold increase and showed no significant variances.

3.5 Comparing *M. intracellularae* and *M. avium* infection in BALB/c MPI.

A)



B)

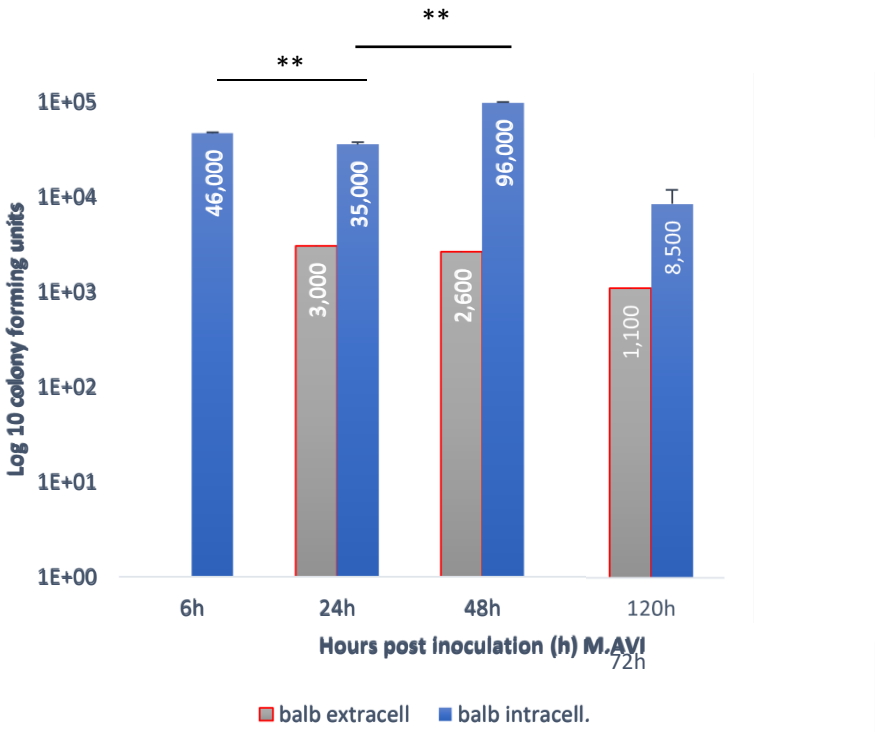


Figure 3.4 Log CFU from 5-day Extracellular killing assay using 400µg/ml kanamycin and 200µg/ml gentamycin (k/g) antibiotic combination, with live M. INT: NCTC 10682 MOI (22) and M. AVI: NCTC 13034 MOI (52) in MPI cells from BALB-C mice

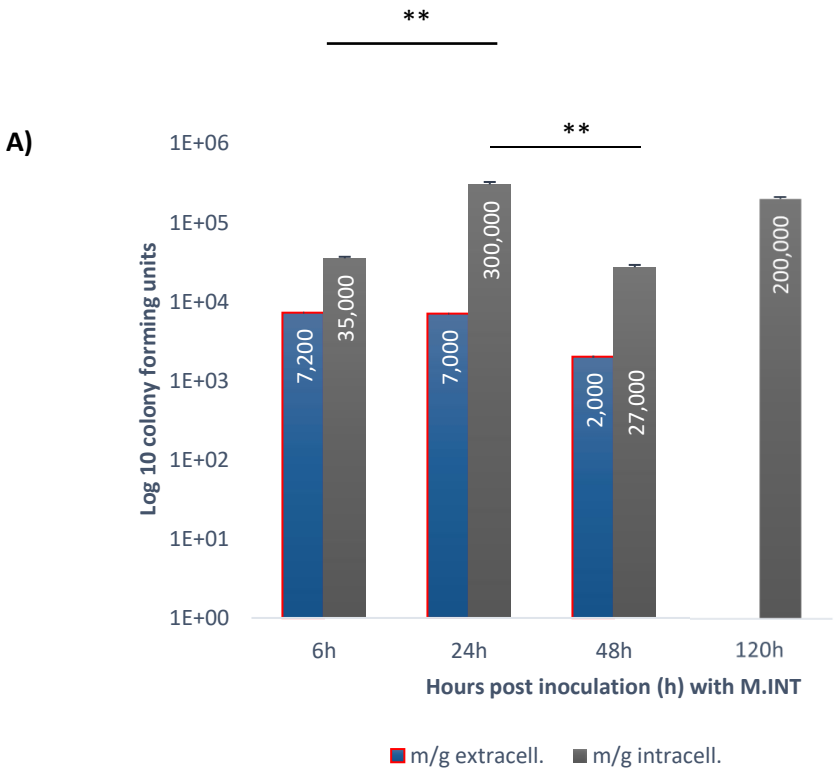
MPIs from BALB-c mice were infected with strain A) *M. intracellularae* (M. INT) NCTC 10682 for MOI (22) and B) BALB-c macrophages infected with strain *M. avium* (M.AVI) NCTC 13034 for MOI (52). Graphs highlight comparisons between timepoints within each bacterial group. Data provided for 5-day extracellular killing assay with infection spread across 6, 24, 48 and 120 hours. CFUs were plotted to show the change in bacterial colony number in both the intracellular and extracellular environment post antibiotic treatment for 6-hours. Points represent mean from 3 technical replicates from one experiment specific to the aforementioned MOIs. Two sample Student t-test was used to identify significant differences between sequential timepoints, and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

Pilot experiments with BALB-c and the rough and smooth strain of MAC were conducted to investigate the effect of the M2 polarity on the behaviour of the bacteria. At 6 hours post anti-biotic treatment. M. INT shows a steady decline in intracellular bacteria from hours 6 – 24 (p=0.001), 24 – 48 (p=0.000). However, at 120 hours there was an 8-fold spike in the number of intracellular bacteria. During this period, extracellular bacteria mimicked the intracellular trend by declining until the final experiment day where none were detected. M.AVI did not display a solid trend in

intracellular numbers and neither in extracellular numbers. M.AVI Intracellular

bacterial CFU fluctuated by significantly decreasing from hours 6-24 (p=0.007), with a 3-fold increase at 48 hours (p=0.000). M.AVI Extracellular bacteria were not present at initially at the uptake phase of 6 hours but rose at 24 hours and declined until 120 hours. Between 48h and 72h, M.INT and M.AVI experienced a mould growth in the agar growth plate whilst incubation which led to plate disposal and inconclusive results at 72 hours.

3.6 Comparing *M. intracellularae* and *M. avium* infection in low GM-CSF ratioed MPIs.



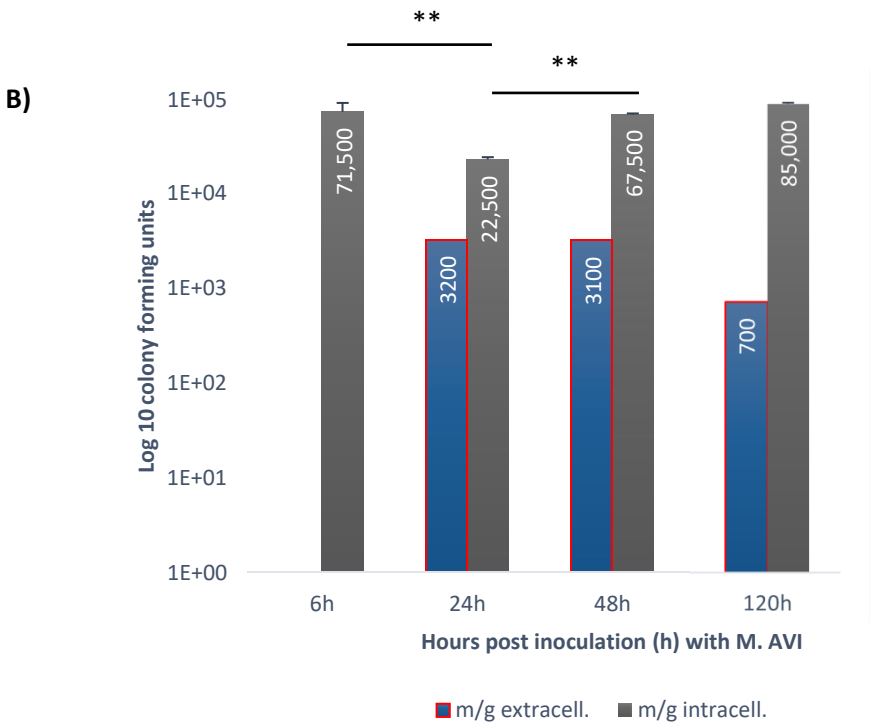


Figure 3.5 Extracellular killing assay using 400µg/ml kanamycin and 200µg/ml gentamycin (k/g) antibiotic combination, with live M. INT: NCTC 10682 MOI (22) and M. AVI: NCTC 13034 MOI (52) in M/G MPI (low GM-CSF MPI culture).

MPI macrophages supplemented with 1:1600 ratio of GM-CSF in media were infected with A) *M. intracellularae* (M. INT) NCTC 10682 for MOI (22) and B) *M. avium* (M.AVI) NCTC 13034 for MOI (52). Graphs highlight comparisons between timepoints within each bacterial group. Data provided for 5-day extracellular killing assay with infection spread across 6, 24, 48 and 120 hours. CFUs were plotted to show the change in bacterial colony number in both the intracellular and extracellular environment post antibiotic treatment for 6-hours. Points represent mean from 3 technical replicates from one experiment specific to the aforementioned MOIs. Two sample Student t-test was used to identify significant differences between sequential timepoints, and P-values were calculated to detect any significance. * = $P<0.05$, ** = $P<0.005$. Groups are non-significant unless explicitly presented on graph.

In order to model the chronic infection that presents in MAC pulmonary disease, pilot experiments using MPI were conducted for both M.INT and M.AVI. In order to maintain healthy viable cells over a period of 18 days in vitro, a lower dilution of GM-CSF was used (1:600) instead of the standard protocol recommended 1:100 ratio. In low GM-CSF culture, M.INT showed inconsistent fluctuations over the experimentation period. In the initial phase, there was a significant rise in intracellular

colonies between 6 and 24 hours for both M.INT (p= 0.001) and M.AVI (p=0.001) and likewise a significant 11-fold decrease at 48 hours (p=0.001). From that point, intracellular colony numbers continued to rise at 120 hours for M.INT. The rough strain M.AVI had a non-significant drop in intracellular numbers between 6 to 24 hours but a significant rise at 48 hours (p= 0.000). The extracellular CFUs for M.AVI peaked at 24 hours and dropped 4-fold towards the end of the experiment at 120 hours. Between 48h and 72h, M.INT and M.AVI experienced a mould growth in the agar growth plate whilst incubation which led to plate disposal and inconclusive results at 72 hours.

