

The Immune Response to *Mycobacterium avium* induced in Avian and Human Macrophage-like Cell Lines and in Chickens

By

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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The University of Nottingham

February 2016

Abstract

Avian tuberculosis remains a significant economic problem for the poultry industry in many countries. An improved understanding of the interaction between host and mycobacteria is important in designing more efficient vaccine strategies. This study investigated the immune responses to *M. avium* (Ma.) strains isolated from different sources, Ma strains 1-2 isolated from chickens, Ma.3-5 from cattle and Ma.6-8 from humans, using human cells *in vitro* and avian cells *in vitro* and *in vivo*. The responses were characterised by expression levels of pro-inflammatory cytokines tumour necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 and chemokines CXCLi1 (K60) and CXCLi2 (IL-8) and nitric oxide production.

A differential immune response was observed between the strains in terms of induction of cytokine and chemokine production in both human THP-1 and avian HD11 cells. With the exception of the avian strains 1 and 2, all strains induced increases in pro-inflammatory cytokines in THP1 cells. Two of the 3 bovine strains also had this effect in HD11 cells but the other strains had little effect.

Use of specific inhibitors indicated that in THP-1 cells the extracellular regulating kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways were important in inducing pro-inflammatory cytokines whereas the p38 pathway did not appear to be involved particularly in regulating TNF-α and IL-1β production. The results also indicate an interaction between the mitogen activated protein

kinases (MAPK) pathways studied in regulation of cytokine production in HD11 cells.

Although layer chickens were resistant to extensive *M. avium* multiplication in the tissues, significant expression of pro-inflammatory cytokines was observed in the liver of infected birds towards the end of the period of observation of 21 days as granulomata had started to appear, characterised by lymphocyte accumulation.

In conclusion, the findings of this study provide further understanding of the host-mycobacteria interaction and that different strains of *M. avium* might behave differentially regardless of their source of isolation.

Declaration

I declare that the work presented in this thesis is my own work and no part has been submitted for any other degree at the University of Nottingham or elsewhere.

Mohammed Shukri Shukur

Conferences, Posters and Oral Presentations

1- Genome bioinformatics Cambridge, United Kingdom, 6-9 September 2012, attending.

2- Shukur M.S., Barrow P.A., Jones M.A. "Immune responses to mycobacteria", Postgraduate research forum Faculty of Medicine, University of Nottingham, June 2013, Poster Presentation.

3- Shukur M.S., Barrow P.A., Jones M.A. "Immune responses to mycobacteria", Kurdistan student conference September 2013, Poster Presentation.

4- Pathology conference Nanjing, China August 2013, attending with lab work in China Agricultural University (CAU), Beijing, China 1st August – 1st September 2013.

5- Shukur M.S., Barrow P.A., Jones M.A. "Differential immune responses to *M. avium* isolates by human and chicken macrophages-like cells." 35th Annual Congress of the European Society of Mycobacteriology, Vienna, Austria, July 2014, Oral Presentation.

6- Shukur M.S., Tötemeyer S, Issa N, Barrow P.A., Jones M.A. "Differential modulation of cytokine expression by ERK and p38 MAP kinases in response to *M. avium* in human and chicken cells." TB Summit London, UK, 24-26 March 2015, Poster Presentation.

7- Shukur M.S., Barrow P.A., Jones M.A. "Chicken *in vitro* and *in vivo* immune responses to *Mycobacterium avium* infection." Global Alliance for Research on Avian Diseases (GARAD) conference, London, UK, 29 June – 1st July 2015, Oral Presentation.

8- Flow Cytometry meeting Medical School, University of Nottingham, 12 November 2014, attending.

9- Research day (first year) School of Veterinary Medicine and Science, University of Nottingham October 2012, Oral Presentation.

10- Research day (second year) School of Veterinary Medicine and Science, University of Nottingham, October 2013, Oral Presentation.

11- Research day (third year) School of Veterinary Medicine and Science, University of Nottingham, January 2015, Oral Presentation.

12- Shukur M.S., Tötemeyer S, Issa N, Barrow P.A., Jones M.A. "Differential modulation of cytokine expression by ERK and p38 MAP kinases in response to *M. avium* in human and chicken cells." Infection and Immunity theme meeting, School of Veterinary Medicine and Science, University of Nottingham, June 2015 (Poster Presentation).

13- Immunology Showcase, Medical School, University of Nottingham, 22 January 2015, attending.

14- Shukur M.S., Tötemeyer S, Issa N, Barrow P.A., Jones M.A. "Immune responses to *M. avium* infection and the role of MAPK in regulation of signal transduction during the infection." 3rd International Congress on Bacteriology and Infectious Diseases, Valencia, Spain, 4-6 August 2015, Oral Presentation.

Acknowledgments

First and foremost, I would like to express my deep gratitude to my supervisors, Prof. Paul Barrow and Assoc. Prof. Michael Jones for their scientific supervision, advice, guidance, and support throughout my PhD study. I have gained so much knowledge from them during my study. Their inspirational enthusiasm motivated me and working with them has been one the greatest honour of my professional life.

I would like to acknowledge all staff members and lab-mates in the Veterinary School that in various ways helped me during my research study.

I am very grateful to Ministry of Higher Education and Scientific Research in Kurdistan for providing a scholarship to me and for financial support.

I would like to thank my wonderful family, my father, mother, brothers and sisters and extended family in Kurdistan for their encouragement and supports during my study.

Finally, I would like to express my deepest thankfulness to my wife Nazeera, my daughters Aysan, Shadan and Sazan for their help and support.

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List of abbreviations

°C	Degree Celsius
µg	Microgram
µl	Micro liter
µM	Micromole
AIDS	Acquired immune deficiency syndrome
AP-1	Activator protein-1
APC	Antigen presenting cells
Ara-LAM	Arabinosylated LAM
ATF	Activating transcription factor
BCG	Bacille Calmette-Guérin
Ca^{2+}	Calcium
cAMP/PKA	Cyclic AMP/protein kinase A
cDNA	Complementary DNA
CFU	Colony forming units
CR	Complement receptor
CREB	Cyclic AMP response element binding protein
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intracellular Adhesion molecule-3-grabbing non-integrin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immuonosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GM-CSF	Granulocyte monocyte colony stimulating factor
GPL	Gylcopeptidolipid
HIV	Human immunodeficiency virus
HPAI	highly pathogenic avian influenza
HSP65	Heat shock proteins
Ig	Immunoglobulin

IL	Interleukin
IL1R1	Interleukin 1 receptor, type I
INF-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinases
JNK	c-Jun N-terminal kinase
LAM	Lipoarabinomannan
LDH	Lactate dehydrogenase
LM	Lipomannan
LPS	Lipopolysaccharide
M.	*Mycobacterium*
MAC	*Mycobacterium avium* complex
Man-LAM	Mannose-capped lipoarabinomannan
MAPK	Mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MD	Marek's disease
MDP	Muramyl dipeptide
MEK	ERK kinase
MHC	Major histocompatibility complex
MKK	MAPK Kinase
ml	Milliliter
MOI	Multiplicity of infection
MR	Mannose receptor
mRNA	Messenger RNA
MyD88	Myeloid differentiation 88
NBF	Neutral buffered formalin
NED	*N*-1-napthylethylenediamine dihydrochloride
NFκB	Nuclear Factor-Kappa B
NO	Nitric oxide
NOD-like receptors	Nucleotide-binding oligomerisation domain receptors
NTM	Non tuberculous mycobacteria
OADC	Oleic acid albumin dextrose catalase
OD	Optical density
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffered saline
PDIM	Phthioceroldimycocerosate
pg	Picogram
PILAM	Phosphoinositol-capped LAM
PIM	Phosphatidyl-myo-inositol mannoside
PMA	Phorbol-12-myristate-13-acetate
PP2A	Protein phosphatase 2A
PPD	Purified protein derivative
PRRs	Pathogen recognition receptors
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpm	Revolutions per minute
SAPK	Stress activated protein kinase
SR	Scavenger receptor
TDM	Trehalose-6, 6 dimycolate
TGF-β	Transforming Growth Factor β
Th	T helper cell
TLRs	Toll like receptors
TNF-α	Tumour necrosis factor-alpha
TRAF	TNF receptor associated factor 6
UBC13	Ubiquitin-conjugating enzyme 13
UK	United Kingdom
USA	United States of America

1 Introduction

1.1 General introduction

Mycobacterial diseases remain significant global issues for livestock and humans as a result of serious economic effects and zoonotic consequences. Bovine tuberculosis and paratuberculosis (Johne's disease) are two major examples of cattle diseases (Biet et al., 2005, Etter et al., 2006, Hasonova and Pavlik, 2006, Fulton and Sanchez, 2013). In addition to these, avian mycobacteriosis can be an important economic problem in the poultry industries of those countries where environmental control is limited by high ambient temperature and where poultry rearing is mainly outdoors resulting in reduced productivity, especially in long-lived birds including layers and breeders. Although avian mycobacteriosis is worldwide in distribution it most frequently occurs in the northern hemisphere, including occasional isolation in those western countries where outdoor, free-range rearing has again become popular (Fulton and Sanchez, 2013). *Mycobacterium avium* (*M. avium*) is not only a major pathogen for chickens (Bartos et al., 2006, Dvorska et al., 2003, Mijs et al., 2002), but also commonly associated with acquired immune deficiency syndrome (AIDS) in humans (Denis, 1994, Falkinham, 1994).

1.2 Taxonomy and microbiology of mycobacteria

The family *Mycobacteriaceae* includes one single genus, *Mycobacterium*, which consists of more than 130 identified species and subspecies (Turenne et al., 2007). Most mycobacterial species are environmental bacteria and are non-pathogenic. However,

several species are pathogenic for both human and animals including *M. tuberculosis, M. bovis* and *M. avium* (Reichenbach et al., 2001, Cosma et al., 2003).

The mycobacteria are acid fast, non-motile, aerobic bacilli. Resistance to some antibiotics, a high level of hydrophobicity and typical immunological characteristics are other features of mycobacteria (Quinn et al., 2011). These characteristics are largely a result of the high lipid content of their cell wall. This also accounts for the slow rate of nutrient uptake and slow growth rate of many mycobacterial species (Palomino et al., 2007).

Mycobacteria can be divided into two major groups according to their growth rate, slow growing and rapidly growing mycobacteria. The slow growing group includes those major species which are pathogenic for humans and animals, such as *M. tuberculosis, M. bovis,* the causative agents of tuberculosis in man and cattle respectively, the *M. avium* complex members and *M. leprae*. The rapidly growing group includes several members which can be pathogenic like *M. chelonae* and *M. abscessus* and non-pathogenic such as*, M. vanbaalenii, M. flavescens* and *M. smegmatis.*

1.2.1 *M. avium* complex

The *M. avium* complex (MAC) is defined as a group of non-tuberculous mycobacteria consisting of genetically related species that differ in their virulence and ecology (Mackenzie et al., 2009). This group is composed of two species, *M. avium* and *M. intracellulare*. The members of MAC can be isolated from the

environment such as water, soil and plants. MAC members are mainly virulent for birds and swine (Falkinham, 2002) and are the most common environmental mycobacteria pathogenic for humans (Falkinham, 2002, Griffith et al., 2007). *M. avium* is commonly associated with disseminated diseases while *M. intracellulare* is commonly associated with respiratory infection (Griffith et al., 2007) in a variety of host species. Specific probes are available to distinguish between *M. avium* and *M. intracellulare*.

M. avium can in turn be divided into four subspecies based on disease produced and genetic similarities (Mijs et al., 2002, Tortoli et al., 2004) including:

I. *Mycobacterium avium* subsp*. avium (*usually simply called *M. avium)* can cause mycobacteriosis in birds in addition to being an opportunistic pathogen and causing disseminated disease in HIV patients. They are also associated with cervical lymphadenitis in children and chronic pulmonary diseases in cystic fibrosis patients (Griffith et al., 2007).

II. *Mycobacterium avium* subsp. *silvaticum,* can cause chronic enteritis in calves, goats and deer similar to paratuberculosis in cattle (Matthews and Mcdiarmid, 1979, Henderson et al., 2000). This subspecies shows mycobactin-dependent growth and is not stimulated by pyruvate (Chiers et al., 2012).

III. *Mycobacterium avium* subsp. *paratuberculosis* (known as *M. paratuberculosis*) is the etiologic agent of granulomatous enteritis in ruminants particularly in cattle known as paratuberculosis or Johne's

disease. Mycobactin is essential for their growth (Thorel et al., 1990). There is growing data that *M. paratuberculosis* is responsible for Crohn's disease in humans (Naser et al., 2004, Mendoza et al., 2009). Although, both *M. avium* and *M. paratuberculosis* are considered as slow growing mycobacteria, the latter grows much slower than the former by the doubling time of 22-26 hrs in comparison to 10-12 hrs for *M. avium* (Bannantine et al., 2003).

IV. *Mycobacterium avium* subsp. *hominissuis* is proposed as a taxon to differentiate the organism isolated from humans and pigs from those found in birds (Turenne et al., 2007). This has been proposed since the genetic profile of *M. avium* isolated from humans is not the same as that found in birds (Bono et al., 1995, Ritacco et al., 1998).

The strains of MAC show variability in their colony morphology. The most common morphological forms of MAC strains are smooth transparent, rough opaque and smooth opaque (Reddy et al., 1996). These variant colony forms of MAC differ in respect to their virulence. Smooth transparent variants show the highest level of virulence among the variants (Pedrosa et al., 1994, Reddy et al., 1996).

M. intracellulare has been isolated from various animal sources and is associated with pulmonary infections in immunocompetent patients (Guthertz et al., 1989, Maugein et al., 2005). *M. avium* is isolated from a wider range of species and is more likely to be responsible for disseminated disease in HIV patients (Tsang et al., 1992, Yakrus and Good, 1990).

Identification of strains of MAC can be done based on serology. From a total of 28 serovars belonging to MAC, 11 serovars including serovars 1-6, 8-11 and 21 have been recognized to belong to *M. avium.* Serovars 7, 12-20 and 25 are regarded as *M. intercellulare* (Dorrestein, 1997). The most virulent pathogenic serovars for birds are serovars 1, 2 and 3 (Fulton and Thoen, 2003) of which serovars 1 and 2 have been recovered from domestic chickens and serovar 3 from wild birds (reviewed by Dhama et al., 2011). Several studies have reported geographical variation in distribution of these serovars (Chew et al., 1994, Torrelles et al., 2000, Askgaard et al., 1994). For instance, serovar 6 occurs predominantly in Scandinavia (Hoffner et al., 1990, Askgaard et al., 1994), serovar 4 is predominant in Spain (Torrelles et al., 2000) and serovars 4, 8 and 9 have been reported as the prevalent MAC serovars in the United Kingdom (UK) (DeSmet et al., 1996).

1.3 Immune responses to mycobacteria

The first line of body defence for all mammals and birds preventing entry of pathogens into the body are the physical barriers of skin and mucous membranes in the nose, throat, respiratory tract and gastrointestinal tract. Although, stomach acid also acts as a body defence mechanism, *M. avium* is able resist low pH and infect the intestine (Bermudez et al., 1992). In addition to their function as a physical barrier, airway epithelial cells also play an immunological function through the release of antimicrobial molecules following infection (Li et al., 2012). In man, *M. tuberculosis* has the ability to

escape this early innate response and lead to progressive disease (Tsolaki, 2009). In man and mouse, *M. avium* shows similar behaviour with inhibition of influx of immune cells (Kim et al., 1998), as a result of inhibition or delay in the release of chemokines such as CXCL8 and CCL5 by epithelial cells (Sangari et al., 1999).

A combination of innate and adaptive immunity mediates the immune response to mycobacteria (Saiga et al., 2011) and the initial resistance is the responsibility of innate immunity which is active before and also initiates the development of adaptive cell-mediated immunity. In protection against intracellular bacteria, cell mediated immune response plays a more important role than humoral immunity (Mastroeni et al., 1993).

In general, the basic principle of the immune responses to invading pathogens in birds is similar to that in mammals. In birds as in mammals, two arms of immunity, innate and adaptive, exist with both cell-mediated and humoral types of adaptive immunity (Degen et al., 2005, Powell et al., 2009). Similarly, the immune response in chickens to intracellular pathogens is predominantly Th1 and infection with extracellular pathogens is predominantly Th2 in nature (Kaiser, 2010). However, chickens differ from mammals in a number of aspects of immunity involving organs (absence of draining lymph nodes), tissues, immune genes, cells and molecules (Wu and Kaiser, 2011) which will be shown later in this chapter.

1.3.1 Innate immunity

The outcome of mycobacterial infection is greatly dependent upon the nature of the innate immune response of the host (Kleinnijenhuis et al., 2011, Ramakrishnan, 2012). This response is initiated by recognition of pathogen microbial components, Pathogen Associated Molecular Patterns (PAMPs) which are recognized by cellular germline-encoded Pattern Recognition Receptors (PRRs) (Akira et al., 2006, Handzel, 2013). Activation of innate immunity in chickens also requires pattern recognition of pathogens via receptors especially TLRs (Wigley, 2013). Macrophages, neutrophils, dendritic cells, and natural killer cells are the major classical cells mediating the innate immune response and initiating the adaptive response to human tuberculosis (Sia et al., 2015).

1.3.1.1 Immune cells involved in innate response

1.3.1.1.1 Macrophages

Mycobacteria are internalised through a number of receptors on the macrophage cell surface. *M. tuberculosis* is recognized and taken up by human macrophages mainly through mannose receptors, scavenger receptors and complement receptors (Hirsch et al., 1994, Roecklein et al., 1992).

It has been shown that a complex of macrophages and dendritic cells strategically exist in avian respiratory tract in order to phagocyte foreign antigens (de Geus et al., 2012). Macrophages are one of the first cells which mycobacteria encounter in lung infection. They play a crucial role in the body defence against tuberculosis

through phagocytosis, intracellular killing and presentation of antigens to lymphocytes (Fenton and Vermeulen, 1996, Pieters, 2008). Activated macrophages are highly bactericidal for phagocytosed mycobacteria (Collins, 1991). Macrophages utilise different mechanisms to kill mycobacteria. The bacteria internalised in phagosomes are exposed to different chemicals including reactive nitrogen and oxygen intermediates, fatty acids, oxygenated lipids and lytic enzymes (Aderem and Underhill, 1999, Moreira et al., 1997). It has been suggested that reactive oxygen and nitrogen species production, phagosome acidification and fusion of the phagosome with lysosomes are the most crucial mechanisms of killing mycobacteria (reviewed by van Crevel et al., 2002). Nitric oxide (NO) is involved in various physiological functions in the cells and during infection plays a dual role in that in addition to playing a central role in immunity it also mediates pathogenesis (Widdison et al., 2007). The phagocytosed mycobacteria are immediately killed if the phagocytic macrophages are activated and systemic immune responses do not develop further (reviewed by Mason and Ali, 2004). Some intracellular bacteria, including mycobacteria, are able to escape from macrophage antimicrobial activity. Chemokines such as CXCL8 and CCL5 are produced by infected macrophages and attract monocytes to the site of infection and the monocytes differentiate into macrophages and ingest the mycobacteria. The interaction between host cells and mycobacteria is a complex process and leads to release of chemokines and cytokines including TNF-α, IL-1β and IFN-γ (Cooper et al., 2011). This cytokine network

plays an important role in the immune response to mycobacteria and outcome of the infection (Cooper and Khader, 2008). A summarised overview of the immune response to mycobacterial infection is shown in Figure 1.1.

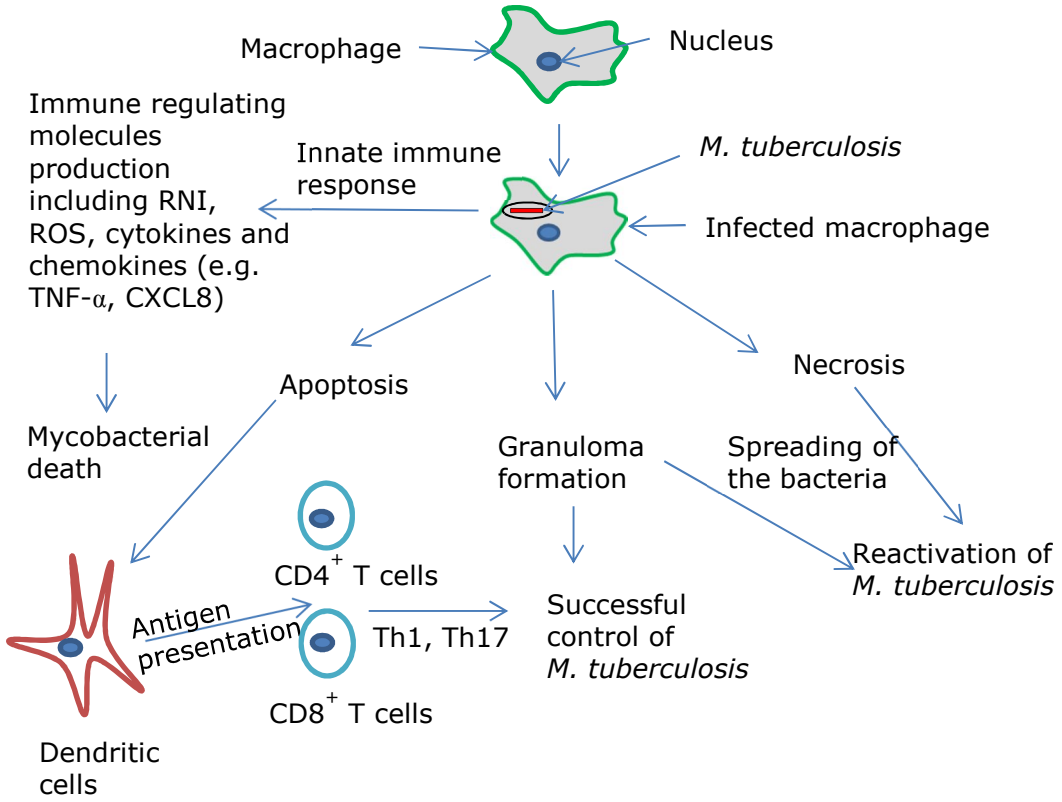


Figure 1.1 Overview of the immune response to mycobacterial infection (modified from Palaga, 2009).

Mycobacteria are up taken by phagocytic cells including macrophages. Following phagocytosis several immune molecules are produced by the phagocytic cells in response to the infection which might lead to mycobacterial death. Antigen presenting cells such as dendritic cells play important role in activation of adaptive immunity through presentation of the bacterial antigens to T cells. Apoptosis of infected macrophages could kill the bacteria and assists the antigen presentation. Necrosis, however might lead to spreading the bacteria in the body.

Intracellular bacterial pathogens, such as mycobacteria, display different strategies for survival and growth within macrophages. *M. tuberculosis* and *M. avium* are able to inhibit maturation of the phagosome and fusion of phagosome-lysosome which is a morphological process required to kill microbes (Vergne et al., 2004, Lambrecht, 2006, Cooper, 2009, Pitarque et al., 2008). They also have the ability to prevent apoptosis of host cells during infection (Keane et al., 2000). Several mechanisms have been suggested for this including manipulation of the Ca^{2+} signalling pathway by the mycobacterial component mannose-capped lipoarabinomannan (Man-LAM) (Rojas et al., 2000), subversion of apoptosis by binding of *M. tuberculosis* to toll-like receptor (TLR)2 (Loeuillet et al., 2006), and control of pro-inflammatory cytokine production as an effect of interleukin (IL)-10 that prevents tumour necrosis factor alpha (TNF-α)-induced apoptosis (Balcewicz-Sablinska et al., 1998).

M. tuberculosis also has the ability to inhibit expression of the major histocompatibility complex (MHC) class II and subsequently prevent processing and presentation of antigen (Ting et al., 1996, Noss et al., 2000). A 19-KDa lipoprotein is known to be the main mycobacterial ligand responsible for inhibiting this process which is done through TLR2 (Noss et al., 2001). The inhibition occurs through MAPK signalling induced through TLR2 producing hypoacetylation of a histone at its class II transactivator pIV promoter. This, results in suppression of transcription of the class II transactivator which is responsible for regulating MHC class II (Pennini et al., 2006).

It is known that stimulation of macrophages and monocytes with mycobacteria or mycobacterial products results in production of TNF-α, an important pro-inflammatory cytokine. It has been reported that macrophages infected with pathogenic mycobacteria produce less TNF-α and other pro-inflammatory cytokines compared with those infected with non-pathogenic mycobacteria (Oliveira et al., 2001, Chakraborty et al., 2013).

Lipoarabinomannan (LAM) has been reported to be able to inhibit maturation of the phagosome when it interacts with the mannose receptor of the host, although this mycobacterial component is mainly recognized by TLR2 (Kang et al., 2005). The ability of mycobacteria to prevent maturation of the phagosome is associated with the p38 pathway (Fratti et al., 2003). It has been reported that Man-LAM inhibits recruitment of phosphoinositide 3-kinases (PI3K) to the mycobacteria-containing phagosome through inhibition of Ca/calmodulin and Rab5 that mediate PI3K recruitment. (Lazarevic and Martinon, 2008).

1.3.1.1.2 Natural killer cells

Natural killer cells also play an important role in the immune response to mycobacterial infection. They express the germ line-encoded receptor NKp46 through which they recognise infected monocytes (Vankayalapati et al., 2002). Natural killer cells appear early in *M. tuberculosis* infection and produce IFN-γ and TNF-α to induce macrophage apoptosis and mycobacterial killing (Allen et al., 2015). These cells have a direct cytotoxic effect through bacterial

lysis by perforin and granulysin produced by cytoplasmic granules (Lu et al., 2014). The role of these cells in bacterial infection in chickens is poorly understood except their antibacterial effect that performed by NK-lysin which is orthologous with human granulysin (Lee et al., 2012).

1.3.1.1.3 Polymorphonuclear cells

Polymorphonuclear cells (PMNs), principally neutrophils, have been shown to be involved in the control of mycobacterial infection in mammals (Fulton et al., 2002). These cells are among the first cells that encounter the bacteria at the site of infection (Pedrosa et al., 2000). They perform an important role in the early immune response through phagocytosis and degranulation by release of granules into the phagosome and subsequent triggering the production of cytokines such as TNF-α which has a number of important immunological functions (see below) (Petrofsky and Bermudez, 1999). Neutrophils also produce reactive oxygen species (ROS) and possess granules which are important in the immune response. Four types of granules have been identified based on their structure, function and content which are produced sequentially during neutrophil maturation, including primary or myeloperoxidase-positive granules, secondary or myeloperoxidase-negative granules, tertiary or gelatinase granules and finally secretory vesicles (Borregaard et al., 2007). Primary granules, which contain antimicrobial peptides and proteolytic enzymes, are involved in killing pathogens when released into the phagocytic vacuoles. Secondary granules contain antimicrobial peptides and

proteases which also have a role in killing pathogens in addition to assessing neutrophil migration. Finally, tertiary and secretory vesicles contain receptors and proteins for adhesion and migration of neutrophils (Borregaard et al., 2007). Neutrophils are thought to extrude a network of chromatin fibres which are associated with antimicrobial peptides and enzymes such as neutrophil elastase and myeloperoxidase (Brinkmann et al., 2004). The structures are called NETs (neutrophil extracellular traps) and have antimicrobial function through trapping the pathogens and exposing them to antimicrobial peptides and enzymes (Mayadas et al., 2014). Neutrophils are also important in the formation of the granuloma during chronic *M. tuberculosis* infection (Seiler et al., 2003).

In the chicken, heterophils are the counterparts of mammalian neutrophils with the capability of killing bacteria via two main mechanisms: degranulation and the oxidative burst (Kogut et al., 2001). Heterophils are also phagocytic; however, their granules are different from mammalian neutrophils as they lack the enzyme myeloperoxidase (Genovese et al., 2013). A network of fibres composed of chromatin and serine proteases can also be released by heterophils (heterophil extracellular traps, HETs) in a similar manner to that by neutrophils. This structure can be used to trap and kill extracellular pathogens (Chuammitri et al., 2009). Expression of cytokines such as IL-1β, IL-6, CXCLi1 and CXCLi2 has also been reported following stimulation of heterophils by pathogens (Kogut et al., 2005, Kogut et al., 2006).