3.7 10-day comparison of *M. intracellularae* and *M. avium* infection in low GM-CSF ratioed MPIs.

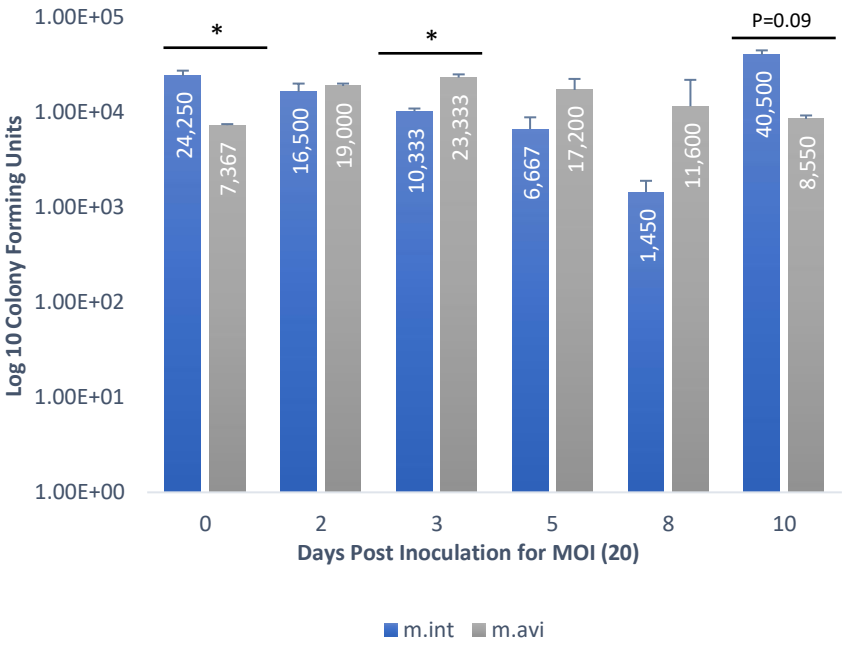


Figure 3.6 Extracellular killing assay using 400µg/ml kanamycin and 200µg/ml gentamycin (k/g) antibiotic combination, with live M. INT: NCTC 10682 MOI (20) and M.AVI: 13034 MOI (20) in low GM-CSF MPI culture for 10 Days.

MPI macrophages supplemented with 1:1600 ratio of GM-CSF in media were infected with *M. intracellularae* (M. INT) NCTC 10682 and *M. avium* (M.AVI) NCTC 13034 for MOI (20). Graphs highlight comparisons at given timepoints between each strain for sequential timepoints. Data provided for 10-day extracellular killing assay with infection spread across days 0, 2, 3, 5, 8 and 10. CFU's obtained by lysing MPIs are plotted on the Left vertical axis. CFUs were plotted to show the change in bacterial colony number intracellularly, post antibiotic treatment for 6-hours. Points represent mean from 3 technical replicates from one experiment specific to the aforementioned MOIs. Two sample Student t-test was used to identify significant differences between sequential timepoints, and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

The low GM-CSF MPI culture was tested for 10 days with both M.INT and M.AVI at MOI (20). There was a significant difference in the uptake of bacteria at day 0 between M.INT and M.AVI (p=0.015) with M. INT having a higher intracellular uptake. However, this subverted at day 3 where M.AVI was significantly higher than M.INT (p=0.022). Both strains appear to behave the same as no significance in intracellular numbers

were detected during days 5 and 8. However, at day 10, M.INT ended the experiment with a higher intracellular bacterial number that could be deemed slightly significant due to chance than true significance (p=0.09). There appears to be a downward trend in both strains with CFU numbers declining from day 0 to day 8. M. INT deviated from this downward trend by a 28-fold increase in intracellular CFU at day 10. Both strains did not appear to disappear in 10 days, therefore the next model shows an infection over 18 days.

3.8 EXPERIMENT 1 Comparing the intracellular CFU of *M. intracellularae* and *M. avium* in low GM-CSF ratioed culture over 18 days

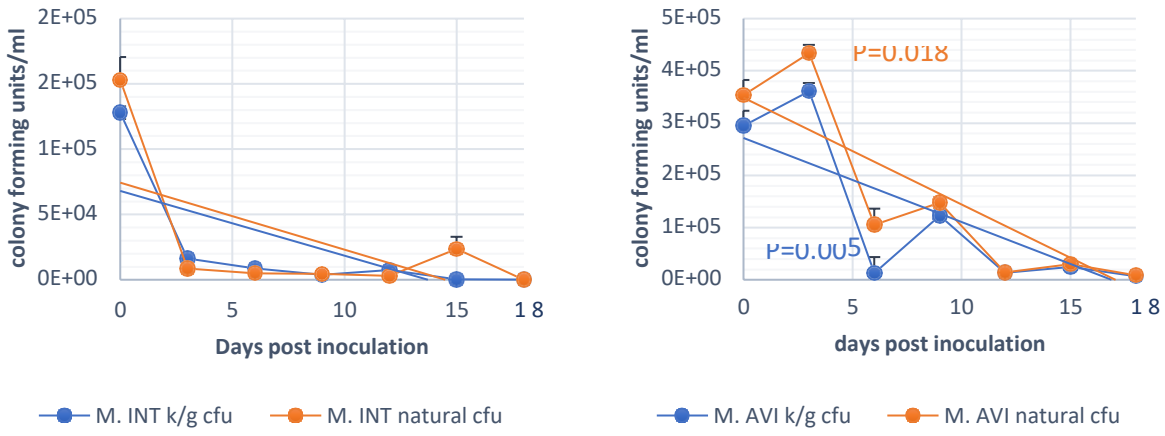


Figure 3.7 Experiment 1: Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days.

MPI macrophages supplemented with 1:1600 ratio of GM-CSF in media were infected with *M. intracellularae* (M. INT) NCTC 10682 for MOI (32) and *M. avium* (M.AVI) NCTC 13034 for MOI (112). Graphs represent change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. Linear regression analyses were performedto determine if the *overall* trend in intracellular CFUs changed across the infection duration and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005 and ns not significant. Groups are non-significant unless explicitly presented on graph.

In the third set of experiments, culture period was extended for 18 days to see how bacteria persisted in the *in vitro* model both with and without the presence of antibiotic treatment to eradicate extracellular bacteria. Both cultures were washed with fresh iRPMI before lysis with 0.2% Saponin. For the two strains *M. intracellularae* (M.INT) and *M. avium* (M. AVI) lysates were procured from lysed MPI cells for every three days post inoculation up to 18 days. Graphs represent the linear regression analysis to show whether the significance in the overall CFU change was different from the 'zero slope'. Two culture types were also assessed; k/g with antibiotics kanamycin and gentamycin being administered every three days in addition to washings whereas well plates under natural infection only had washings performed on them. For all strains and culture types there was an overall decrease across the infection period. Linear analysis showed that P=0.018 for Avi k/g and P= 0.005 for Avi nat. where there was significant deviation from the zero slope for M.AVI for both culture types. However, the same could not be said for the M. Int cultures where no significance was detected.

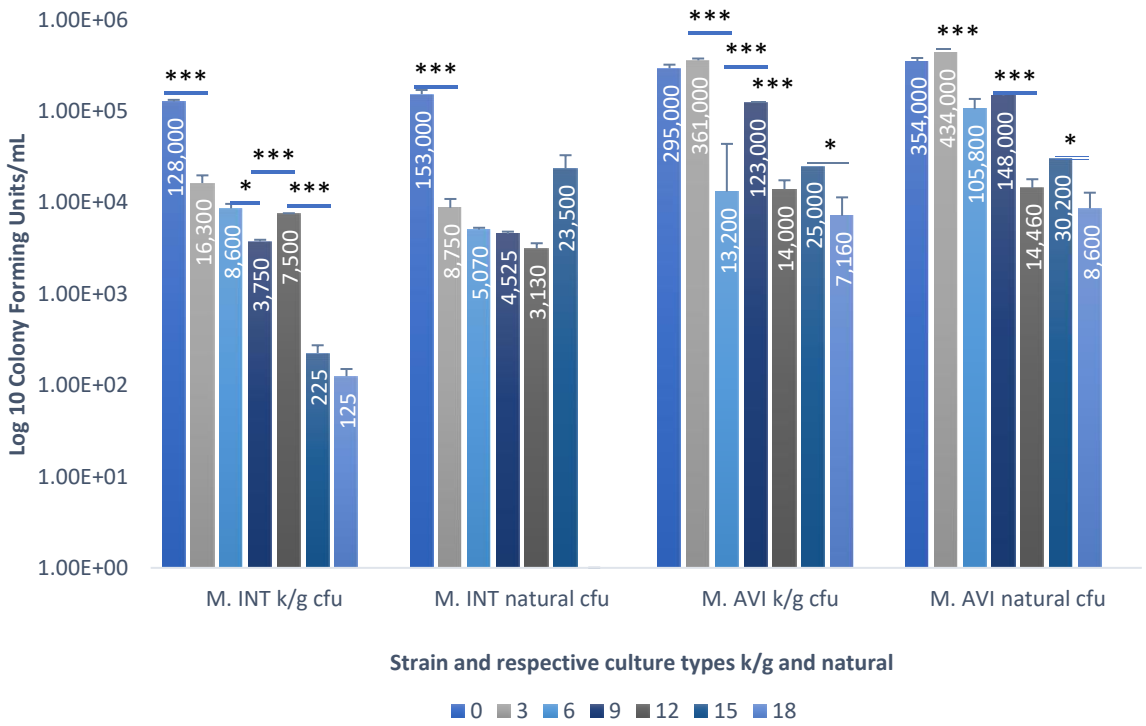


Figure 3.8 Sequential Timepoint analysis of Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days.

MPI macrophages supplemented with 1:1600 ratio of GM-CSF in media were infected with *M. intracellularae* (M. INT) NCTC 10682 for MOI (32) and *M. avium* (M.AVI) NCTC 13034 for MOI (112). Graphs represent change in log intracellular colonies across infection period for each strain and respective culture type. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. Students t-test was used to identify significances between sequential timepoints, and P-values were calculated to detect any significance. * = P<0.05, ** = P<0.005 and ns not significant. Groups are non-significant unless explicitly presented on graph.