Defensins are antibacterial peptides produced by a variety of host cells including PMNs and epithelial cells (Lehrer et al., 1993, Ganz, 2003). A major function of defensins in bacterial infections is through the formation of pores in the bacterial cell membrane. In addition to this role, β-defensins (see below) are able to attract innate immune cells such as macrophages and mast cells to the site of infection (Soruri et al., 2007). There are three classes of defensins produced by mammals. The first two, α-defensins and β-defensins, are found in all mammals and the third class θ-defensins, found only in non-human primates (Kaiser, 2010). Chickens appear to only produce β-defensins (Lynn et al., 2007). In the chicken β-defensins play an important role in immune responses to various pathogens at mucosal surfaces such as infection of the intestine by *Campylobacter* and *Salmonella* (van Dijk et al., 2007) also by a direct microbicidal effect and by chemotaxis of granulocytes.

1.3.1.1.4 Dendritic cells

Dendritic cells (DC) are the major professional antigen presenting cells and play a pivotal role in antigen presentation to T cells (Marino et al., 2004). In the early stages of mycobacterial infection, these cells are present in large numbers at the site of infection (Tascon et al., 2000, Mohagheghpour et al., 2000). Antigens are taken up by immature dendritic cells resident in the tissues. These cells recognise PAMPs via PRRs, including C-type lectin receptors and TLRs, and process the antigens (Uematsu and Akira, 2006, Takeuchi and Akira, 2010). As a result of this DCs become mature and expression of MHC II and co-stimulatory molecules by these cells is

enhanced (Granucci et al., 1999). The mature cells migrate to draining lymph nodes where they initiate the immune response by stimulation and proliferation of naïve T cells (Banchereau and Steinman, 1998). In the chicken, bone marrow-derived DCs have been cultured and as in mammalian DCs express key markers such as CD11, C-type lectin DEC205, MHC II and CD40 following maturation by LPS (Wu et al., 2010). In addition, MHC II^+ and $CD80^+$ phagocytic cells have been identified in the chicken lung as dendritic cell population, characterised by the absence of decrease in pH of their endosomal compartment following uptake of fluorescently labelled beads coated with either LPS or avian influenza virus (de Geus et al., 2012).

1.3.1.2 Pathogen recognition receptors

A large variety of receptors is expressed on macrophages and these receptors have been shown to play major roles in the uptake and recognition of mycobacteria and initiation of the host innate immune response to these bacteria (Akira et al., 2006). These receptors are involved in the generation of signals that activate macrophages (Akira et al., 2006) in response to pathogens including *M. tuberculosis* (Jo, 2008, Hossain and Norazmi, 2013).

A number of PRRs have also been reported to recognise mycobacteria and induce innate immune response in the host (Saiga et al., 2011). These include C-type lectin receptors, NOD-like receptors and TLRs. Here we will briefly mention the role of each

receptor in the process of the immune response to mycobacterial infection.

1.3.1.2.1 C-type lectin receptors

C-type lectin receptors are expressed on the cell surface of a wide range of antigen-presenting cells such as dendritic cells and macrophages and recognise various PAMPs to initiate the immune response (Kerrigan and Brown, 2009). C-type lectin receptors include mannose receptor, Macrophage Inducible Ca^{2+}-dependent C-type Lectin (Mincle), Dectin-1 and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) which are involved in the initial response to mycobacteria (Killick et al., 2013).

Mannose receptors have been reported previously to regulate phagocytosis of mycobacteria (Kang et al., 2005). Several cell wall components of mycobacteria and other bacteria are recognized by these receptors including LAM and Man-LAM glycolipids (Martinez-Pomares, 2012). These receptors have also been reported to recognise virulent strains of *M. tuberculosis* (Schlesinger et al., 1996). Functional mannose receptors and their ultrastructural localisation on the surface of chicken macrophages have also been reported (Rossi and Himmelhoch, 1983). In addition, recently a homologue of mammalian mannose receptor C-type 1 (CD206), called MRC1L-B, has been identified on chicken macrophages (Staines et al., 2014).

Mincle receptor is an essential receptor for recognition of mycobacterial trehalose-6, 6 dimycolate (TDM) (Ishikawa et al., 2009).

Dectin-1 has been shown to be necessary for TNF-α production by murine bone marrow-derived macrophages in response to infection with less virulent mycobacterial species and strains, such as *M. bovis* Bacille Calmette-Guérin (BCG), *M. smegmatis* and *M. tuberculosis* H37Ra but not to virulent ones including *M. tuberculosis* H37Rv and *M. avium* (Yadav and Schorey, 2006).

DC-SIGN (now designated as CD209) is expressed specifically on the surface of immature dendritic cells and macrophages (Soilleux et al., 2002). This receptor differentially interacts with virulent and avirulent mycobacterial species. It interacts with Man-LAM from *M. tuberculosis* but does not interact with Arabinosylated LAM (Ara-LAM) from *M. smegmatis* (Geijtenbeek et al., 2003). In the chicken, the gene for CD209 has been identified with 28.7% similarity to that in human and 34% to mice (Lin et al., 2009). The existence of cDNA for CD209 in the chicken could lead to further characterisation of dendritic cells in this species (Schultz and Magor, 2008).

1.3.1.2.2 NOD-like receptors

There are two main subfamilies of NOD-like receptors, NOD1/NOD2 and the NALPs (NACHT-, LRR- and pyrin domain containing proteins) (Inohara et al., 2005). Both NOD1 and NOD2 can detect peptidoglycan, a component of the cell wall of bacteria (Kanneganti et al., 2007). NOD2 can also detect muramyl dipeptide which can be

found on both Gram positive and Gram negative bacterial cell wall (Girardin et al., 2003). Impaired cytokine production has been observed in response to *M. tuberculosis* in NOD2-deficient mice (Divangahi et al., 2008). The NALP family comprises at least 14 proteins in humans which are thought to recognise bacterial PAMPs (Tschopp et al., 2003). Genes for both NOD1 and NOD2 exist in the chicken genome while, in contrast, the NALP subfamily of NOD-like receptors is absent in this species (Kaiser, 2010).

1.3.1.2.3 Toll like receptors

TLRs are a family of transmembrane proteins that are expressed on various cells including innate immune cells, such as macrophages (Krutzik and Modlin, 2004), dendritic cells (Kadowaki et al., 2001) and epithelial cells. TLRs are not only important in host innate immunity but they also initiate and enhance adaptive immunity (Iwasaki and Medzhitov, 2004). In mammalian cells at least twelve TLRs have been identified, with each of them responding to a variety of PAMPs (Akira et al., 2006, Rock et al., 1998). Some differences exist between TLRs in many aspects such as the type of cells that they are expressed on, species specificity, their specificity to a ligand, induction of cellular response and utilization of signalling adaptors (Iwasaki and Medzhitov, 2004, Keestra et al., 2010).

Several TLRs are involved in recognition and interaction of mycobacteria and their components (Table 1.1). For example, TLR2 interacts with lipomannan, LAM, 19kDa lipoprotein and phosphatidyl-myo-inositol mannoside (PIM) from mycobacteria in

human and mice (Aliprantis et al., 1999, Brightbill et al., 1999, Ferwerda et al., 2005). TLR2 also cooperates with other receptors to recognise PAMPs (reviewed by Coffey and Werling, 2011). This receptor can form a heterodimer with TLR1 or TLR6. The TLR2/TLR1 heterodimer interacts with diacylated lipoproteins and the heterodimer with TLR6 interacts with triacylated lipoproteins (Jones et al., 2001b, Thoma-Uszynski et al., 2001). In addition, mycobacterial DNA is recognized by TLR9 (Hemmi et al., 2000). TLR4 is also assumed to interact with mycobacterial components (Saiga et al., 2011) such as 38KDa glycolipoprotein which can interact with both TLR4 and TLR2 (Jung et al., 2006). Means et al. (1999) found that heat-labile mycobacterial components selectively activate cells via TLR4. In addition, mycobacterial heat shock protein 65 has been reported to activate cells through TLR4 (Bulut et al., 2005).

In chickens, at least 10 TLR genes have been recognised. However, in comparison to mammals, these genes express a different repertoire (Brownlie and Allan, 2011). In addition to TLRs 1, 3, 4, 5, and 7, two types of TLR1 named TLR1LA (TLR16), and TLR1LB and two types of TLR2, TLR2A and TLR2B are expressed in chicken as a result of gene duplication (Temperley et al., 2008). Both TLR2A and TLR2B can form functional heterodimer with both types of TLR1 in the chicken (Keestra et al., 2007, Higuchi et al., 2008). However, the TLR8 gene is disrupted due to insertion of a retroviral-like insertion element (Philbin et al., 2005) and TLR9 is also absent in this species (Keestra et al., 2013). Furthermore, TLR15 appears to

be specifically expressed in avian species (Higgs et al., 2006). In the chicken, TLR21 is a functional orthologue of TLR9 in mammals and is able to recognise unmethylated CpG DNA (Brownlie et al., 2009, Keestra et al., 2010).

Table 1.1: Recognition of mycobacterial ligands by TLR family.

Ligand	Mycobacteria species	TLRs	Reference
Lipoproteins (19, 27,33 kDa)	*M. tuberculosis, M. leprae, M. bovis,*	TLR2	(Noss et al., 2001, Krutzik et al., 2003, Hovav et al., 2004)
Glycolipoprotein (38 kDa)	*M. tuberculosis*	TLR2,TLR4	(Jung et al., 2006)
Gylcopeptidolipid (GPL)	*M. avium*	TLR2	(Sweet and Schorey, 2006)
Heat shock proteins (HSP65, HSP70)	*M. tuberculosis*	TLR2,TLR4	(Bulut et al., 2005)
Lipomannan (LM)	*M. tuberculosis, M. bovis*	TLR2	(Quesniaux et al., 2004)
Ara-LAM	*M. smegmatis*	TLR2	(Wieland et al., 2004)
PILAM	*M. smegmatis*	TLR2	(Heldwein and Fenton, 2002)
PIM 2, PIM 6	*M. tuberculosis, M. bovis, M. smegmatis*	TLR2	(Gilleron et al., 2003)
DNA	All mycobacteria	TLR9	(Hemmi et al., 2000)

Various TLRs are responsible for recognising and interacting with mycobacteria and their components. Abbreviation: Toll like receptor (TLR), lipomannan (LM), glycopeptidolipid (GPL), arabinosylated Lipoarabinomannan (Ara-LAM), Phosphoinositol-capped LAM (PILAM), Phosphatidyl-myo-inositol mannoside (PIM), Deoxyribonucleic acid (DNA).

Engagement of TLRs leads to activation and translocation of transcriptional factors and subsequent transcription of other genes involved in regulation of the immune response. The process can result in production of several pro-inflammatory cytokines, NO, chemokines and defensins (Underhill et al., 1999, Iwasaki and Medzhitov, 2004). Signal transduction is initiated by binding of the myeloid differentiation factor 88 (MyD88) to the Toll/IL-1 receptor (TIR) domains of TLRs. This recruits many other factors including IL-1 receptor-associated kinases (IRAK), TNF receptor-associated factor (TRAF)6, mitogen-activated protein kinase (MAPK) and followed by activation and translocation of transcriptional factors including nuclear factor (NF)κB (Takeda and Akira, 2004, Akira et al., 2003) (Figure 1.2). A MyD88 adaptor is necessary for most TLR signalling pathways; however TLR3 and TLR4 can transmit signals in a MyD88-independent pathway (Kawai et al., 1999, Hoebe et al., 2003). TLR4 can use alternative adaptor molecules including the TIR domain–containing adapter inducing IFN-β (TRIF) and the TRIF-related adapter molecule (TRAM) which can also lead to activation of NFκB and responses which differ from those elicited by the MyD88-dependent pathway (Doherty and Arditi, 2004).

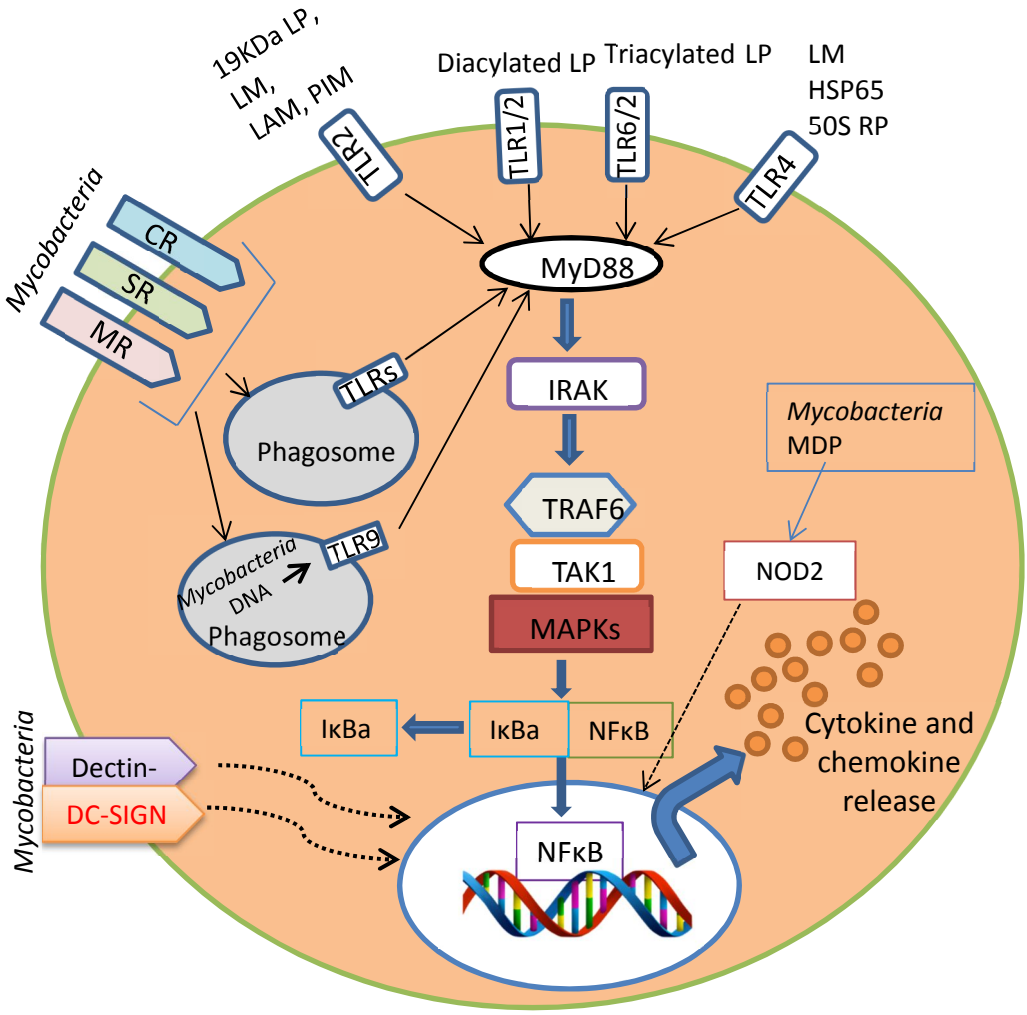


Figure 1.2: Immune recognition of *M. tuberculosis* during phagocytosis and TLRs activation by *M. tuberculosis* and its ligands (modified from Hossain and Norazmi, 2013).

Mycobacteria and its microbial components are recognised by TLRs. The signal transduction is initiated by binding of the MyD88 to the TIR domains of TLRs. This recruits many other factors including IRAK, TRAF6, MAPK and followed by activation and translocation of transcriptional factors including NFκB. Translocation of NFκB into the nucleus leads to transcription of genes involved in regulation of the immune response and subsequent production of several pro-inflammatory cytokines, NO, chemokines and defensins. Abbreviations: Toll like receptors (TLRs), myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor (TIR), IL-1 receptor-associated kinases (IRAK), TNF receptor-associated factor (TRAF)6, mitogen-activated protein kinase (MAPK), nuclear factor (NF)κB.

1.3.1.2.4 Complement receptors

Complement receptors (CRs) interact with complement components that are important for mycobacterial opsonisation before the process of phagocytosis. CR1, CR3 and CR4 are the main CRs involved in phagocytosis of mycobacteria (Ferguson et al., 2004). C3 promotes the uptake of mycobacteria through binding to the mycobacterial surface and phagocytosis occurs via CR1, CR3 and CR4 (Schlesinger, 1993). It is likely that the key role in the process of mycobacterial phagocytosis through CRs is played by CR3 as its absence results in reduction of *M. tuberculosis* uptake by macrophages of up to 80% (Schlesinger et al., 1990). The involvement of non-activating macrophage CRs in mycobacteria cell entry is an advantage to the bacteria since using these receptors does not lead to production of reactive oxygen intermediates (Wright and Silverstein, 1983). In chicken heterophils, CR1 and CR3 are the major CRs that play an important role in phagocytosis of opsonised particles by complement (Kogut et al., 2003). CRs play an important role in degranulation by heterophils, a process that is regulated by tyrosine kinase-dependent phosphorylation (Kogut et al., 2001).

1.3.1.2.5 Scavenger receptors

Scavenger receptors (SRs) are transmembrane glycoproteins that have the ability to bind a wide range of ligands including lipopolysaccharide (LPS) from Gram negative and lipoteichoic acid from Gram positive bacteria (Krieger et al., 1993, Dunne et al., 1994). Although Zimmerli et al. (1996) found that class A SRs might

play a role in uptake of *M. tuberculosis* by human macrophages, SRs generally play a relatively less important role in phagocytosis of mycobacteria.

1.3.1.3 Cytokine and chemokine production by immune cells during mycobacterial infection

Recognition and phagocytosis of mycobacteria by macrophages initiates a series of complex events in order to control intracellular bacillary growth. This is performed through a complex signalling cascade and production of a number of innate immune mediators (Kaisho and Akira, 2000). These mediators play a crucial role in the immune response to mycobacteria both at early and more chronic stages of the infection (Flynn and Chan, 2001). Several studies have indicated the importance of the cytokines produced by macrophages such as TNF-α, IL-6, IL-1α, IL-1β, IL-12 and IL-10 in control of *M. tuberculosis* infection (Wang et al., 2010, Sarkar et al., 2012, Chakraborty et al., 2013, Guirado et al., 2013). In addition, the importance of the production of different cytokines and chemokines by human THP-1 and chicken HD11 cells in response to other bacteria have also been shown previously (Jones et al., 2003, Smith et al., 2005, Setta et al., 2012). A schematic of the interaction between cytokines and the relevant cellular components of the immune system, as described in the following sections, is shown in the following Figure 1.3.

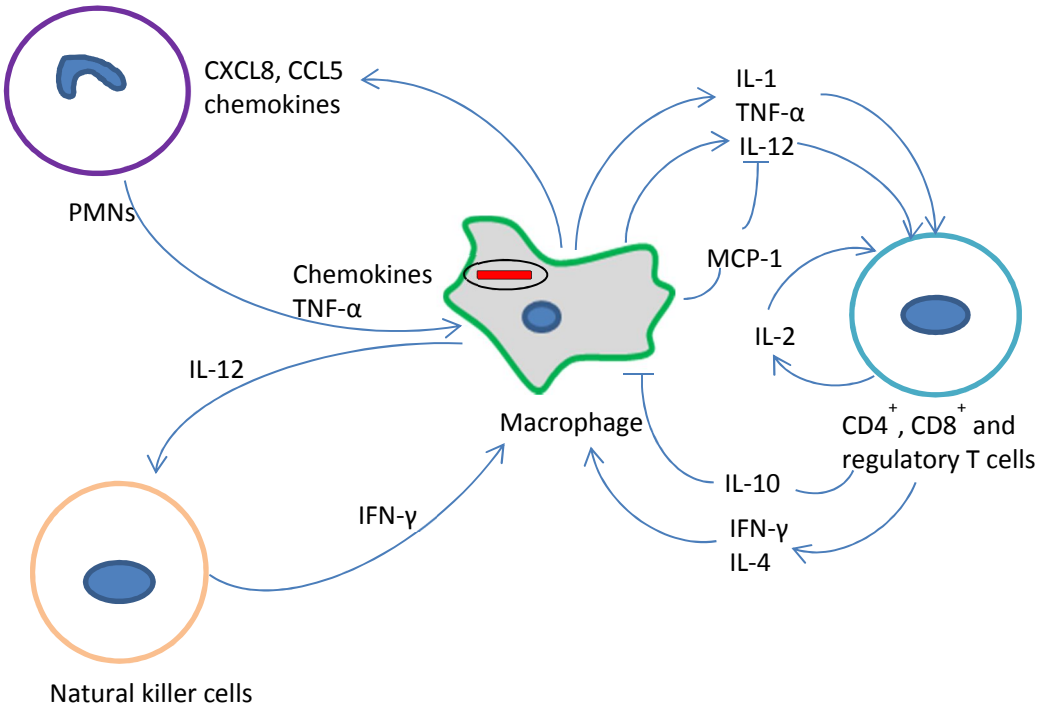


Figure 1.3: Immune responses to *M. tuberculosis* at the cellular level (modified from Berrington and Hawn, 2007).

Following mycobacterial infection, various cytokines are released from activated macrophages which in turn activate other immune cells including T cells, macrophages, natural killer cells and neutrophils. Abbreviations: polymorphonuclear cells (PMNs), tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), interferon gamma (IFN-γ), monocyte chemotactic protein-1 (MCP-1).

1.3.1.3.1 Tumour necrosis factor alpha (TNF-α)

The mammalian TNF superfamily is composed of TNF ligands and their corresponding receptors which include 19 ligands and 29 receptors, with identification of only 10 ligands and 15 receptors in chickens (Sutton et al., 2015). TNF-α is one of the most crucial pro-inflammatory cytokines secreted during the early immune response to *M. avium*. It is predominantly produced by macrophages, monocytes and dendritic cells in response to stimulation with mycobacteria or their products (Ottenhoff et al., 1998). This

cytokine has been suggested to play an essential role in phagocyte activation (Orme and Cooper, 1999, Cooper, 2009), the induction of apoptosis (Balcewicz-Sablinska et al., 1999) and formation and maintenance of granulomata in mycobacterial infections (Roach et al., 2002, Algood et al., 2005). Inhibited granuloma formation, increase in number of mycobacteria and subsequently reduction in survival time have been observed in mice deficient in TNF-α (Bean et al., 1999, Flynn et al., 1995). The production level of this cytokine is inversely related to the level of mycobacterial pathogenicity (Roach and Schorey, 2002). In comparison to mammals, a decreased repertoire for the TNF superfamily has been recognized following genome analysis and an avian homologue of TNF-α has not been identified in the chicken genome (Kaiser et al., 2005). However, a TNF-α-like response has been demonstrated by avian macrophages in response to stimulation by *Eimeria tenella* or LPS (Zhang et al., 1995, Rautenschlein et al., 1999). In addition, a similar response has been detected in chicken following *Clostridium perfringens* infection through expression of TNF-α-inducing factor homologue (Lu et al., 2009). This inconsistency could be due to the identification of TL1a which is a TNF-α-related member of TNF family (Kaspers et al., 2008). The recombinant TL1a production in chicken cells has been reported in response to protein LPS-induced TNF-α factor (Hong et al., 2006). The absence of lymphotoxin genes and TNF-α in the chicken genome could be linked to the absence of lymph nodes in this species (Kaiser, 2012).

1.3.1.3.2 Interleukin 1 beta (IL-1β)

This cytokine is produced by macrophages, monocytes and dendritic cells. Signals mediated by IL-1 are crucial components of the host defence mechanism to mycobacteria (Fremond et al., 2007, Yamada et al., 2000). In addition, deficiency in IL-1 receptor in mice leads to their rapid death after challenge with low doses of *M. tuberculosis* (Fremond et al., 2007, Mayer-Barber et al., 2010). This cytokine may also have a role in regulation of production of inducible nitric oxide synthase (iNOS) (Chan et al., 2001), maturation and acidification of the phagosome and expression of adhesion molecules (Dinarello, 2009). In addition, it can regulate activation of various enzymes including phospholipase A and cyclo-oxygenase (Hernandez-Pando et al., 2006). Enhanced mycobacterial outgrowth and defective granuloma formation were observed in mice deficient in IL-1R type I infected with *M. tuberculosis* (Juffermans et al., 2000). A homologue in the chicken of mammalian IL-1β has also been cloned (Weining et al., 1998) and enhanced expression of mRNA level of this pro-inflammatory cytokine has been reported in chicken in response to *Salmonella* (Beal et al., 2004).

1.3.1.3.3 Interleukin 6 (IL-6)

Both pro- and anti-inflammatory properties have been reported for IL-6 (VanHeyningen et al., 1997). This cytokine is produced by macrophages and many other cell types including T cells and fibroblasts (Kishimoto et al., 1992). Although IL-6 can suppress TNF-α and IL-1β production (Schindler et al., 1990) and enhance *in vitro* growth of *M. avium* (Shiratsuchi et al., 1991), this cytokine has

a protective role in mycobacterial infection. Mice deficient in IL-6 showed an increase in *M. tuberculosis* susceptibility (Ladel et al., 1997). Production of this cytokine by macrophages infected with *M. avium* is essential for a T cell-mediated immune response (Appelberg et al., 1994). cDNA encoding IL-6 has been identified in the chicken (Schneider et al., 2001) and a strong IL-6 response to infection with *Salmonella* has been reported in chicken kidney epithelial cells (Setta et al., 2012).

1.3.1.3.4 CXCL8

A number of chemokines are released from macrophages infected with mycobacteria. Several reports have focused on the role of CXCL8 (IL-8). This chemokine plays a central role in attracting and activating many other immune cells including neutrophils (Oppenheim et al., 1991), T lymphocytes, eosinophils and basophils (Baggiolini et al., 1994) and possibly monocytes (Gerszten et al., 1999). An *in vivo* study suggested an essential role for this chemokine in the immune response to *M. tuberculosis*, as anti-CXCL8 antibody inhibits formation of granuloma (Larsen et al., 1995). This chemokine also plays an important role in the immune response to bovine tuberculosis (Widdison et al., 2009). Recruitment of leukocytes to the site of *M. tuberculosis* infection particularly to the site of formation of the granuloma is the main role of this chemokine (Gerszten et al., 1999). In chickens, CXCLi1 and CXCLi2 have been reported as two homologues of human CXCL8 with similar biological functions (Poh et al., 2008).

1.3.1.3.5 Interferon gamma (IFN-γ)

Interferon (IFN)-γ is mainly released from activated T cells and natural killer cells and is important in activation of the anti-mycobacterial activity of macrophages (Sugawara et al., 1998). This cytokine activates iNOS synergistically with TNF-α (Saito and Nakano, 1996). It also stimulates acidification of the phagosome and is necessary to control replication of mycobacteria (Schaible et al., 1998). High levels of this cytokine are associated with the clearance of intracellular bacteria such as *Salmonella* (Beal et al., 2004) and a strong T cell response (Beal et al., 2005). IFN-γ promotes the antigen presentation process by induction of expression of MHC class I and II and enhancing differentiation of $CD4^+$ T cells into T-helper cell-1 (Th1) (Flesch et al., 1995, Fenton et al., 1997). Inactivation of the IFN-γ gene in mice results in severe disseminated *M. tuberculosis* infection (Cooper et al., 1993).

1.3.1.3.6 Inducible nitric oxide synthase (iNOS)

The iNOS enzyme is responsible for producing NO which is bactericidal (MacMicking et al., 1997, Garcia et al., 2000). It has been reported that production of NO by macrophage is a critical requirement to control growth of mycobacteria in mice (Olleros et al., 2005) and human alveolar macrophages (Nozaki et al., 1997, Rockett et al., 1998). In addition, the *iNOS* gene plays a critical role in the control of tuberculosis in the murine model of infection since a mouse strain with a genetic disruption in *iNOS* ($iNOS^{-/-}$) is highly susceptible to infection with *M. tuberculosis* (MacMicking et al., 1997). Furthermore, Jagannath et al. (1998), demonstrated that

inhibition of iNOS by *N*-monomethylarginine resulted in an increase in *M. tuberculosis* growth in human monocytes. Recently, Landes et al. (2015), found that expression of iNOS and NO in human macrophages in response to *M. tuberculosis* infection is dependent on expression of the NOD2 receptor.