The decrease in intracellular CFU's between day 0 and day 3 were significant for both M.INT k/g (P=0.000) and M. INT nat (P=0.004). This was not the case for any M.AVI culture as intracellular numbers appeared relatively the same for the initial part of the infection. The intracellular colony number of the M. INT culture treated without antibiotics had no significant variations for the remaining days of the experiment. Comparatively, there was overall downward fluctuating trend for the antibiotic treated M. INT culture and both of the M.AVI cultures. Both M.AVI k/g and nat reflected the same degrees of significance for the decreases observed between days

3 and 6 (P=0.000) , 9 and 12 (P=0.000) as well as 15 and 18 (P=0.04) with the first two decreases more significant than the one between 15 and 18.

The M. INT culture treated with antibiotics did not have any significance between days 3 and 6 however M. INT k/g experienced a decrease between days 6 and 9 (P=0.021) and an increase between 9 days and 12 days (P=0.000) and a final significant decrease between days 12 and 15 (P=0.03).

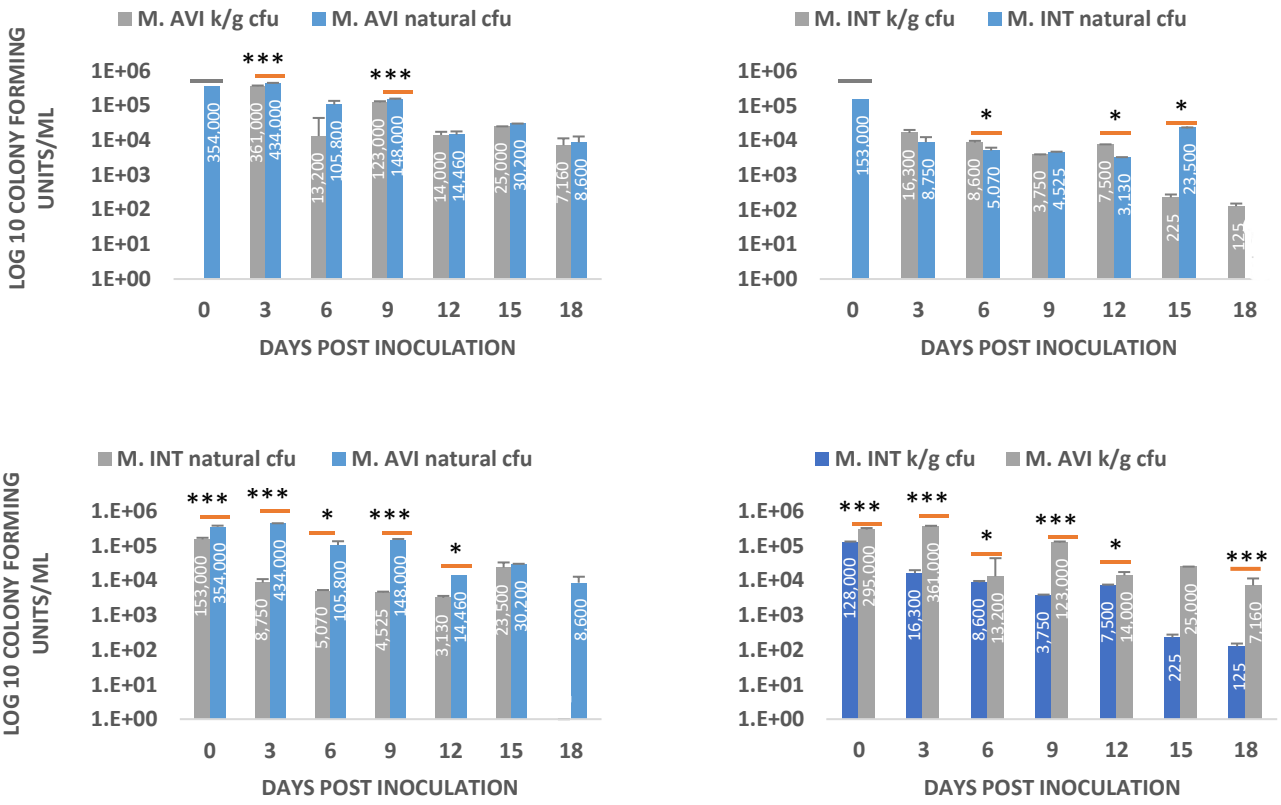


Figure 3.9 Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days.

MPI macrophages infected with two different strains with MOI (32) for M. INT and MOI (112) for M. Avi. Chronic infection was mimicked with infection spread across days 0 to day 18 with CFU's from lysed MPIs for every three days. Log CFUs were plotted to show the change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences between strains and culture types across individual time points. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

The comparisons of the strains and culture types for days post inoculation is outlined below.

0 days

This timepoint reflects the uptake of intracellular bacteria and survival. Irrespective of culture type, M.AVI had a relatively higher number of intracellular bacterial uptake than M. INT. This difference was significant between M. INT and M.AVI for both natural (p=0.000) and antibiotic treated (p=0.002) cultures.

3 days

When considering culture types there was only a significant difference between M.AVI k/g and nat (p=0.002), with the natural culture type having a higher intracellular uptake number. There was also a 4-fold strain difference between M. INT vs M.AVI natural cultures (p=0.000) where M.AVI was significantly higher than M. INT. The antibiotic treated strain difference meant that M.AVI had a significant uptake of bacteria compared to M. INT k/g (p=0.000). M. INT k/g and M. INT Nat experienced no statistical differences on day 3.

6 days

On day 6 post-infection there was a slight significance in culture type of M. INT where the antibiotic administered set of wells had a higher CFU than the naturally treated ones (p=0.048). There was no difference between either culture type at day 6 for the rough strain, M. AVI. Additionally, strain differences existed for both culture types, although day 6 variances were relatively less significant than the previous days for M. INT and M.AVI nat (p=0.047) as well as M. INT k/g and M.AVI k/g (p=0.049).

9 days

There was a relatively high significant variance between culture types of the rough strain where M.AVI natural CFUs were higher than the antibiotic treated ones (P=0.005). However, this culture type significance at day 9 was only experienced by the rough strain. In contrast to the previous timepoint at day 6, it was observed that the strain differences were as highly significant as days 0 and 3 between m. int and M.AVI k/g (p=0.000) as well as M. INT and M.AVI natural (p=0.000)

12 days

Slight culture type variances were observed between M.INT k/g and M.INT natural on day 12 (p=0.014) with the k/g culture having the high CFU number between the two. The rough strain had virtually similar intracellular CFU numbers which lacked sufficient difference to convey a significance. Strain differences on day 12; M.INT kg and M.AVI k/g (p=0.023), M.INT natural and M.AVI natural (p=0.034) were similar to day 6 and the degree of significance was markedly less severe than that of day 9.

15 days

At this timepoint there was the least variances between both cultures and strains in comparison to other days. No differences that involved int k/g culture type could be identified as the agar plate for bacterial colonies had to be discarded due to mould contamination. On the whole there were no variances for the rough strain k/g and natural cultures or when M.INT and M.AVI natural cultures were compared.

18 days

For the antibiotic treated cultures, M.AVI produced relatively more colonies compared to M.INT even though overall the CFU numbers were minimal ($p=0.002$). No comparisons could be made between M.INT natural culture regarding strain or culture due to development of mould on the agar plate. M.AVI did not have a significant difference of culture type.

3.9 Experiment 1 Comparison of IL-6 levels stimulated by rough and smooth varieties of MAC over 18 days in low GM-CSF ratioed culture

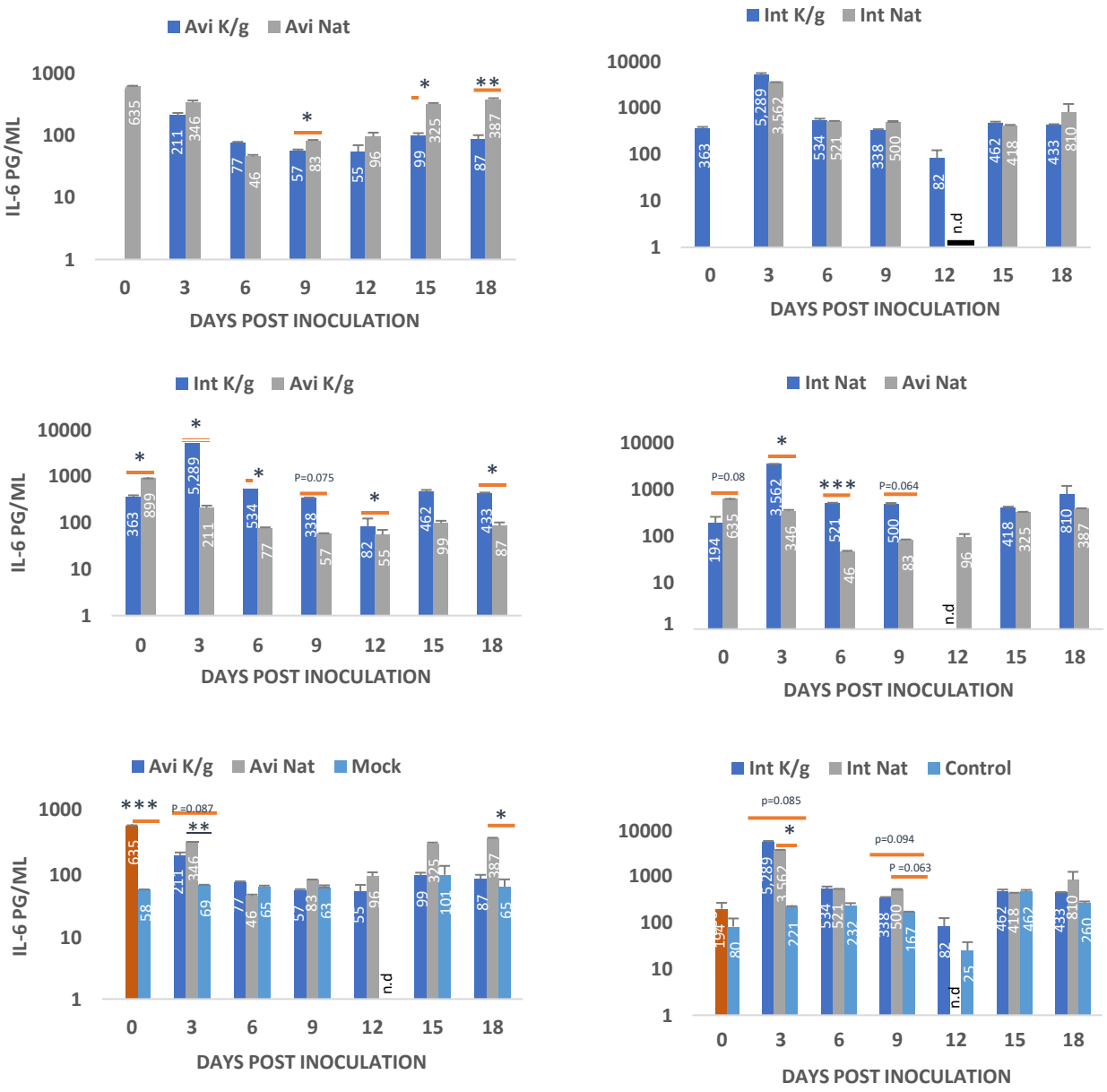


Figure 3.10 Experiment 1 Comparison of M.INT and M.AVI induced IL-6 cytokine production from Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days

IL-6 pg/mL levels induced by MPI macrophages infected with two different strains with MOI (32) for M. INT and MOI (112) for M. Avi. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences between strains and culture types in comparison to each other and mock

control across individual time points. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

The following section focuses on the soluble effector response, in particular, the amount of IL-6 pg/ml produced by MPI cells in response to MAC infection by M.INT and M. AVI. Differences were also noted between IL-6 levels when one in-vitro culture was treated with antibiotics in addition to washing as opposed to just washing alone. Mentioned in previous sections, inflammation is the hallmark of PD caused by MAC. IL-6 being a well-known pro-inflammatory cytokine was of high interest as detected levels can convey a reliable understanding about the inflammation inducing process in-vivo as represented by MPIs which closely resemble the primary AM's.