1.3.1.3.7 Interleukin 12 (IL-12)

This cytokine is mainly produced by macrophages and dendritic cells following phagocytosis of mycobacteria. It consists of two subunits, p35 and p40. Production of IFN-γ by $CD4^+$ and $CD8^+$ T cells is mainly induced by IL-12, suggesting a crucial role for this cytokine in development of a Th1 response to mycobacterial infection (Flynn and Chan, 2001, Ngai et al., 2007). Reduction in IFN-γ production and increased susceptibility to infection with BCG and environmental mycobacteria has been observed in humans with mutations in the IL-12p40 and IL-12 receptor genes (Casanova and Abel, 2002). Mice deficient in IL-12 have also been found to be highly susceptible to infection with mycobacteria (Cooper et al., 1997). In chickens, the cDNA sequences for both IL-12 subunits have been identified with homologies in their amino acid sequences to the corresponding subunits from various species such as human and mice ranging between 21% to 42% for IL-12p35 and IL-12p40, respectively. However, a remarkable degree of functional similarity has been reported between chicken and mammalian IL-12 (Degen et al., 2004).

1.3.1.3.8 Interleukin 10 (IL-10)

This is an important anti-inflammatory cytokine which, with transforming growth factor beta (TGF-β), is released to counter the effect of pro-inflammatory cytokines (reviewed by van Crevel et al., 2002). These anti-inflammatory cytokines inhibit activation of T cells which results in inhibition of IFN-γ production through prevention of IL-2 transcriptional activation which is essential in proliferation of T cells (Rojas et al., 1999).

In contrast to the numerous detailed studies on the human immune response to *M. tuberculosis*, relatively very little is known about the chicken immune response to *M. avium,* particularly the role of each cytokine and chemokine which needs to be further elucidated using cells from the target host.

1.3.1.4 Apoptosis

It is known that in *M. tuberculosis* infection host cell death is important in controlling the infection by macrophages and defining the outcome of the disease (Lee et al., 2009). Different types of cell death can be observed in macrophages infected with *M. tuberculosis* including apoptosis and necrosis (Butler et al., 2012). Apoptosis or programmed cell death is an ordered degradation and energy-dependent process and is mediated by cleavage of and activation of pro-caspase3 into caspase3 (Riedl and Shi, 2004). Apoptosis has been reported in the chicken-derived HD11 cells resulting from infection with *Salmonella* Typhimurium (Babu et al., 2009, Xie et al., 2003) and stimulation with synthetic CpG (Xie et al., 2003).

Apoptosis of peripheral blood lymphocytes has also been reported in chickens as a result of infection with infectious bursal disease virus (Vasconcelos and Lam, 1994).

1.3.1.5 Necrosis

Necrosis, in contrast, is energy independent and a disordered cell-death process that does not involve activation of caspase, although recently it has been suggested that necrotic cell death can also follow a programmed event (Golstein and Kroemer, 2007). It is known that in macrophages infected with *M. tuberculosis*, apoptosis results in death of the intracellular mycobacteria (Molloy et al., 1994, Lopez et al., 2003b). Apoptotic cells infected with mycobacteria are phagocytosed by healthy macrophages to control further bacterial growth (Molloy et al., 1994). This event can also enhance T-cell responses through antigen presentation (Winau et al., 2004). So, in infection with intracellular bacteria such as mycobacteria, apoptosis might be beneficial for the host through the removal of a suitable environment for bacterial growth (Fratazzi et al., 1999). This process occurs more readily in alveolar macrophages infected with avirulent mycobacteria such as *M. tuberculosis* H37Ra, BCG and *M. kansasii* than in those infected with virulent mycobacteria such as *M. tuberculosis* H37Rv and Erdman (Keane et al., 2000). Chen et al. (2008) demonstrated that eicosanoids regulate cellular death during mycobacterial infection; prostaglandin E2 (PGE2) stimulates apoptosis and LipoxinA4 induce necrosis. They found that virulent *M. tuberculosis* are able to induce LipoxinA4 and inhibit PGE2 (Chen et al., 2008). This suggests that

control of apoptosis in mycobacterial infection is associated with the virulence of mycobacterial species and strains (Keane et al., 2000). Autophagy is another type of cell death in which cytoplasmic components are sequestered in a double membrane to form an autophagosome which then fuses with a lysosome and resulting in degradation of the enclosed contents (Levine and Deretic, 2007, Klionsky et al., 2007). In the chickens, cell death through necrosis has been reported in a hepatoma cell line in response to infection by low pathogenic influenza virus (Mosavi et al., 2015).

1.3.1.6 Autophagy

Autophagy is regulated mainly by class I and class III PI3K, serine/threonine kinases and the mammalian target of rapamycin (Levine and Deretic, 2007). In mycobacterial infection induction of autophagy stimulates phagosome maturation and leads to inhibition of survival of *M. tuberculosis* and BCG in macrophages (Gutierrez et al., 2004, Delgado et al., 2008). Activation of macrophages with IFN-γ can induce autophagy resulting in elimination of mycobacteria (Gutierrez et al., 2004). Autophagy has been demonstrated in chicken DF-1 cells and in primary chicken embryo fibroblast cells following Newcastle disease virus infection which was shown to be beneficial for the viral replication (Sun et al., 2014).

1.3.1.7 Mitogen activated protein kinesis signalling pathway superfamily

The mitogen-activated protein kinases (MAPK) pathway superfamily plays a crucial role in initiating the control of mycobacterial growth

in macrophages. This signaling pathway superfamily is composed of at least seven distinct subfamilies including; extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 MAPK (p38 α/β/γ/δ), ERK3/4, ERK5, stress activated protein kinase/c-Jun N-terminal kinase (SAPK/ JNK1/2/3), ERK7/8 and Nemo-like kinase pathways (Arthur and Ley, 2013, Peti and Page, 2013). Among these, ERK1/2, p38 and JNKs are the most important and extensively studied pathways (Peng et al., 2014). Activation of these pathways occurs through phosphorylation by a group of MAPK kinases (MKK) (Means et al., 2000). These pathways are important in cytokine gene expression and are activated upon binding of macrophage growth factor and specific cytokine receptors. In addition, they play an important role in various cellular functions including biosynthesis of cytokines (Kovalovsky et al., 2000), reorganization of cytoskeleton (Guay et al., 1997), cellular proliferation (Pages et al., 1993) and apoptosis (Cross et al., 2000). Furthermore, MAPK pathways play a pivotal role in the regulation of many antimicrobial mechanisms including acidification of the phagosome and antigen presentation via expression of MHC class II (Jo et al., 2007). Whole mycobacteria or their components can stimulate activation of MAPK pathways through TLRs which subsequently phosphorylate and activate transcriptional factors such as NFκB and activator protein-1 (AP-1) (Wang et al., 2001, Pathak et al., 2004) (Figure 1.4). However, pathogenic and non-pathogenic mycobacteria can differentially activate MAPK (Roach and Schorey, 2002). There are accumulating data that production of immune effector molecules

such as TNF-α, NO and IL-1β is greatly dependent up on activation of the MAPK pathways in stimulated cells (Roach and Schorey, 2002, Yadav et al., 2004). MAPK is one of the main signalling pathways which activation in macrophages results in production of pro-inflammatory cytokines and chemokines and pathogenic mycobacteria may modulate this pathway and thus modulate cytokine production (Reiling et al., 2001, Roach and Schorey, 2002). The ability of mycobacteria to prevent phagosome maturation is associated with the p38 pathway and inhibition of this pathway has been reported to elevate acidification of the phagosome (Fratti et al., 2003). In addition, this inhibition has increased the ability of macrophages to kill intracellular *M. paratuberculosis* (Souza et al., 2006).

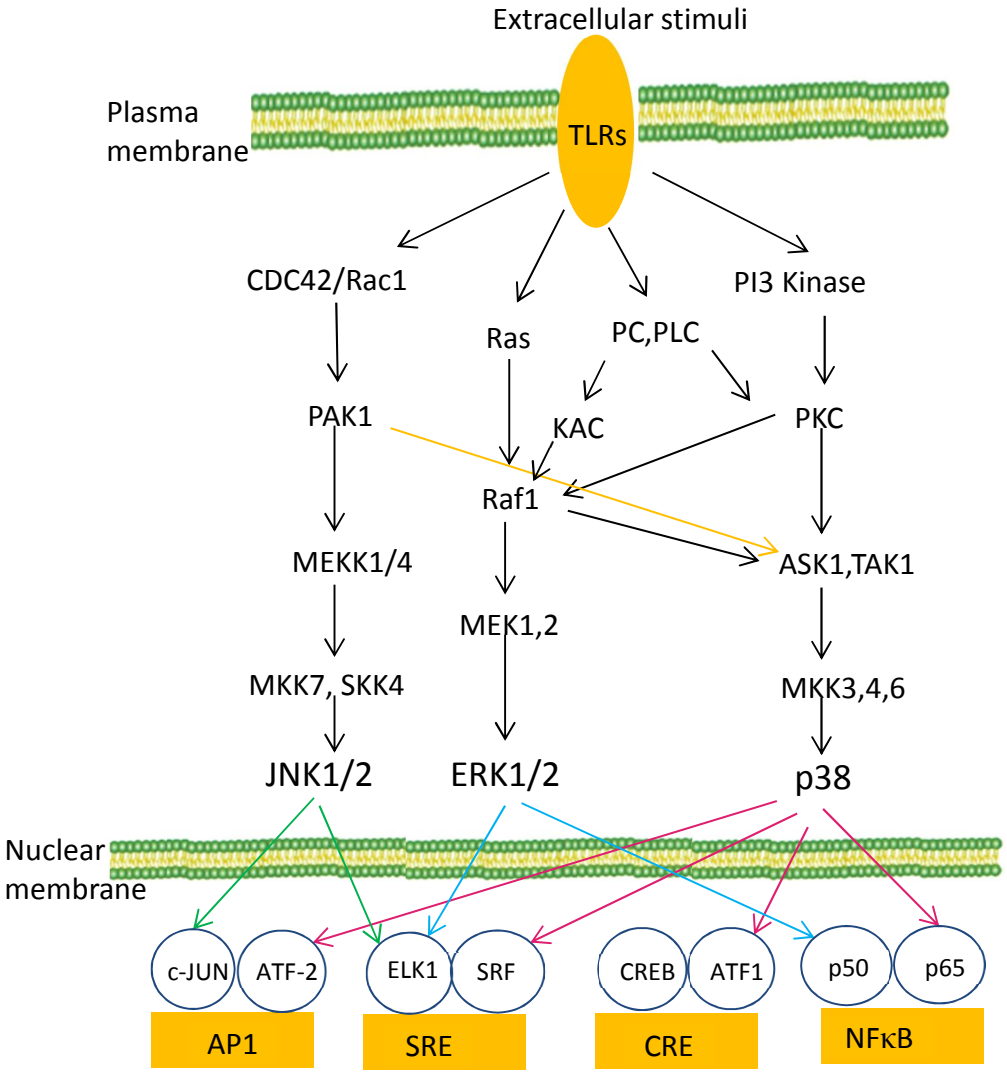


Figure 1.4: Activation of mitogen activated protein kinases through activation of TLRs (modified from Schorey and Cooper, 2003).

Abbreviations: activated protein 1 (AP1); apoptosis signal regulating kinase (ASK); activating transcription factor (ATF); Cell division cycle protein 42 (CDC42); cAMP response element (CRE); cAMP response element binding protein (CREB); Ets domain protein (ELK); extracellular signal-regulated kinase (ERK); Jun N-terminal kinase (JNK); ERK kinase (MEK); MEK kinase (MEKK); MAPK kinase (MKK); nuclear factor kB (NFκB); p21 activated kinase (PAK1); phosphatidylcholine-specific phospholipase C; PI-3-kinase, phosphoinositide-3-kinase; PKC, protein kinase C (PC-PLC); Raf protooncogene S/T protein kinase (Raf1); serum response element (SRE); serum response factor (SRF); TGFβ-activated kinase (TAK1); Toll like receptors (TLRs).

The main three members of MAPK are regulated by various upstream kinases. ERK kinase (MEK) and MKK2 activate ERK, MKK3, MKK4 and MKK6 activate p38 and finally, MKK4 and MKK7 activate JNK pathway (Raingeaud et al., 1996, Pearson et al., 2001). Cyclic AMP/protein kinase A (cAMP/PKA) and Ca^{2+}/calmodulin/Ca^{2+}-calmodulin-dependent protein kinase are two main pathways that regulate macrophage MAPK activity during mycobacterial infections (Liebmann, 2001, Torgersen et al., 2002). Activation of cAMP/PKA can lead to phosphorylation of a number of proteins including other kinases, phosphatases and transcriptional factors (Kopperud et al., 2003). This pathway has also been reported to activate and suppress a number of cytokines (Galea and Feinstein, 1999). The Ca^{2+}/calmodulin/Ca^{2+}-calmodulin-dependent protein kinase pathway is also able to activate several transcriptional factors that are involved in immune regulation including ATF-2, c-JUN and ELK-1 (Enslen et al., 1996). These two pathways, upstream of the ERK1/2 pathway, can be activated in macrophages infected with *M. avium* and *M. smegmatis* (Yadav et al., 2004). It has also been found that constant activation of ERK1/2 and elevation of TNF-α production in macrophages infected with *M. smegmatis* is dependent upon activation of these two pathways (Yadav et al., 2004).

The signals from receptors on the cell surface can activate the ERK1/2 pathway (Marshall, 1995). This pathway is important in activation of cellular immunity that leads to regulation of cytokine gene transcription, translation and many other functions (Karin, 1995). The signals to this pathway are produced by different

upstream stimuli that act on the Ras family of small G proteins (Campbell et al., 1998). Ras activates downstream effectors of the Raf-MEK-ERK cascade (Rausch and Marshall, 1999). PD98059 is a specific inhibitor of the ERK pathway. This inhibitor is known to block ERK activation via upstream ERK kinases MEK1 and MEK2 (Dudley et al., 1995). U0126 is also a selective inhibitor of ERK with higher affinity to MEK1 (Favata et al., 1998). SB203580 inhibits activation of p38 through binding to the ATP binding site and subsequently prevents phosphorylation of downstream MAPK-activated protein kinase 2 which is an important physiological substrate of p38, and inhibits activity of ATF-2 transcription factor (Young et al., 1997). SP600125 competes with ATP and subsequently inhibits phosphorylation of c-Jun (Bennett et al., 2001).

Engagement of IRAK4 to MyD88 occurs after binding of the microbial ligands to TLRs. Then a complex is formed by IRAK4 with the related kinases IRAK1, IRAK2, TRAF6 and E2 ubiquitin-conjugating enzyme 13 (UBC13). Then MAPK and NFκB are activated as a result of catalyzing the formation of k63-linked polyubiquitin chains by UBC13 and TRAF6 (Newton and Dixit, 2012). NFκB activation plays an important role in transcription of pro-inflammatory cytokines and fusion of phagosome and lysosome (Yamamoto and Takeda, 2008). Activation of this transcriptional factor is dependent upon TLR2 and MyD88, and does not occur in mice deficient in TLR2 (Sampaio et al., 2008). Stimulation of TLR2 can activate signal transduction pathways including MAPK.

Activation of MAPK may lead to phosphorylation of IκBα, the inhibitor of NFκB. This could lead to separation of IκBα from the IκBα-NFκB complex and release into the cytoplasm. In the cytoplasm, IκBα degradation occurs by the proteasome followed by NFκB translocation to the nucleus (reviewed in Rocco and Irani, 2011).

In production of TNF-α several transcriptional factors are involved. Many of these transcriptional factors are activated by MAPK. For instance, ERK1/2 can mediate activation of Ets-1 (Seidel and Graves, 2002), JNK is required for phosphorylation of c-JUN and finally activation of ATF-2 and CREB require activation of p38 or JNK (Whitmarsh and Davis, 1996, Roach et al., 2005). There is a great deal of published information on the regulation of signaling transduction in response to LPS (Kawai et al., 1999, Lomaga et al., 1999, Guha and Mackman, 2001, Heldwein and Fenton, 2002) but very little is known on regulation in response to *M. avium* infection.

It has been reported that ligation with TLR2 of PIM isolated from *M. tuberculosis* and AraLAM isolated from avirulent mycobacteria can lead to activation of ERK1/2 pathway and subsequently phosphorylation and activation of the NFκB and AP1 transcriptional factors in the RAW264.7 murine macrophage cell line (Jones et al., 2001a).

1.3.2 Adaptive immunity

Although tubercle bacilli can be removed by the innate immune system, if this does not occur then a cell mediated response will

begin. In the case of *M. bovis*, the dose may affect the type of immunity. High numbers of the pathogen may lead to a cell mediated immune response and subsequently production of antibodies against *M. bovis*, whereas a lower dose of bacteria may lead to a more gradual development of the cell mediated immunity with little or no antibody production (Boddu-Jasmine et al., 2008). Normally, adaptive immunity initiates with contributions from three processes, namely presentation of antigen by different immune cells, co-stimulation and production of cytokines (Sohal et al., 2008).

T cells are also effector cells in the immune response to mycobacterial infection. In mycobacterial infection both $CD4^+$ and $CD8^+$ cells play a role in combating the bacteria and regulating the adaptive immunity (Grotzke and Lewinsohn, 2005). Previously two subsets of $CD4^+$ cells were known, Th1 which are mainly responsible for producing IFN-γ and Th2 which produce IL-4 and IL-10 (Broere et al., 2011). Another subset of these cells is Th17 cells which are able to produce IL-17 (Lyakh et al., 2008). These cells stimulate an influx of other immune cells including Th1, monocytes and neutrophils to the site of infection to be involved in the process of immunity against tuberculosis (Khader et al., 2007). Recently, Gopal et al. (2014) showed that IL-17 is important role in the early immune response to hypervirulent strain HN878 of *M. tuberculosis*, while this cytokine in not necessary for protection against normally virulent strains such as *M. tuberculosis* H37Rv.

A further subset, Th9, of $CD4^+$ cells has been described that are distinct from other Th cells and potentially produce IL-9 and IL-10 (Veldhoen et al., 2008). Th9 cells can be differentiated from naïve T cells by combined action of IL-4 and TGF-β (Dardalhon et al., 2008). In immunity, these cells seem to play similar functional role as Th2 cells including the promotion of allergic inflammation and the immune response to helminthic parasites (Chang and Kaplan, 2015). An increase in Th9 cells with the phenotype of effector memory cells in human tuberculosis has been reported with a collaborative immune regulation function between these cells and pleural mesothelial cells (Ye et al., 2012).

Th1 is one of the important cell types involved in immune response in mycobacterial infection (Delogu and Fadda, 2009). Following secretion of IL-12 from macrophages and dendritic cells, naïve T cells are differentiated into Th1 and these cells perform their action by production of IFN-γ which activates macrophages to become more bactericidal (Flynn et al., 1993, Cooper et al., 1997).

T regulatory cells are a further subset of $CD4^+$ T cells that express CD25 on their surface in addition to expression of FoxP3 as a transcriptional regulator (Hori et al., 2003). Proliferation and influx of these cells to the site of *M. tuberculosis* infection have been reported (Scott-Browne et al., 2007) with the function of prevention of efficient clearance of *M. tuberculosis* infection in mice (Kursar et al., 2007). These cells have capability to produce IL-10 and TGF-β and inhibit effective immune response through inhibition of expression of IFN-γ by $CD4^+$ and $CD8^+$ T cells (Garg et al., 2008).

These have a predominantly anti-inflammatory effect which reduces tissue damage.

$CD4^+$ and $CD8^+$ T cells are significant lymphocytes in controlling growth of *M. tuberculosis* (Grotzke and Lewinsohn, 2005). IFN-γ is produced by both $CD4^+$ and $CD8^+$ T cells (Lalvani et al., 1998, Mikkelsen et al., 2011). Depletion of $CD4^+$ cells in mice infected with *M. tuberculosis* has been reported to result in increase in the number of bacteria and subsequent death in spite of expression of IFN-γ and NOS2 (Scanga et al., 2000). IFN-γ is considered as an important activator cytokine in immune response to mycobacterial infection. This cytokine is necessary in protection against tuberculosis in mice (Tascon et al., 1998) and human (Dorman and Holland, 2000). $CD8^+$ T cells also play an important role in immune response to *M. bovis* in cattle. They contribute to the production of IFN-γ and immunopathology in bovine tuberculosis (Villarreal-Ramos et al., 2003). Stimulation of macrophages by IFN-γ can lead to activation of NFκB and subsequent release of pro-inflammatory cytokines (Flynn and Chan, 2001). This transcription factor is essential in immune defense against tuberculosis in mice (Yamada et al., 2001).

IFN-γ can activate macrophages and induce mechanisms that enable the activated macrophage to control the mycobacteria via increasing production of reactive oxygen and nitrogen intermediates (Flynn et al., 1993, Boehm et al., 1997). However, *M. tuberculosis* is able to inhibit the macrophage response to IFN-γ through a TLR-dependent mechanism (Kincaid and Ernst, 2003, Banaiee et al., 2006). A *M.*

tuberculosis outer membrane component, 19KDa lipoprotein, prevents IFN-γ induced macrophage activation (Gehring et al., 2003, Pennini et al., 2006) through inhibition of expression of a subset of IFN-γ-induced genes such as class II transactivator and chemokine CXCLi1 (Kincaid and Ernst, 2003). This process is mediated by gene-specific mechanisms through disruption of binding of transcriptional factors to the promoters (Benson and Ernst, 2009). However the precise mechanism by which *M. tuberculosis* inhibit responses to IFN-γ it remains to be elucidated. 19KDa lipoprotein is also capable of inhibiting antigen presentation by MHC class II and thus subsequently preventing activation of adaptive immunity (Noss et al., 2001).

MHC class I and II are both able to recognise bacterial antigens. They bind microbial antigens and carry them to the cell surface to be presented to specific T lymphocytes (Sohal et al., 2008). Mycobacteria-infected macrophages can present antigens associated with both MHC class II and I on their surface, to be recognized by $CD4^+$ and $CD8^+$ cells, respectively (Mikkelsen et al., 2011). In addition, CD1-restricted T lymphocytes can recognise mycobacterial lipoproteins presented by CD1 molecules. These molecules are non-polymorphic types of MHC class I and mainly expressed on phagocytic cells including dendritic cells (Sohal et al., 2008). The CD1-restricted T cells have cytotoxic ability and can also produce IFN-γ (Sieling et al., 2000).

The process of MHC expression is mainly stimulated by IFN-γ (Pai et al., 2004). However, mycobacteria have been reported to be able

to down-regulate expression of MHC molecules. For instance, Weiss and colleagues (2001) reported that *M. paratuberculosis* can down regulate expression of both MHC class I and class II in infected bovine macrophages *in vitro* (Weiss et al., 2001).

The complex of MHC class I-antigen can activate $CD8^+$ cells and consequently differentiate into cytotoxic T cells. These cytotoxic T cells have the ability to lyse macrophages infected with *M. bovis in vivo* (Skinner et al., 2003) and cause destruction of the mycobacteria *in vitro* (Stenger et al., 1997). Following activation, a set of cytokines, including IFN-γ, are produced by these cells (Mikkelsen et al., 2011).

γδ T cells are further sub-set of T cells which can regulate or stimulate host defence against mycobacteria (Waters et al., 2011). γδ T cells can recognise non-classical antigens of mycobacteria such as heat shock protein (Haregewoin et al., 1989), and produce IFN-γ at the site of infection in addition to their ability to lyse the infected cells (Plattner and Hostetter, 2011). These cells are found abundantly in the chicken intestinal tract (Berndt and Methner, 2001) and may play an important role in the immune response during systemic salmonellosis in this species (Berndt et al., 2006).

A balance between Type 1 and Type 2 immune responses to tuberculosis is hugely important. Protective immunity has been reported as a result of balance between IL-4 and IFN-γ production while more prominent IFN-γ could lead to increased pathology and reduced bacterial numbers (Widdison et al., 2006); the importance of IL-4 in mycobacterial infection is a controversial subject. A

negative role for IL-4 in mycobacterial infection has been suggested which indicated more severe disease with increase cytokine levels (Ordway et al., 2004). However, Sugawara et al. (2000) suggested this cytokine is essential for protection as IL-4 deficient mice showed an increase in the number of mycobacteria in their lungs. This beneficial role for IL-4 is made clearer by understanding that Th2 responses can counterbalance the undesirable consequences of strong Th1 cytokines. Since an increased IFN-γ level is more likely to be associated with granuloma formation and the pathology in mycobacterial infection (Ehlers et al., 2001), the balance between this cytokine and IL-4 could be important in protective immunity in tuberculosis.

In contrast to the crucial role of cell mediated immunity in mycobacterial infection, it is argued that the humoral immune response is usually considered as insignificant in protection against these intracellular bacteria (Beal et al., 2006, Maglione and Chan, 2009, Casadevall and Pirofski, 2012). However, there are accumulating data that B cells and antibody formation might also play a protective role in clearance of intracellular bacteria including *Salmonella* (Nanton et al., 2012) and mycobacteria (Bosio et al., 2000, Kozakiewicz et al., 2013). B cells have been suggested to have an effect on progression of the inflammation in lung infected with *M. tuberculosis* (Maglione et al., 2007). Reduction in influx of macrophages, $CD8^+$ cells and neutrophils have been observed in mice deficient in B cells suggesting that B cells may regulate production of chemokine and adhesion molecules (Bosio et al.,

2000). In addition, it has been found that deficiency of immunoglobulin A (IgA) (Rodriguez et al., 2005) or polymeric Ig receptor (Tjanlund et al., 2006) in mice could result in increased susceptibility of these animal models to infection with tuberculosis compared to wild type as these animals are not able to actively transport IgA or IgM. Furthermore, mice infected with *M. bovis* showed an increase in survival time due to presence of monoclonal antibodies bound on the surface of the bacteria (Chambers et al., 2004). Also enhanced survival time of mice infected with *M. tuberculosis* has been reported following administration of IgG3 monoclonal anti-arabinomannan antibody (Teitelbaum et al., 1998).

1.3.3 Granuloma formation

In mycobacterial infection, formation of the granuloma is a crucial phenomenon to constrain the bacteria in a protective environment in order to restrict their growth and prevent their further dissemination in the body. Granulomata provide a unique microenvironment where phagocytic cells and T lymphocytes can accomplish their bactericidal effect and increase contact between cells in order to eliminate the bacteria (Widdison et al., 2008). In addition, by formation of this structure the host is able to limit bacterial access to nutrients and oxygen and expose the bacteria to acidic pH and other immune effectors including NO (Rustad et al., 2009). On the other hand, the granuloma also provides a suitable niche for mycobacteria where they can survive and continue to modulate the immune response to limit bacterial damage during chronic infection (Sandor et al., 2003). As a characteristic of cell

mediated immunity, granulomata are formed at the site of infection. These structures are mostly composed of macrophages, multinucleated giant cells generated by fusion of macrophages, epithelioid-like macrophages, foamy cells that contain lipid bodies and dendritic cells and the structures are surrounded by several types of lymphocyte including $CD4^+$, $CD8^+$ and γδ T cells (Silva Miranda et al., 2012) (Figure 1.5). In chickens the granulomata due to bacterial infection are mostly composed of heterophils with a granulomatous periphery. The most hepatic granulomata in avian mycobacteriosis show central caseous necrosis (Supartika et al., 2006).