Analysed above were the colony forming units recovered from day 0 to day 18 for every three days when MPIs were infected with two phenotypically different strains M.INT and M.AVI. The supernatants were also procured before lysis and were put through post-infection analysis to be quantified by sandwich ELISA.

Day 0

All cultures were treated the same on day 0, where bacteria were administered antibiotics and then washed. M.AVI significantly differed from M. INT in both culture types, however only M.AVI significantly differed from the mock (p=0.000) in the uptake phase. M.AVI induced significantly higher IL-6 levels in antibiotic treated cultures k/g (p=0.023). However, significant differences between naturally treated cultures of M. INT and M.AVI were likely to be due to chance than true significance (p=0.08).

Day 3

Induction levels at day 3 were for both strain culture types were not significant although there were slight significances between the strains themselves from M.INT k/g vs avi k/g (p=0.02) and M.INT nat vs avi nat (p=0.024). When evaluated against the mock, the antibiotic treated wells of both m. int (p=0.085) and M.AVI (p=0.087) were above the confidence level and deemed significant due to chance with m. int having the greater degree of significance than m.avi. Out of the naturally treated wells, both M.AVI (p=0.003) and M. int (p=0.02) showed true significance to mock il-6 levels.

Day 6

Generally, there was an observable peak of IL-6 during day 3, which declined at day 6. IL-6 levels appear to be non-significantly induced between k/g and nat cultures of both the rough and smooth strain. Strain differences only exist between the natural cultures (p=0.001) of M.INT and m.avi. Compared to mock, there were no differences across any culture of M.INT but M.AVI had a slight significance above the confidence level (p=0.068).

Day 9

day 9 appears to take on the same trend as day 6 with very little cytokine induction across the board. There was significance between antibiotic and naturally treated culture types for M.AVI (p=0.03) but none for m.int. Strain differences existed for both cultures between M.INT and M.AVI k/g: (p=0.075) and nat (p=0.064) that could possibly be due to chance than true significance. In contrast to day 6 mock, day 9 showed chance significance between both culture types k/g (p=0.094) and nat (0.063) for M.INT but no significance true or otherwise was present when M.AVI cultures were compared to mock.

Day 12

Somewhat like day 6, day 12 exhibited the least variances between culture, strain or mock. IL-6 pg/mL were below detectable levels for the naturally treated smooth strain. However, comparative to mock, M.AVI nat was significantly different (p=0.004).

Day 15 and Day 18

Day 15 had no variance across the culture types for each strain or when otherwise compared to mock. On the final day of the experiment, day 18, there was a high degree of significance between the antibiotic and naturally treated cultures for M.AVI (p=0.009) with strain differences that were significant to int k/g and avi kg (p=0.05). Avi nat was the only significance observed in comparison to mock (p=0.016).

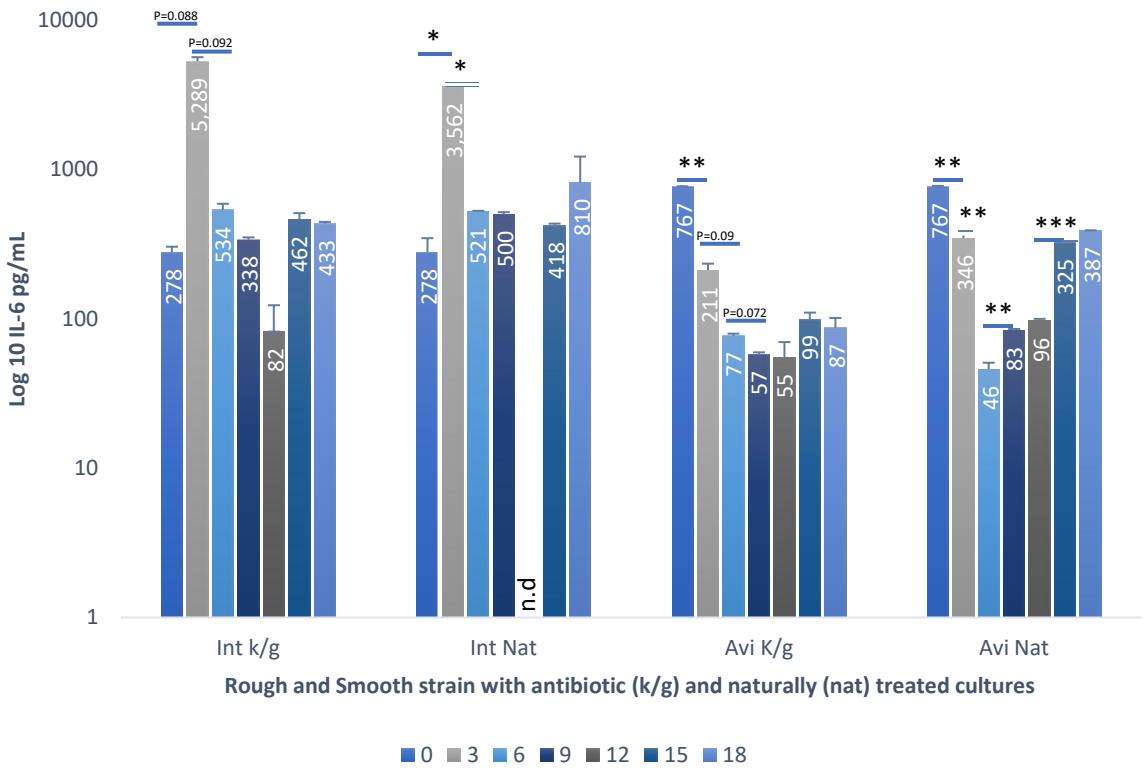


Figure 3.11 Sequential timepoint comparison of M.INT and M.AVI induced IL-6 cytokine production from Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days

MPI macrophages infected with two different strains with MOI (32) for M. INT and MOI (112) for M. Avi. Chronic infection was mimicked with infection spread across days 0 to day 18 with CFU's from lysed MPIs for every three days. CFUs were plotted to show the change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences in sequential timepoints. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

In the initial infection phase, there was only true a significant difference between days 0 and 3 IL-6 levels with M.AVI treated k/g (p=0.004) and nat (p=0.000). M.AVI had the highest il-6 levels at day 0 which decreased significantly at day 3. With the smooth strain M. int there was a observed 14- and 17- fold increase between day 0 and 3 for naturally (p=0.033) treated versus antibiotic treated (p=0.088) cultures respectively. This increase appeared to be more due to chance than true significance for the antibiotic treated culture as p-values were above the confidence level. Following on from day 3, day 6 experienced a significant dip in IL-6 levels across the board with

M.INT nat (p=0.022) and M.AVI nat (p=0.003). For the antibiotic treated cultures of both strains, significances were more likely due to chance with M.INT k/g (p=0.092) and M.AVI (p=0.09). No IL-6 could be detected for day 12 for the k/g culture of M. Int. The natural treated rough strain culture was the only group that experienced a significant rise in IL-6 towards the end of the experiment between days 12 to 15 (p=0.000).

3.10 EXPERIMENT 2 Comparing the intracellular CFU of *M. intracellularae* and *M. avium* in low GM-CSF ratioed culture over 18 days

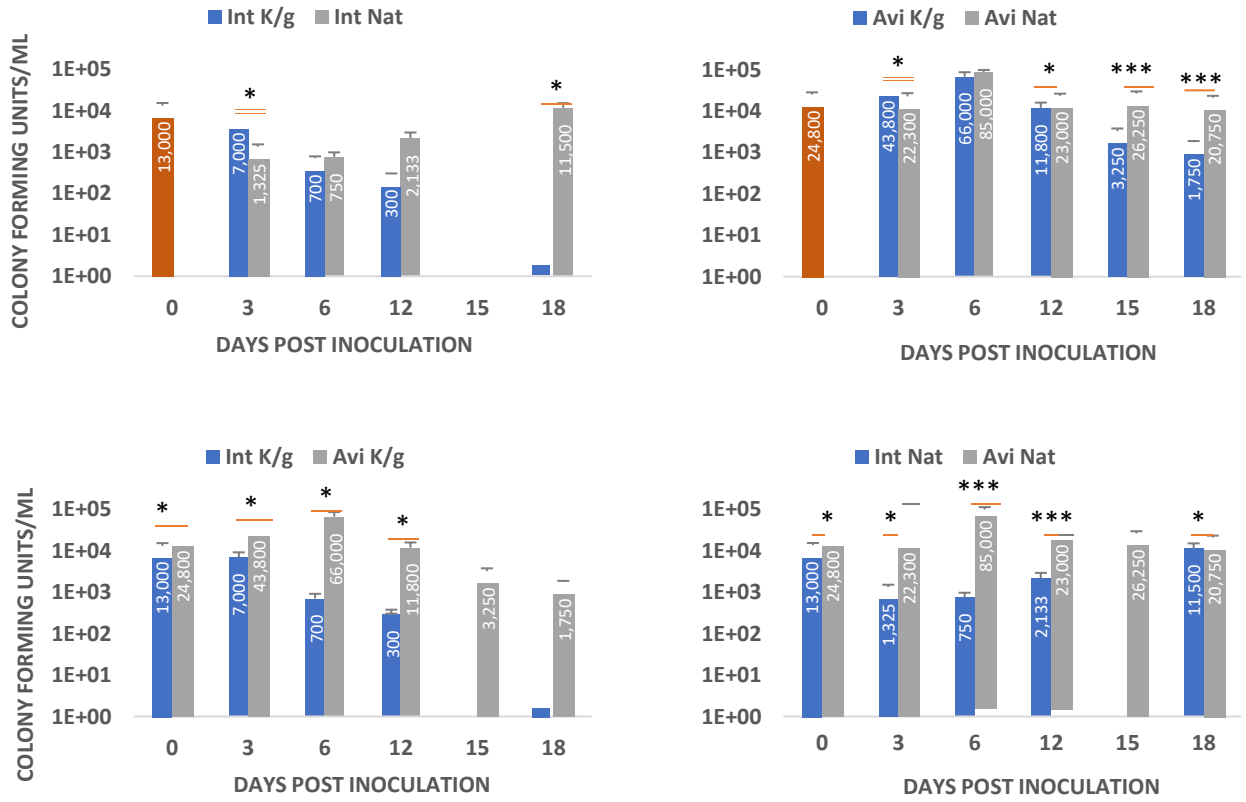


Figure 3.12 EXPERIMENT 2 Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (27) and M.AVI: 13034 MOI (8) in low GM-CSF MPI culture for 18 Days

MPI macrophages infected with two different strains, MOI (27) for M. INT and MOI (8) for M. AVI. Chronic infection was mimicked with infection spread across days 0 to day 18 with CFU's from lysed MPIs for every three days. CFUs were plotted to show the change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences between strains and culture types across individual time points. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

There was a significant strain difference in the uptake of bacteria at day 0 between M.INT and M.AVI for both antibiotic k/g (p=0.042) and untreated culture types (p=0.037). Between the culture types for the strains themselves, there were significant intracellular CFU variances that presented at day 3 with the antibiotic treated cultures for both M.INT (p=0.03) and M.AVI (p=0.022) being significantly higher than their untreated counterparts. Between M.AVI k/g and M.AVI natural, the natural culture appeared to have higher intracellular colony numbers than k/g for days 12 (p=0.024), 15 (p=0.001), and 18 (p=0.003). Contrastingly, apart from significant differences -presented at day 3, M.INT presented only one more significant between culture types at day 18 (p=0.04). This experiment had mould contamination on the solid agar plates for Day 15 for M.INT and Day 9 for both M.INT and M.AVI and therefore these counts were not shown on the graph. In comparison to M.INT, M.AVI had significantly more intracellular colonies taken up in both antibiotic and non-antibiotic treated cultures. In the antibiotic treated cultures, these differences were significant for days 6 and 12. In the natural cultures, there were significant strain variances at days 6, 12 and 18. Despite M.AVI having the lower MOI relative to M.INT, there appeared to be a higher bacterial uptake in both antibiotic and natural cultures for the rough strain. M.AVI culture types peaked at 6 days non-significantly. In these sets of experiments, M.INT persisted less than M.AVI and had an inclination to disappear on the final day of the experiment. This tendency was seen more with the antibiotic treated culture than the non-antibiotic treated one for both strains. The peak

CFU points for both strains were different with M.AVI peaking much later at day 6 and M.INT peaking very early on at Day 0.

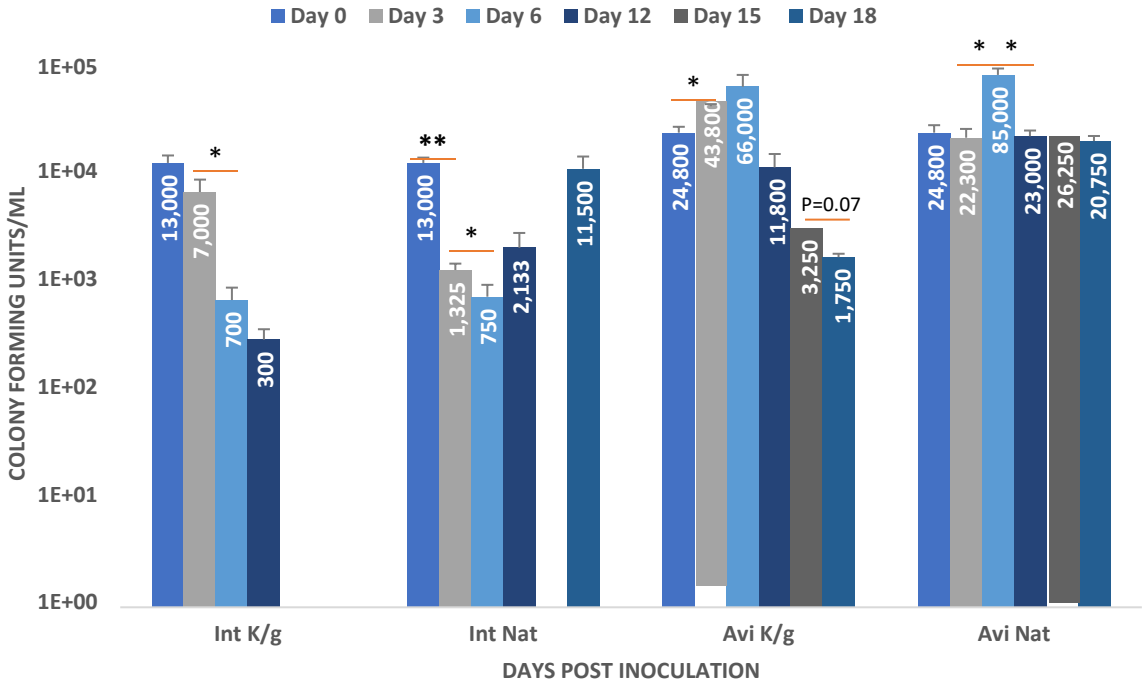


Figure 8a EXPERIMENT 2 Sequential timepoint analysis of colony forming units extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (27) and M.AVI: 13034 MOI (8) in low GM-CSF MPI culture for 18 Days

MPI macrophages infected with two different strains with MOI (27) for M. INT and MOI (8) for M. Avi. Chronic infection was mimicked with infection spread across days 0 to day 18 with CFU's from lysed MPIs for every three days. CFUs were plotted to show the change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences in sequential timepoints. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

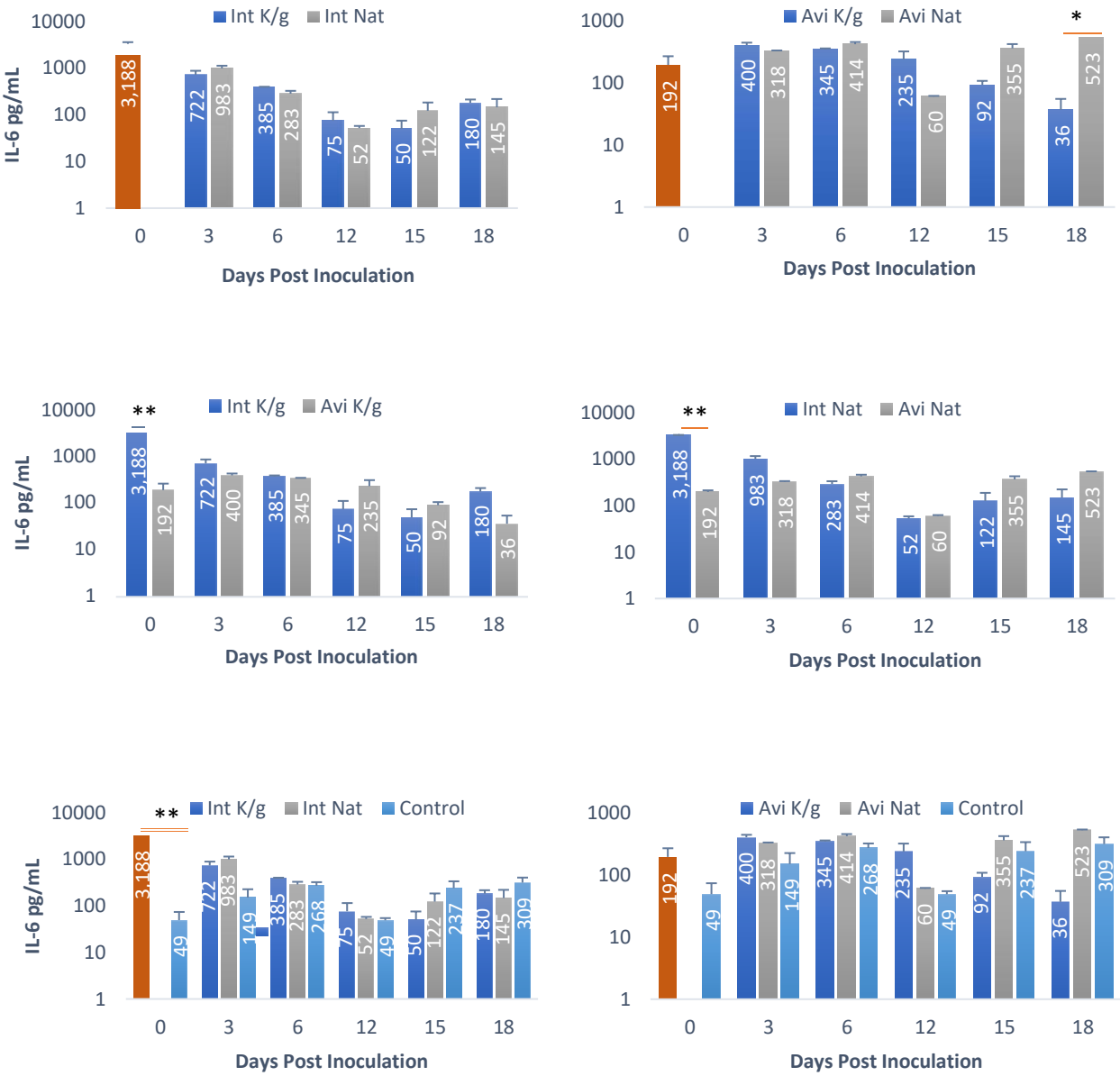


Figure 3.14 Experiment 2 Comparison of M.INT and M.AVI induced IL-6 cytokine production from Extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (32) and M.AVI: 13034 MOI (112) in low GM-CSF MPI culture for 18 Days

IL-6 pg/mL levels induced by MPI macrophages infected with two different strains with MOI (27) for M. INT and MOI (8) for M. Avi. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences between strains and culture types in comparison to each other and mock control across individual time points. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph

INT k/g and natural culture types had no significant differences in IL-6 throughout the whole experiment. AVI k/g and AVI Nat followed the same trend but deviated at day 18 where the natural culture had a 14.5-fold difference from antibiotic treated one (p=0.038). When comparing antibiotic treated behaviour between strains on day 0, M.INT proved to have a 16.6-fold significantly higher induction of IL-6 pg/mL than M.AVI (p=0.001). As both bacteria were treated the same on day 0, counts from these cultures were combined to give a representation of both natural and antibiotic treated cultures. Neither the strains nor their respective culture types differed significantly from the mock induction of IL-6 apart from the antibiotic treated M.INT culture that had a 65-fold higher IL-6 level than the mock (p=0.001).