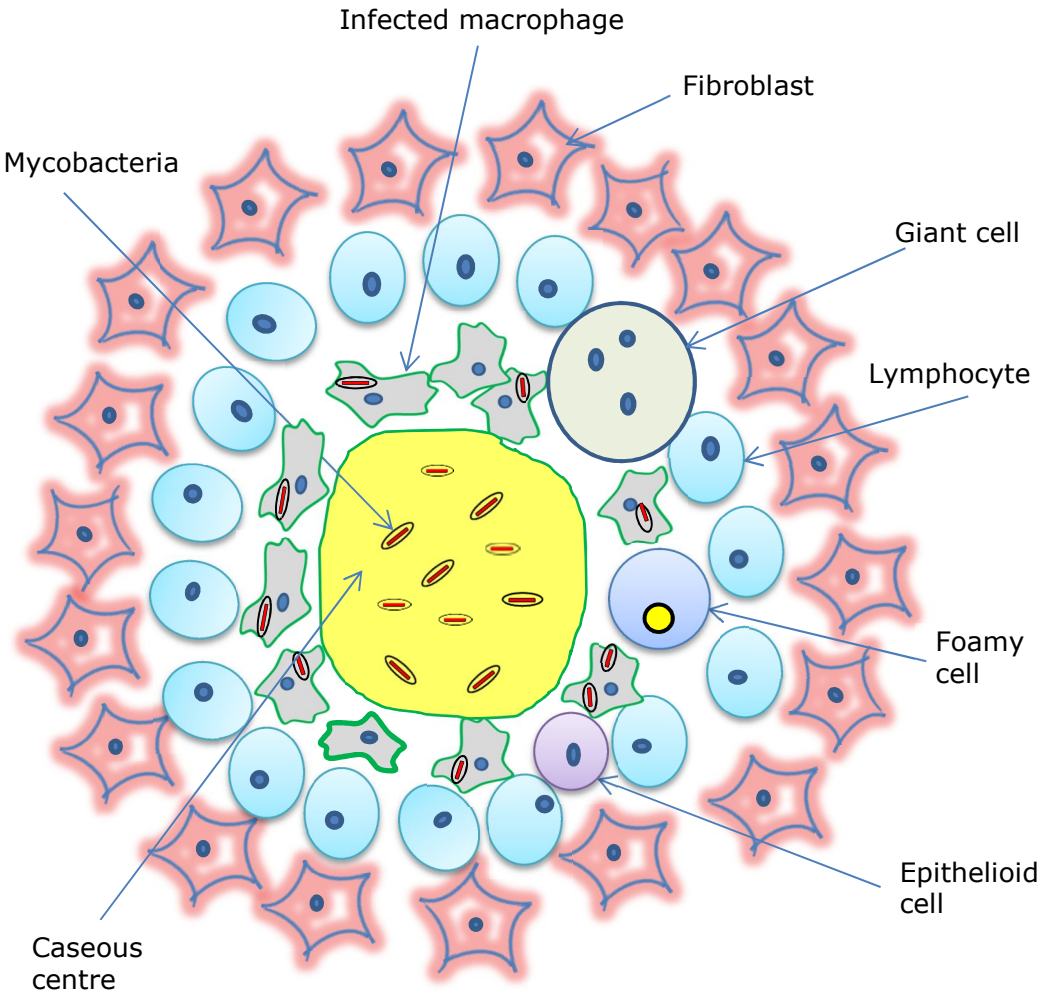


Figure 1.5: Schematic diagram of a tuberculous granuloma (modified from Huynh et al., 2011).

In many species granulomata are formed at the site of infection and are mostly composed of macrophages, multinucleated giant cells generated by fusion of macrophages, epithelioid cells, foamy cells that contain large lipid bodies and dendritic cells. The structures are surrounded by several types of lymphocyte including $CD4^+$, $CD8^+$ and γδ T cells.

1.4 Avian mycobacteriosis

Avian mycobacteriosis is a chronic, contagious disease affecting a wide range of animals, particularly domestic and wild bird species. (Hejlicek and Treml, 1995). The major significant pathogenic species of this disease in birds is *M. avium* (Fulton and Sanchez, 2013). However, several other mycobacterial species have been associated

with the disease in birds including *M. genavense* (Shitaye et al., 2008).

1.4.1 Epidemiology

Avian mycobacteriosis is widely distributed in the world (reviewed by Dhama et al., 2011). Among different domestic bird species, domestic fowls show high susceptibility to this disease, domestic turkey and guinea fowl are less susceptible, duck and goose moderately resistant and domestic pigeon are highly resistant to this disease (Hejlicek and Treml, 1995).

The major sources of avian mycobacteriosis are infected birds and contaminated soil and water because of long survival of mycobacteria in the environment (Fulton and Sanchez, 2013). The mycobacteria are released from ulcerated intestinal lesions of infected birds into droppings. The alimentary tract is the most frequent route of infection (Dvorska et al., 2007) with the respiratory route being less important. Birds are infected by ingestion or inhalation of contaminated aerosols (reviewed by Dhama et al., 2011). Birds suffering from progressive disease are thought to be the most significant risk to spread infection as they also contaminate the environment heavily (Dvorska et al., 2007). Chronicity, long term persistence in flock, may lead to a wasting disease with mortality (Biet et al., 2005).

It has been reported that the disease affects male and female birds equally (Keymer et al., 1982). Avian mycobacteriosis more commonly affects young birds than adults (Mutalib and Riddell, 1988, Cromie et al., 1991). However, a higher mortality rate in

adult swans (33%) in comparison to young ones (Brown et al., 1992), indicates the chronic nature of the infection with infection at a young age and the appearance of significant disease much later in adult birds (Soler et al., 2009, Dhama et al., 2011).

1.4.2 Clinical signs

Infected birds show no or few specific signs unless the disease progresses. Some nonspecific signs might appear for a few days to months prior to death including weight loss, diarrhoea and difficulty in breathing (Tell et al., 2001). Breathing difficulties in infected birds may be the result of either enlargement of liver and spleen or leakage of fluid from infected liver into abdomen and subsequently air sacs collapsing and abdominal distension (Saggese et al., 2010, Dahlhausen et al., 2012). Unlike human tuberculosis, in avian mycobacteriosis lesions are less frequently observed in the lungs (Tell et al., 2001, Fulton and Thoen, 2003). Clinical signs only appear during the advance stages of avian mycobacteriosis (Biet et al., 2005). This can lead to difficulties in diagnosis (Kaevska et al., 2010). The disease progresses during three phases including the latent period, and the lesion development and cachexia stages. Development of huge numbers of bacilli and large tubercles occur during the cachexia phase (Fulton and Sanchez, 2013). There are no data available regarding the latent stage of avian mycobacteriosis. In humans, during the latent stage of tuberculosis bacterial growth is controlled and there are no clinical symptoms. However, there is a major risk of progression of the disease by reactivation of the bacteria (Ronacher et al., 2015). There are also low tissue bacterial

numbers during this stage and this can thus complicate investigation of the mechanism of bacterial reactivation (Flynn and Chan, 2001). The mycobacteria in the latent stage can reactivate resulting in disease, for example when the immune response of the host is suppressed or becomes weak. In humans several conditions might lead to reactivation of infection during the latent stage of the disease, for instance, in co-infection with other pathogens, physical stress, malnutrition, ageing and chemotherapy (Tsolaki, 2009). Co-infections of tuberculosis with HIV has been reported in humans (Shen et al., 2004). Mohan et al. (2001) found that TNF-α plays an important role in preventing reactivation of tuberculosis in mice. How far this relates to avian mycobacteriosis is unclear.

Three forms of avian mycobacteriosis have been reported, the first being the typical form characterized by the development of granulomata or tubercles in several organs, the second form characterized by the development of lesions in the intestinal tract and the third form manifest as infection with no tuberculous lesions (reviewed by Dhama et al., 2011). Lameness might also be observed in birds as a result of infection of the leg bones or joints (VanDerHeyden, 1997a).

1.4.3 Pathology

The gross lesions are commonly observed as different size pale yellow or white nodules appearing in internal organs including the intestine, liver, lung, bone marrow, air sacs and spleen (Biet et al., 2005, Fulton and Sanchez, 2013). The nodules are firm and caseous necrotic debris with various small yellowish granules can be

observed after sectioning (Bougiouklis et al., 2005, Tell et al., 2001). The typical histopathological lesions in birds are granulomata which may be caseous and necrotic with eosinophilic debris at the centre and a layer of multinucleated giant cells at the edges. These can also be surrounded by many other inflammatory cells, mostly macrophages and lymphocytes (Shivaprasad and Palmieri, 2012).

1.4.4 Diagnosis

Due to the variable and non-specific clinical signs of the disease, which can be observed in many chronic diseases, diagnosis of avian mycobacteriosis is difficult (Dahlhausen et al., 2012). Biochemical tests, and serology can be suggestive of mycobacterial infection. However, definite diagnosis of avian mycobacteriosis depends on culture or other methods of identification of the organism in faeces and tissues including microscopy with the acid-fast stain (Shitaye et al., 2008, Saggese et al., 2010). In addition, molecular techniques including PCR could be used as confirmatory diagnosis of avian mycobacteriosis (Turenne et al., 2007). Using PCR to amplify mycobacterial 16S rRNA could also be used as a diagnostic test for identification of *M. avium* (Pavlik et al., 2000). In addition, the injection of purified protein derivative (PPD) of *M. avium* into the comb or wattle of birds and assessment of the reaction 48 to 72 h later, as done in cattle, has also been used for diagnosis of the disease (Tell et al., 2001).

1.4.5 Treatment

Treatment of avian mycobacteriosis is not recommended in the poultry industry due to generally antibiotic resistant nature of the causative agent in addition to the high cost of prolonged regimen of therapy (Tell et al., 2001, Buur and Saggese, 2012). Hydrophilic antibiotics including macrolides, fluoroquinolones and aminoglycosides cannot easily cross the highly lipophilic cell wall barrier of *M. avium*. Moreover, the slow growing nature of the bacteria means the chemotherapy is required to be applied over a prolonged period (VanDerHeyden, 1997a). Since *M. avium*-infected birds continuously spread the organisms into the environment and become sources of new infection, euthanasia of infected birds is recommended (VanDerHeyden, 1997b).

1.4.5.1 Control

Mycobacteria are able to survive for months or years in soil and water and they are highly resistant to environmental conditions, so the control of the disease requires identification and elimination of the infected birds, providing good sanitation and nutrition and reduction of stress factors (Pollock, 2006, Dhama et al., 2011).

1.5 Cells used in this study

1.5.1 THP-1 cells

The THP-1 cell line is a human promonocytic cell line that can be differentiated to macrophage-like cells using Phorbol-12-myristate-13-acetate (PMA) (Tsuchiya et al., 1980). The differentiated cells are adherent and morphologically similar to macrophages (Tsuchiya

et al., 1982). Upon differentiation, THP-1 cells behave more like human monocyte-derived macrophages in comparison to other myeloid cell lines, including U937, HL-60, HEL or KG-1 cell lines (Auwerx, 1991). These cells have a high phagocytic capacity for latex beads in addition to expressing a similar cytokine profile as monocyte-derived macrophages in response to TLR2 ligands (Daigneault et al., 2010). Furthermore, similar to human macrophages, PMA-differentiated THP-1 cells show increased adherence and expression of surface markers of differentiation of macrophages (Schwende et al., 1996). Following differentiation, increased NADPH-oxidase activity and expression of cell membrane CD11b receptor have been reported in THP-1 cells (Schwende et al., 1996). Thus, because of the difficulties associated with producing human primary macrophages, THP-1 cells can be used to model macrophage function. These cells have been reported to respond to infection with *M. tuberculosis* by production of various cytokines such as TNF-α, IL-1β, IL-6 (Chakraborty et al., 2013).

1.5.2 HD11 cells

The HD11 cell line is a chicken macrophage-like cell line that has been derived from chicken bone marrow and transformed by replication-defective avian leukaemia viruses (Beug et al., 1979). These slightly adherent cells are macrophage-like both in morphology and function with phagocytic capacity (Beug et al., 1979). HD11 cells have been used widely as a model to investigate pathogen interactions with avian macrophages. These cells have been shown to produce NO (He et al., 2006), cytokines such as IL-6

and IL-1β (Smith et al., 2005) and chemokines including CXCLi1 and CXCLi2 (Setta et al., 2012) in response to *Salmonella* and *Campylobacter*.

1.6 Aims and objective

No studies have reported in depth on the immune response to infection with *M. avium* in chickens. Therefore, it is worthwhile to further elucidate the nature of the interaction between the host and *M. avium* to improve our understanding perhaps leading towards designing an efficient vaccine strategy.

The objectives of this study were to:

1- Compare the immunological interactions of *M. avium* from different human and animal sources in avian and human infection cell models *in vitro*.

2- Study the role of various MAP kinases in cellular regulation associated with *M. avium* infection.

3- Evaluate the immune response to *M. avium* strains during infection of chickens.

2 Materials and Methods

2.1 Bacterial cultures and strains

Avian, bovine and human strains of *Mycobacterium avium* subspecies *avium* were used in this study (Table 2.1). *M. avium* NC.08562-02 (Ma.1) and *M. avium* NC.0855-07 (Ma.2) were isolated from laying chickens with avian mycobacteriosis (H. W. Smith, unpublished information). *M. avium* strains 61.3623.05 (Ma.3), 61.4627.08 (Ma.4) and 61.3728.08 (Ma.5) were isolated from cattle lymph nodes (donated by C. Wray, Animal and Plant Health Agency, Weybridge). Three other strains 430/330 (Ma.6), 446/301 (Ma.7) and 460/132 (Ma.8) were isolated from human disease and obtained from Public Health England, Colindale, London. Bacterial stocks were stored at -80°C in 15% glycerol. Bacterial stocks were thawed and aliquots of them were used to inoculate 7H10 Middlebrook agar (Difco, Sparks, USA) and 7H9 Middlebrook broth (Difco, USA). Both these were supplemented with 10% oleic acid albumin dextrose catalase (OADC; Difco, USA), 0.5% glycerol and 0.05% Tween 80 (Sigma, Poole Dorset, UK). Cultures were incubated at 37°C for 3-4 weeks and, in case of broth, in a shaking incubator (Forma Orbital Shaker-Thermo, UK) at 150 rpm. 37°C. The optical densities (ODs) of all bacterial strains were measured at 600 nm using a spectrophotometer (Libra S22 Biochrom, Scientific Laboratory Suppliers, UK) and serial dilutions were done to count the number of bacteria. The numbers of colony forming units (CFU) were determined by plating the bacteria on 7H10 agar plates cultured for 3-4 weeks.

Table 2.1: Strains of *M. avium* subspecies *avium* used in this study.

M. avium strain	Abb.	Source	Reference
NC.08562-02	Ma.1	Chicken	H. W. Smith, unpublished information
NC.0855-07	Ma.2	Chicken	H. W. Smith, unpublished information
61.3623.05	Ma.3	Cattle	C. Wray, Animal and Plant Health Agency, Weybridge
61.4627.08	Ma.4	Cattle	C. Wray, Animal and Plant Health Agency, Weybridge
61.3728.08	Ma.5	Cattle	C. Wray, Animal and Plant Health Agency, Weybridge
430/330	Ma.6	Human	Public Health England, Colindale, London
446/301	Ma.7	Human	Public Health England, Colindale, London
460/132	Ma.8	Human	Public Health England, Colindale, London

M. avium subspecies *avium* strains were isolated from different species. *M. avium* NC.08562-02 (Ma.1) and *M. avium* NC.0855-07 (Ma.2) were isolated from laying hens with avian mycobacteriosis. *M. avium* strains 61.3623.05 (Ma.3), 61.4627.08 (Ma.4) and 61.3728.08 (Ma.5) were isolated from cattle lymph nodes. Three other strains 430/330 (Ma.6), 446/301 (Ma.7) and 460/132 (Ma.8) were isolated from human disease. No information on serotypes was available.