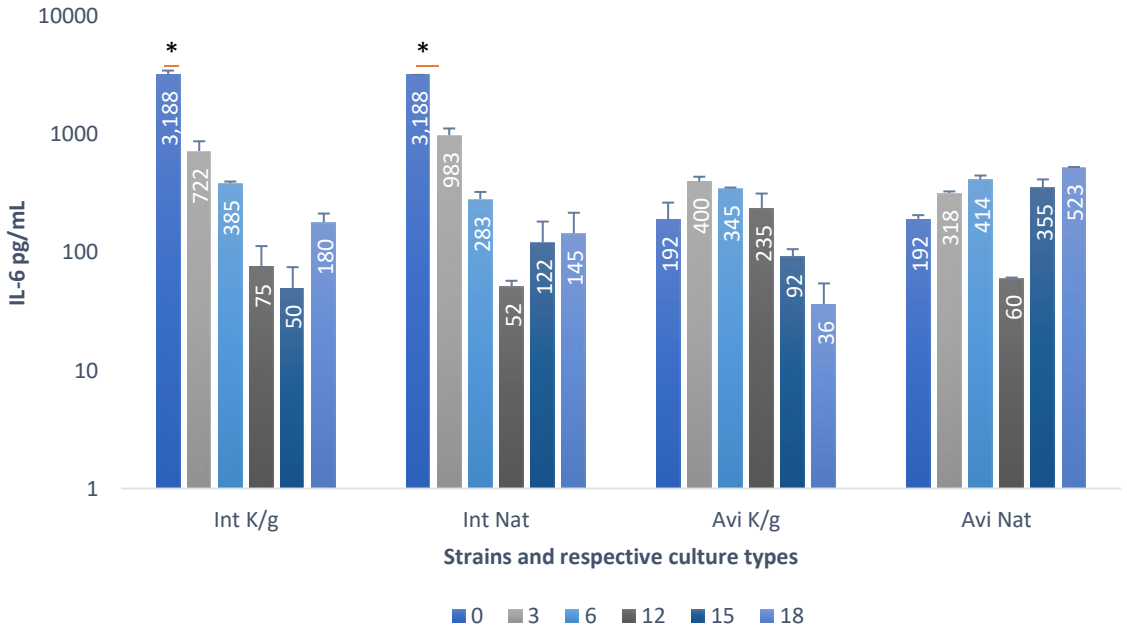


Figure 3.15 EXPERIMENT 2 Sequential timepoint analysis of IL-6 cytokine induction from extracellular killing assay with 400µg/ml kanamycin and 200µg/ml gentamycin, (k/g) and without antibiotics (natural infection), live M. INT: NCTC 10682 MOI (27) and M.AVI: 13034 MOI (8) in low GM-CSF MPI culture for 18 Days

MPI macrophages infected with two different strains with MOI (27) for M. INT and MOI (8) for M. Avi. Chronic infection was mimicked with infection spread across days 0 to day 18 with CFU's from lysed MPIs for every three days. CFUs were plotted to show the change in intracellular colonies across infection period. Points represent mean from three technical replicates from one experiment specific to the aforementioned MOIs. 2-sample T-test was performed to detect significant differences in sequential timepoints. Accept no differences between strains and culture unless otherwise stated on the graph. * = P<0.05, ** = P<0.005. Groups are non-significant unless explicitly presented on graph.

Chapter 3: Discussion

4.1 Novel Alveolar Lung Macrophage Model is Suitable for Studying Long Term MAC Intracellular Growth and Persistence for 18 days.

Alveolar macrophages are an example of tissue-resident macrophage that are responsible for host defence against respiratory pathogens (Luque-Martin et al., 2021). Due to its implication and role in disease, it is key to develop an in vitro model that is relevant to mimic in vivo macrophage behaviour to further our understanding of MAC infection. Currently a variety of human and murine cell types and lines are used. Most common are murine BMDMs, THP-1 and peritoneal macrophages (Skinner et al., 1994; Bermudez et al., 1999; De Logu et al., 2005; Zaru et al., 2009; Andrejak et al., 2015; Das et al., 2019). Models that use pluripotent stem cells from different sources, although widely used, present ethical issues and the addition of exogenous substrates and cytokines to tailor the culture to represent a specific microenvironment is a laborious process (Luque-Martine et al., 2021). In the analysis of cell surface markers F4/80 and CD11b, it appeared that alveolar macrophages were functionally different from peritoneal and BMDMs due to their high expression of F4/80 and CD11b compared to the low levels of F4/80 and CD11B elicited by alveolar macrophages (Zhan et al., 2008). Therefore, it can be argued that peritoneal or BMDM macrophages may not be the most ideal representation of alveolar macrophages in MAC infection studies. Additionally, BMDMs express CD64 plus receptors TL2 and TLR4 with a leniency for M1 which indicates its use in responding to infections in the early phase (Zajd et al., 2020). Therefore, we can assume that MAC studies using BMDMs are less suitable to observe a long-term infection as they are only representative of a particular macrophage subset. Moreover, the use of human THP-1 macrophages reduces the interspecies variability of results but often utilise the aid of phorbol 12-myristate 13-acetate (PMA) to induce maturation (Rampacci et al., 2020). This can alter the physiological conditions of the cells which could include changes to cell surface markers

and the profile of cytokines induced (Rampacci et al., 2020). Therefore, THP-1 cells may not be the ideal cell line to use in experiments that rely on a true immune response that best reflects the lung alveolar macrophage (Rampacci et al., 2020). The present study uses MPI cells that are phenotypically and functionally similar to alveolar macrophages. Herein, we observed that MAC was able to replicate successfully in MPI cells and induce the production of the cytokine IL-6. MPIs have also been used effectively to study drug susceptibility and intracellular replication of Mtb and another related NTM, MAB (Fejer et al., 2013; Woo et al., 2018; Kelly et al., 2022). In these studies, researchers compared MPIs and alveolar macrophages and observed strongly similar response pro-inflammatory responses to the respiratory pathogens. The findings in the present study corroborate the theory, that MPIs are an optimal cell line to model MAC infections.

Within the literature there exists models of *in vitro* NTM infection that have successfully observed the intracellular behaviour of MAC and drug susceptibility up to 96 hours at MOI (10) and below. However, they are unrepresentative of the long-term intracellular persistence that MAC is known to exhibit (Sousa et al., 2019), and these models often produce variable results due to the methodology and cell lines used (Skinner et al., 1994; Bermudez et al., 1999; De Logu et al., 2005; Zaru et al., 2009; Andrejak et al., 2015; Das et al., 2019). The lack of cultures extending beyond a certain timepoint might be due to the increasing intracellular burden of mycobacterial bacilli which would result in macrophage apoptosis and subsequent extracellular contamination (Sturgill-Koszycki et al., 1994; Early et al., 2011; Blumenthal et al., 2015). Experiments in the present study lowered the concentration of GM-CSF for the MPI cells, which extended the life of the *in vitro* culture for 18 days of infection by controlling cell growth and proliferation. Murine and human GM-CSF share 70% of nucleotide and 56% amino acid sequences and therefore have modest structural homology (Shi et al. 2006). GM-CSF is

present at low or non-detectable levels in the human lung environment under normal homeostasis (Trapnell et al., 2002). Beltan et al. (1999) further observed that GM-CSF levels, responsible for cell recruitment and differentiation, were low in the presence of opportunistic and pathogenic strains of *Mycobacterium* such as *M. avium* and *M. intracellularae,* compared to non-pathogenic strains. Fejer et al. (2013) explains that GM-CSF is necessary for alveolar macrophage function and at reduced levels, macrophages may not function optimally. Therefore, MAC might use the opportunity of impaired alveolar macrophage function due to low GM-CSF levels to establish infection within the macrophage. Overall, murine GM-CSF used in this model is fairly representative of human GM-CSF and its low concentration is what is naturally observed in the lung environment as well as in the case of MAC infection (Shi et al., 2006). The low GM-CSF *in vitro* system used in this project sustained the MAC infection within MPI cells, with minimal MPI death and no extracellular contamination observed.

Mycobacterium have been observed to replicate vigorously within macrophages, burst macrophage cells, and spill intracellular bacteria into the extracellular space (Early et al., 2004). For the purpose of studying purely intracellular behaviour, extracellular bacteria needed to be controlled. Previous studies have utilised washing cells post-infection, but often bacterial regrowth was observed 5-7 days later (Ferro et al., 2015; Raajmakers et al., 2021). Furthermore, as observed during the optimisation experiments of this project, aggressive, or numerous washings with DPBS might cause *in vitro* monolayer disruption. Additionally, once ingested by macrophages, intracellular bacteria might adopt different pathological tendencies than their extracellular counterparts, as observed by (Early et al., 2011). They described this phenomenon whereby MAC that originated from apoptotic macrophages were more efficient at invading fresh macrophages. There is a possibility that strains of *Mycobacterium avium* may adopt different phenotypes in the intracellular and extracellular

environments during infection (Bermudez et al., 2004). Therefore, it is vital that optimal methods such as the one used herein are applied to experiments that investigate the impact of intracellular bacteria, regarding how they interact with host cells and how effective drug combinations are against intracellular MAC.

Mycobacterium intracellularae (smooth) and *Mycobacterium avium* (rough) growth analyse herein confirmed that the two morphologically different strains had varying growth rates, with *M. intracellularae* doubling under 24 hours and *M. avium* which doubled in CFU over 50 hours. Based on these observations, the initial infection experiments utilised the smooth strain alone due, its faster replication rate and colony formation on agar making it more favourable to optimise experimental models in a reasonable time frame. The pilot experiment model was based on studies that proved amikacin to be efficacious against extracellular MAC (Brown-Elliot et al., 2013) and Mtb in a culture of RAW264.7 or differentiated THP-1 cells at a MOI of 2 (Song et al., 2017). Brown-Elliot et al. (2013) conducted an amikacin drug susceptibility testing of 462 clinical isolates of MAC. Variable MIC breakpoints were obtained for different strains ranging from 8µg/ml to >64ug/ml. Of the seven MAC isolates for >64µg/ml MIC, notably *M. intracellularae* and *M. avium* studied herein were two of them. This implied that an amikacin concentration above 65µg/ml was needed to inhibit the MAC strains of the present study.

Liposomal amikacin *in vivo* has reduced mycobacterial CFUs in spleen and liver of C57BL/6 by 3 to 4 logs with effects extending when administered at the chronic stage of infection as well. However, in the same study 60µg/ml of liposomal amikacin was more effective than 2000ug of aqueous amikacin (Stephan et al., 1996). The published literature on amikacin effectiveness was taken as a guideline to use aqueous amikacin 200µg/ml in the present study. MPI

cells ,also from C57BL/6 mice, infected with MAC and treated with amikacin, experienced regrowth of MAC within 3 days. Although results herein did confirm findings of previous studies where amikacin inhibited MAC growth, it was a temporary effect as *M. intracellularae* had rapid regrowth at 72-120 hours post-infection and lawn of extracellular bacteria that could have spilled over into intracellular CFU counts. Therefore, amikacin might not be effective in its aqueous form against MAC as corroborated earlier (Stephen et al., 1996). This effect appeared to diminish in studies where amikacin was used synergistically with clarithromycin (Beatriz et al., 2015; Wu et al., 2019). However, this synergistic use would not be valuable to the present study hypothesis to investigate intracellular bacteria due to the permeability of clarithromycin into the macrophage (Scaglione et al., 1993).

Gentamycin protection studies are a popular method to investigate the intracellular behaviour of pathogens (Tabrizi and Robins-Browne, 1992; Auster et al., 2019). Kanamycin has also showed effectiveness in MAC patient studies where it lowered rates of sputum relapse and improved clinical symptoms. Kanamycin was effective at concentrations higher than 8µg/ml in patients (Suzuki et al., 2008). Additionally, *in vitro* killing assays with another intracellular pathogen, *Burkholderia pseudomallei,* saw significant 5-log reduction of extracellular CFUs with kanamycin concentrations above 100 µg/ml (Mulye et al., 2014). In the present study, extracellular MAC growth was inhibited in cultures that were administered a combined kanamycin/gentamycin antibiotic treatment at concentrations 400µg/ml and 200ug/ml, respectively. With the added benefit of extracellular killing in *combination* with washing it could be valuable to maintain a long-lasting culture without losing any cells due to monolayer disruption. Bermudez et al. (1997) observed that macrophage cell monolayers infected with M. avium were prone to cell detachment and death which would eventually lead to bacteria release into neighbouring macrophages. Due to the lack of extracellular

contamination in these cultures we can assume that no apoptosis occurred, and cells were intact with replicating mycobacteria held within.

Comparing the antibiotic treated cultures with the natural treated cultures (i.e., 'natural' being washing two times only), was to indicate whether the improper inhibition of extracellular bacteria might have a knock-on effect towards the intracellular CFU counts. The naturally treated culture was representative an of uncontrolled *Mycobacterium* growth with minimal external influence from any experimentally imposed antibiotic treatments. The findings herein show that the antibiotic treatment is more beneficial for the later phase of the experiment as bacteria might be more likely to regrow during this period as previous studies have also shown MAC regrowth in culture after 3-7 days (Ferro et al., 2015; Raajmakers et al., 2021). Neither cultures experienced any extracellular growth; current data indicates that antibiotic treated cultures of *M. intracellularae* and *M. avium* showed steady decline in intracellular CFUs without elimination until day 18 post infection. In contrast, although the naturally treated cultures showed a decline up until day 12-15 post infection, they experienced a rapid spike in intracellular CFU numbers post infection day 15-18. From the behaviour of the naturally treated cultures we can predict that there is possibility of eventual macrophage apoptosis beyond the 18 days studied in this project if the MAC continues to rapidly replicate within macrophages and cause membrane rupture. Therefore, in this model, we can conclude that the kanamycin/gentamycin treated cultures are necessary for the success of the long-term infection model in MPIs especially in the later infection phase. This would be beneficial for test drug susceptibility in both early and chronic stages of infection.

4.2 Macrophage polarity has differential effects on MAC uptake and intracellular replication

To demonstrate the possible polarity of macrophages in an M1 and M2 context, MPIs derived from BALB/c mice (M2) were compared with wild-type MPIs from C57BL/6 mice (M1) in MAC experiments. Fejer et al. (2015) demonstrated that wild-type MPIs from C57BL/6 mice were more inclined to have a pro-inflammatory (M1) nature. In the present study, comparison to BALB/c MAC infection under the same experimental conditions, wild-type MPIs showed higher levels of smooth and rough MAC uptake. Previous studies have shown a lack of killing from macrophages of BALB/c mice compared to C57BL/6 mice in addition to impaired bactericidal activity (Watanabe et al., 2004). Primary macrophages that were polarised for M1 or M2 phenotype with GM-CSF and M-CSF respectively, showed that there was no difference in intracellular bacterial CFU when macrophages were infected with *M. avium* MOI 10 (Kilnic et al., 2022). Additionally, the same study showed *mycobacteria* were eliminated at 144h post infection and used gentamycin to remove extracellular bacteria (Kilnic et al., 2022). Contrasting evidence is observed in the present study where BALB/c deemed M2 polarised MPI macrophages were seen to have less efficient uptake than wild type MPI macrophages (M1) over 5 days. However, the present study used MOIs that were 2- to 5- fold higher which could be more representative of bacterial persistence rather than elimination as higher bacterial numbers would not easily be cleared intracellularly.

In another study where MAC persistence in BALB/c was demonstrated, peritoneal macrophages obtained from BALB/c mice were infected with phenotypically undefined MAC strains which showed persistent growth in experiments lasting up to 8 days (Sarmento and Appelberg, 1995). Peritoneal BALB/c macrophages *in vitro* had $1x10^7$ CFU/ml at day 5 which is approximately 250-fold higher than the CFU observed in the present study for the same

timepoint which demonstrates that BALB/c might be more susceptible to MAC. (Sarmento and Appelberg, 1995; Watanabe et al., 2004) Although, they do show a similar trend in persistence to the results herein, CFU numbers were well above the range of the present study implying that exact conclusions cannot be compared. The mentioned study perhaps did not consider bacterial regrowth and presented CFU counts based on what they assumed to be purely intracellular bacterial numbers, but external growth contamination might have occurred due to their use of washing cell monolayers to clear extracellular bacteria instead of antibiotics (Sarmento and Appelberg, 1995). Notably, peritoneal macrophages are usually obtained in insufficient quantities and growth agents such as Brewers thioglycolate broth or proteose peptone are used to increase the yield (Zhang et al., 2008). However, these agents might also change the physiology of the peritoneal cells obtained therefore the varying results between peritoneal BALB/c macrophages and MPIs might be due to the altered physiology due to the growth agents.

4.3 MAC evades host immune defences and replicates inside MPI macrophages.

Bermudez et al., (1997) investigated viability of *M. avium* uptake of bacteria MOI (100) in primary human monocytes from donors and declared that there was a significant decrease in cell viability seen as early as day 2 of the infection. Bermudez et al. (1999), later published findings that MOI 10 induced apoptosis in primary human macrophages after 5 days. Contrastingly, we report here that our model was able to contain an MOI 112 infection and sustain it until 18 days. *Mycobacterium avium* was studied in human monocytic cell line (MM6-Mfs) which was chosen for how characteristically similar it was to mature monocytes on a cytochemical and immunological criteria. This criteria included markers such as My4, M42, LeuM3, 63d3, Mo2 and UCHNI in addition to the phagocytic ability which was also comparable to mature macrophages (Sato et al., 2000). At an MOI of 1, MAC was observed to grow by 3-logs from day 0 to day 7 (Sato et al., 2000). Contrastingly, the present study shows the overall declining CFU of MAC in MPIs for both *M. intracellularae* (MOI 32 and 27) and *M. avium* (MOI 112 and 8) antibiotic and naturally treated cultures at day 6. This might be due to the cell type used as MPIs exhibit more cell surface markers that are similar to alveolar macrophages, and MM6-Mfs used by Sato et al. (2000) are more representative of a non-specific mature macrophage, which might not be characteristic of the lung alveolar macrophage due to the dissimilarity in markers.

In this project, IL-6 levels were analysed due its notable role in *Mycobacterium* infection pathogenesis. However, studies have speculated whether raised IL-6 coincides with a promotion or inhibition of *M. avium* intracellularly (Dennis 1992; Shiratsuchi et al., 1991). When treated with recombinant IL-6, studies have observed that enhanced *M. avium* growth

is seen in macrophages (Denis, 1992). Furthermore, in cultured spleen cells infected with *M. avium*, IL-6 was present at week 2 rather than week 1. This delayed response was thought to be rooted in an immune suppressive mechanism from MAC to establish intracellular colonisation (Champsi et al.,1995). Contrastingly, the present study observed that IL-6 produced by infected cells was significantly different from non-infected cells in the *first week* post-infection. There is a possibility that in the Champsi et al. (1995) study, they could not control the extracellular contamination of mycobacteria from apoptotic cells, and this resulted in the spike in IL-6 in week 2. Additionally, research shows low levels were induced by MAC compared to any other NTM strain in PMBC (Sampio et al., 2008). This response was corroborated in another investigation that investigated cytokine responses induced by the related species MAB in MPIs (Kelly et al., 2022), which showed high levels of significant IL-6 induction overall from MAB compared to the present study where IL-6 levels induced by MAC were comparable to that of mock.

In published studies of pathogenic, non-pathogenic, and opportunistic mycobacteria, cytokine levels of IL-6 were markedly lower in opportunistic and pathogenic species such as *M. avium* and *M. tuberculosis* as opposed to non-pathogenic species (Fang et al.,2020; Beltan et al., 1999). Evidence in the present study corroborated these findings by establishing that IL-6 was not significant from mock levels for the majority of the experiment, despite challenge with opportunistic MAC pathogens. Furthermore, Greenwell-Wild et al. (2002), claims that the pro-inflammatory response of MAC infected human macrophages dies down in 24 hours and remains at a low level up to 4 days post-infection. This is corroborated herein where high IL-6 levels are seen initially in the beginning at day 3 for *M. intracellularae* and 6 hours post infection for *M. avium* but decreases and is maintained at low levels on the continuous days.

It is possible that, the relatively low induction of cytokines from MAC, observed in this project could reflect a beneficial mechanism towards its intracellular survival. In support of this hypothesis, another published study showed that, more virulent strains elicited limited macrophage effector mechanisms such as low TNFα levels which is supposedly how it escapes elimination (Blumenthal et al., 2005).

The study herein observed that the low levels of IL-6 may be a significant finding, and may influence the growth of intracellular MAC. In an infection scenario, if the IL-6 levels were high, it would alert the recruitment of other cells and macrophage effectors that might lead to the killing of intracellular mycobacteria. Therefore, ending their colonisation and persistence which would be unfavourable for their survival.

Both *in vivo* and *in vitro* studies have aimed to analyse cytokine levels of supernatant cultures and intracellular bacteria as separate experiments, although few have suggested a link between either. In some studies that wanted to establish a relationship with intracellular CFUs and cytokines, results are contradictory (Champsi et al., 1995) to the ones herein. This could be due to the difference in cell type used. Additionally, the methodology did not employ the appropriate clearance of extracellular bacteria which could have accumulated and led to higher IL-6 response and intracellular CFU growth. The present study proposes that IL-6 levels are maintained at low concentrations, but intracellular MAC populations thrive and persist in MPIs at relatively high levels thus proposing an inverse relationship (Figures 3.9, 3.10, 3.12, 3.14). This phenomenon was corroborated by previous literature that proposes MAC dampens the pro-inflammatory response to maintain its intracellular foothold within the macrophage (Blumenthal et al., 2005). Therefore, we may suggest from our observations that MAC favourably optimises low IL-6 levels to promote intracellular bacterial replication but not

activate a robust pro-inflammatory response that would lead to intracellular killing. This is the suggested mechanism by which MAC evades the immune response and persists in the host.

4.4 Comparisons of MAC Smooth and Rough Strain in MPI cell infections

Several studies observe a more aggressive infection presented from smooth strains of MAC as opposed to the rough (Tateishi et al., 2009; Michelini-Norris et al., 1992). Pedrosa et al. (1994) claimed that smooth variants had an established virulence in BALB/c mice and rough variants could either have similar virulence levels as the smooth variant or be entirely avirulent. Further, Sampaio et al., (2008) found no significant differential effects between rough and smooth colonies of MAC when BMM from C57BL/6 mice were infected for up to 20 hours. Findings in the present study are contradictory as the lower MOI 8 in MPIs for the rough strain, *Mycobacterium avium*, replicated and persisted more efficiently than the higher MOI 27 of *M. intracellularae*. Therefore, we can assume that there is an underlying mechanism of rough strain persistence that is not dependant on the amount of bacteria that is present in the initial stages of infection.

In human monocyte derived macrophages, Blumenthal et al., (2005) investigated the gene expression profile when these macrophages were infected with different colony morphotypes of MAC. Over a 7-day culture with low MOI and vigorous washing to remove extracellular bacteria, a smooth opaque colony type was seen to slowly be eliminated post-macrophage uptake, with a 1-log reduction from day 0 to 7 post infection. Additionally, genetic analysis showed early phase upregulation of genes pertaining to a pro-inflammatory macrophage response (IL-1β, IL-6, TNF-α, lymphotoxin α) (Blumenthal et al., 2005). The smooth opaque, *M. intracellularae* MPI infection experiments of this project corroborate this

by showing a sufficient uptake in MPIs and slow elimination therein, with IL-6 induced early and overall declining from day 0 to 18. Therefore, evidence shows that MAC pathogenesis could be tied to the efficient uptake in the early phase of the experiment, its persistence and non-activation of host cell immune mechanisms (Blumenthal et al., 2005).

Moreover, Michelina-Norris et al. (1992), claimed that a more virulent smooth strain of MAC does not initiate pro-inflammatory macrophage cytokines as an escape mechanism from the immune system. We can assume from this that virulence is possibly correlated to the strength of the pro-inflammatory response induced post-infection. The results within corroborate this theory as IL-6 levels were comparable to mock for both MAC strains which indicate that the smooth *M. intracellularae* and rough *M. avium* might be equally virulent which supports aforementioned results by Sampaio et al. (2008) who observed no differential effects between the rough and smooth MAC colonies.

Morphotype differences were more pronounced in MAB than MAC. This is corroborated in work similar to this project by Fejer lab colleagues, where the MAB rough strain induced a more robust pro-inflammatory cytokine response in MPIs than the smooth (Kelly et al., 2022).

Results are also known to vary from *in vitro* to *in vivo* experiments with claims that the smooth variant *in vivo* is more aggressive compared to the rough (Tateishi et al., 2009; Michelini-Norris et al., 1992). This may be due to the continuous stimulation provided by an active *in vivo* model that encourages the mycobacteria to behave differently when faced with a more active immune environment that is not isolated to macrophages alone.

Despite the close morpho typical relationship between the strains of the same species, there is evidence that suggests that virulence differs between them (Blumenthal et al., 2005). There is more investigation into smooth variants of MAC than rough due to its supposed virulence

and persistence correlated with a more aggressive form of the disease. Blumenthal et al. (2005) concluded that virulence is not dependent on the intracellular replication of each strain nor the ability of lack thereof to activate macrophages. They argued that virulence should be measured as an exclusive event for each strain isolate and generalisation should be avoided. However, in the present study, an observation shared by both the smooth *M. intracellularae* and the rough *M. avium*, is the IL-6 induction that failed to be significantly different to that of non-infected cells. This implies that despite the differences in intracellular CFUs due to variable MOI's, the smooth and the rough strain possibly share an underlying mechanism of immune evasion to survive within the host cells.

4.5 Areas of Improvement and Future Considerations

The present study could benefit from improvement in methodology, especially with regards to the gentamycin protection assay. It is vital that intracellular bacteria are precisely enumerated to estimate the invasive potential and pathogenesis of bacterial species. When considering an appropriate antibiotic for the clearance of extracellular bacteria, it should be assessed for its ability, or lack thereof, to permeate through into the intracellular space. Gentamycin was thought to not permeate into the intracellular space and moreover, another aminoglycoside antibiotic, kanamycin, also demonstrated efficacy in extracellular clearance of *Burkholderia* in an MPI cell model (Chen et al., 2019). In addition to the antibiotic treatment, washing the plated cells with fresh infection media proved to significantly reduce the number of contaminating extracellular bacteria present when lysing infected macrophages. If the antibiotic were to enter the intracellular space, it may influence the internalised bacterial numbers and affect their persistence and replication. Kim et al. (2019) suggested an alternative clearance method where a 5-fold difference in the intracellular CFU recovery was observed when antistaphylococcal endopeptidase was used in an extracellular killing assay for *Staphylococcus aureus* instead of gentamycin. Researchers identified the possibility that gentamycin was internalised during the bacterial killing and reduced the internal CFUs. Therefore, Kim et al. (2019) highlights the benefits of an enzyme-protection assay rather than a gentamicin which might alter host physiology. However, this process might not be so easily applied as it is necessary to discover an enzyme that kills other bacterial species like MAC.

High MOIs were used to represent the severe forms of infection as most studies have a limit of the highest MOI being 10. The study herein uses a range of MOI's which could represent differential infection load correlating to low and high bacterial numbers. It would be beneficial

for comparison purposes to have achieved a similar or same MOI between both the smooth and rough strain however this was not possible as clump dispersion in the rough strain by syringe was ineffective. There are several mentions of NTM strains and their nature to aggregate and clump with the rough stain proven to be phagocytosed in clumps (DePas et al., 2019; Brambilla et al., 2016). This could explain the MOI variances between the smooth and rough strain in the experiments herein. Nishimura et al., (2020) describes that the rough variant aggregates more than the smooth phenotype in the log phase, which might explain the differences in MOI in the present experiments. Although disruption by syringe was utilised in the methodology, and tyloxapol was also used in the 7H9 growth media, the clumps might benefit from a more disruptive approach such as sonication which was used to successfully disperse clumps in *M. smegmatis* (DePas et al., 2019).

The enumeration of MAC by CFUs is a laborious process where variability is observed, and colonies can take up to several weeks to develop fully on agar plates. This method could benefit from improvement and PCR quantification could be used for proper enumeration. Real-time multiplex PCR has demonstrated high specificity and sensitivity towards *M. avium* by being able to detect as few as 100 to 1000 mycobacterial CFU per gram (Sevilla et al., 2015). Furthermore, when paired with luminescence-labelled MAC to observe a light sensitive viability in both the extracellular and intracellular space, a low cost and effective system can be utilised in place of CFU counting (Larson et al., 2014).

Another limitation is that it would be beneficial to analyse a host of other cytokines such as IL-10 and TNF-α which are often seen to have important roles in MAC infection. TNF-α is seen to inhibit M. avium intracellular replication which might indicate its importance in colonisation in the early phase. This bacteriostatic effect is seen to extend to all isolates and

it would give an insight into the intracellular mechanisms of phagosome-lysosome inhibition, and how TNF-levels would correspond with intracellular CFU (Appelberg and Orme, 1993). Additionally, IL-10 has immune dampening effects and has been identified to increase in levels during MAC infections. It would be beneficial to observe IL-10 levels in conjunction with IL-6 across the infection period to see if there is an inverse relationship between the pro-inflammatory IL-6 and anti-inflammatory IL-10 (Hussain et al., 2016).

The results collected from experiments herein are derived from technical replicates. Historically, technical replicates only tend to show variation within the bounds of materials and methodology (Bell, 2016). Technical replicates indeed, increased the methodological accuracy of the experimental protocol and were essential in fine tuning processes; in turn they restricted the applicability of the results to a sample MAC isolate from another growth cycle. Biological replicates of the MAC isolates could have overcome this issue by building confidence in the consistency of results from samples obtained under different conditions thus increasing the validity of results overall. Regrettably, time-constraints regarding the experiments did not provide capacity to obtain biological replicates here-in. Future experiments would benefit from a mixture of biological replicates, obtained from experimenting with MAC isolates grown at different points in time, and technical replicates to give a bird's eye view of the experimental process to highlight any discrepancies in the methodology as well as maintain the validity of results.

5. General Conclusions

Researchers claim that it is more vital to measure drug susceptibility in the intracellular environment which corresponds directly to the pathogenesis whereas the extracellular environment is more representative of a relapse rate after treatment (Larson et al., 2014). Therefore, the development a robust system for NTM drug analysis is vital as bacterial inhibition is the primary indication of drug efficacy (Rampacci et al., 2020). We propose that MPIs used in the long-term infection model is the best representative of human lung alveolar macrophages and the model herein, uses kanamycin/gentamycin as a means to eradicate extracellular bacteria and allow exclusively intracellular behavioural insights.

Overall, the measure of virulence is either the cytokine profile or the bacterial uptake and apoptotic reinfection. As this measure is not standardised across models, it is difficult to make conclusions as to what strains are more pathogenic. It is also dependent on the cell line and infection model used as strains may be more or less virulent depending on the cell type. Published studies have conflicting results, showing that both rough strains and smooth opaque strains might be less pathogenic in mice and macrophage models. However, the varying MOIs in the present experiments leave it open to interpretation as to the root of the uptake variation between strains. Applying the aforementioned improvements to this cell model, it would be possible for this long-term infection model to be established as a standardised method in the drug susceptibility and intracellular behaviour analysis of MAC and other respiratory pathogens.

6. References

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