2.2 Cell culture

2.2.1 THP-1 cells

THP-1 human leukaemic monocytic cells (Tsuchiya et al., 1980) were grown in 75 cm flasks (Thermo Fisher Scientific, Roskide, Denmark) containing RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% foetal calf serum (FCS; Gibco, UK) and 2mM L-glutamine (Gibco, UK) and incubated at 37°C in 5% CO2. Every 3-5 days the cells were centrifuged at 1000 g, for 10 min at 20°C and re-suspended in fresh media. Viable cell counting was performed using Trypan blue staining and counting in a haemocytometer (Marienfeld-Superior, Germany).

2.2.2 HD11 cells (chicken macrophages-like cells)

Chicken macrophage-like HD11 cells (Beug et al., 1979) were cultured in 75 cm flasks in RPMI 1640 containing 5% FCS, 5% chicken serum (Sigma-Aldrich, USA), 10% tryptose phosphate broth (Sigma-Aldrich, UK) and 2mM L-glutamine at 37°C, 5% CO2. For harvesting the cells, after removing old medium, the cells were washed twice with sterile phosphate buffered saline (PBS; Gibco, UK) and then 5 ml trypsin/EDTA (10 mM) in PBS 10% v/v were added to cells and incubated for 5 min at 37°C in 5% CO_2 in order to release the cells from the flask surface. The contents were transferred to a Falcon tube (Scientific Laboratory Supplies, UK) and 3 ml of culture media were added to it before centrifugation at 300 g for 5 min. The cell pellet was re-suspended into 3 ml medium and reseeded at a density appropriate to whichever experiment was carried out.

2.3 Cell invasion

Cells were seeded in 24-well plates at a density of $5x10^5$/ml prior to infection. THP-1 cells were differentiated into macrophages with 50ng/ml PMA (Sigma-Aldrich, Louis, USA) (Cossu et al., 2012). After 48 h of incubation, supernatants were removed and cells were washed 3 times with sterile pre-warmed PBS and replaced by fresh 37°C RPMI containing FCS and L-glutamine.

Broth cultures of the bacterial strains were diluted in PBS and cells were infected at a multiplicity of infection (MOI) of 2:1 and 10:1 (Jones et al., 2001c). The optical densities of all bacterial strains

were checked by spectrophotometer. One ml of each bacterial broth was centrifuged at 1000 g for 10 min and the pellets re-suspended with an appropriate volume of PBS to obtain the appropriate MOI ($5x10^6$/100µl per well). *Salmonella enterica* serotype Enteritidis LPS (Sigma-Aldrich, USA) was used as a positive control, as it is a potent cytokine inducer, and PBS alone was used as a negative control. In THP-1 experiments, negative control samples were THP-1 cells differentiated by PMA with RPMI cell culture medium, supplemented by 10% FCS and 2mM L-glutamine, without added bacteria. Similarly, in HD11 experiments the cells without bacteria were used as the negative control.

After 1 h incubation, the supernatants were removed and the cells were washed three times with pre-warmed sterile PBS. In order to remove extracellular bacteria, fresh RPMI supplemented with 10% FCS containing gentamicin (100 µg/ml, 37°C; Sigma-Aldrich, USA) was added to culture and incubated at 37°C in 5% CO_2 (Jones et al., 2001c, Smith et al., 2005). At various time points post-infection (2, 6 and 24 h) supernatants were collected and kept at -20°C, for estimation of NO concentration in THP-1 and HD11 cells and different pro-inflammatory cytokines and chemokines in THP-1 cells.

The cells were lysed by adding 200µl 0.5% (vol/vol) Triton X-100 (Fisher Scientific, New Jersey, USA) to release intracellular bacteria. Decimal dilutions were plated on 7H10 agar to estimate viable numbers (Weiss et al., 2002) and cultured for at least 3-4 weeks.

2.4 Griess assay

To investigate NO production by infected cells, the level of nitrite, which is a more stable metabolic product, was measured in the tissue culture supernatants, collected after different time points (2, 6 and 24 h) post-infection, using the Griess assay (Promega, Madison, USA). The Griess reagent system is established by chemical reaction between 0.1% sulfanilamide and 1% *N*-1-napthylethylenediamine dihydrochloride (NED) in 5% phosphoric acid to provide acidic conditions for the reaction. Aliquots of 50 µl of the samples from tissue culture were transferred to a 96-well plate (flat-bottom; Thermo Fisher Scientific, Denmark) in triplicate for each strain and each time point. Then 50 µl of the sulfanilamide solution were dispensed to all wells and incubated for 10 min at room temperature. After that, 50 µl of the NED solution were added to all wells and incubated for 10 min at room temperature. The concentration of nitrite was determined by measuring the absorbance at 540 nm using a Microplate reader (Microplate Absorbance Reader, LT-4000, Labtech, Sussex, UK). In order to accurately measure nitrite concentration a standard reference curve of nitrite was prepared for each assay. For this, seven two-fold serial dilutions were made (100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µM) and performed in triplicate. For the positive control cell cultures stimulated with LPS were used and cells without bacteria were used as the negative control.

2.5 ELISA quantification of cytokines

The supernatants from the invasion assays were stored at -20°C until used. The concentration of TNF-α, IL-1β, IL-6 and CXCL-8 cytokines in THP-1 cell culture supernatants were measured by ELISA assays using commercially available kits (R and D Systems, Abingdon, UK) and the procedures carried out in accordance with the manufacturer's instructions. The samples were measured in triplicate and by using a standard curve the concentrations of the cytokines were calculated in picograms per millilitre (pg/ml).

The sandwich type of ELISA was used to detect the cytokines analysed in this study. Briefly, a 96-well plate was coated with 100 µl of capture antibody per well and incubated overnight at room temperature. Each well was aspirated and washed with wash buffer three times. The plate was blocked with 300 µl of reagent diluent per well and incubated at room temperature for one hour. Wells were aspirated and washed three times with wash buffer (0.05% Tween 20 in PBS). Then, 100 µl of each sample was added to wells in triplicate. A standard reference curve was prepared for each assay. For this a seven point two-fold serial dilution series of the standard was performed in triplicate. For the positive control cell cultures stimulated with LPS was used and differentiated cells in RPMI without bacteria and stimulated with PBS were used as negative control. After 2 h incubation at room temperature, aspiration and washing was repeated and 100 µl of diluted detection antibody added to each well and incubated for 2 h. Wells were aspirated and washed three times and 100 µl of streptavidin-HRP

added to each well and incubated for 20 min at room temperature. The aspiration and washing were repeated and 100 µl of substrate solution added to each well and incubated for 20 min at room temperature. Finally, 50 µl of Stop solution (2 N H_2SO_4) were added to each well, gently mixed and the optical density determined at 450 and 540 nm using a Microplate reader. Readings at 540 nm were subtracted from those at 450 nm.

2.6 Inhibition of signal transduction

Both HD11 and PMA-differentiated THP-1 cells were pre-treated with highly specific inhibitors of each MAPK sub-family pathway for 30 min at 37°C prior to the infection with the *M. avium* strains. All inhibitors were reconstituted in sterile dimethyl sulfoxide (DMSO (Fisher Scientific, Loughborough, UK)) and used at a concentration of 50 µM in the experiments. PD98059 (2-amino-3-methoxyflavone) (Cell Signaling Technology, UK) or U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) (Cell Signaling Technology, UK) were used to inhibit ERK1/2 pathway. SB203580 [4- (4-fluorophenyl)-2- (4-methylsulfinylphenyl)-5- (4-pyridyl) imidazole] (Tocris, Bristol, UK) was used for inhibition of the p38 MAPK pathway and SP600125 (anthra [1,9-cd] pyrazol-6(2H)-one) (InvivoGen, SanDiago, USA) used for inhibition of the JNK pathway. The concentrations used in this study (50 µM) were chosen based on previous research published with human and chicken cells (He and Kogut, 2003, Crippen, 2006, Kogut et al., 2008, Leyva-Illades et al., 2012).

2.7 Cytotoxic effect of inhibitors on THP-1 and HD11 cells

To evaluate the cytotoxic effect of selected concentrations of the inhibitors on the viability of THP-1 and HD11 cells, the lactate dehydrogenase (LDH) liberation assay was performed using a CytoTox 96 kit (Promega, UK) following the manufacturer's instructions. The LDH enzyme is released from cells following cellular membrane damage and subsequent cell death. The cell culture supernatants from infection experiments were collected at different time points following addition of the inhibitors. The cytotoxicity percentage is determined as the percentage of the LDH liberated from lysed cells into the supernatants. To determine this, the optical density of the cell supernatants was measured at 490 nm using a spectrophotometer and the values expressed as a percentage using the following equation for cells infected with each of *M. avium* strains: % of cytotoxicity = ((experimental release - spontaneous release - effector rerelease) / (maximum release-spontaneous release)) X 100 (Daim et al., 2011).

In the equation, spontaneous release is LDH released from non-infected cells, maximum release is release of LDH from cells following incubation at -80°C for 30 min before thawing at 37°C and effector release is LDH released from *M. avium* strains alone.

2.8 *In vivo* chicken studies

For the chicken study, a total of forty eight one-day-old unvaccinated, mixed sex, commercial layer chickens were obtained

from Hy-Line UK Ltd. The birds housed on solid floor with litter and were given access to antibiotic-free feed and water *ad libitum*. At three weeks of age the birds were divided into 4 groups, twelve birds each in separate rooms. Three groups were infected with different *M. avium* strains (one strain per group). Bacterial suspensions were inoculated intravenously into the wing vein of each bird with 0.1 ml containing *ca*. $1x10^5$ CFU of the corresponding *M. avium* strain (Ma.3, Ma4 and Ma.5). The bacteria were diluted in PBS with 0.05% Tween 80 to reach *ca.* $1x10^5$ CFU. The control group was mock-infected with 0.1 ml of sterile PBS-Tween 80. The chickens were inoculated intravenously, because previous work has shown the same patterns of resistance and susceptibility between chicken lines for *Salmonella* or *M. avium* (Barrow and Bumstead, unpublished). For *Salmonella* the main site of expression of this phenotype is in the spleen rather than in the intestine (Bumstead and Barrow, 1988). In a similar way those authors found that expression of resistance or susceptibility to *M. avium* strains in inbreed chicken lines was also expressed in the spleen (Barrow and Bumstead, unpublished).

Three birds from each group were euthanized on the day of infection (30 min after inoculation) and at 1, 2 and 3 weeks post-infection with the killed birds sprayed with 70% alcohol to decontaminate the surface of the carcass. Blood samples (0.2 ml) were taken post-mortem from heart and added to 1.8 ml sterile distilled water in Eppendorf tubes. Previously weighed sterile 20 ml universal tubes were used to collect spleen and liver samples from individual

euthanized chickens for further bacteriological examination and placed on ice before using. In addition, tissue samples of liver and spleen were placed directly in RNA-later (Sigma-Aldrich, UK), placed on ice, before being stored at -20°C for RNA extraction. Simultaneously, liver and spleen tissue samples were collected from euthanized birds and placed into 10% neutral buffered formalin (NBF) for further histopathological examination.

For bacteriological examination, blood samples were decimal diluted using sterile PBS-Tween while liver and spleen were homogenized using Griffith's tubes in an appropriate volume of sterile PBS-Tween 80. A seven point serial dilution was performed on the homogenized samples and 50 µl of each dilution inoculated on 7H10 agar plates and incubated at 37°C. After 14 days mycobacterial colonies were enumerated and expressed as Log_{10} CFU/g.

To determine cytokine expression, RNA was extracted from homogenized samples and reverse-transcribed to cDNA before running in quantitative real-time polymerase chain reaction (qRT-PCR) all of which will be explained later in this chapter.

The experiment was approved by the school ethical review panel under Home Office Project License 40/3412.

2.8.1 Histopathology

Using a routine histological procedure the liver and spleen samples were processed. The tissue samples were removed from chickens and placed in 10% NBF for 24 h at room temperature to be fixed. Then NBF was removed and tissue samples were rinsed with PBS

and then placed in 70% ethanol until they were processed and embedded.

For paraffin embedding of tissue samples, the fixed samples were placed in embedding cassettes (Thermo-Scientific, UK) and then for processing they were placed in a tissue processer (Leica Microsystems, Germany). The paraffin embedding program for processing was performed for a total of 17 h as follows:

1. ethanol 70% 1 h
2. ethanol 80% 1.30 h
3. ethanol 95% 1.30 h
4. ethanol 100% four changes, first two for 1 h and the other two for 1.30 h
5. Histoclear (Fisher Scientific, UK) three changes, 1 h followed by 1.30 h and then 30 min
6. paraffin two changes, first one 2 h followed by 3 h

Then tissues were embedded in paraffin blocks.

Sections of 6-µm were cut using a microtome (Leica Microsystems, RM2255, Germany) after trimming the paraffin blocks. The sections were placed on a 30% ethanol-moistened small glass before placing them in a water bath at 40°C. Then the paraffin sections were mounted onto superfrost glass slides (Fisher Scientific, UK) and placed on a hot plate at 42°C overnight.

2.8.2 Haematoxylin and eosin staining

For staining the sections the following procedure was applied. First, the sections were deparaffinised by placing the slides twice into Histoclear each for 5 min. For rehydration of the sections, they were

placed in 100% ethanol for 2 min and then in 95% ethanol for another 2 min followed by 70% ethanol for 2 min. Then the slides were rinsed in running tap water thoroughly before staining with haematoxylin (Sigma-Aldrich, UK) for 3 min and washing with tap water. The sections were placed in acidified industrial methylated spirits (IMS; Sigma-Aldrich, UK), prepared by adding 3 ml HCl (Acros, Belgium) in 300 ml IMS, 2 dips each of 1-2 seconds and then dipped in ammoniated water (3 ml ammonia in 300 ml water) (Fisher Scientific, UK) and then washed thoroughly with tap water. The slides were checked under the microscope to observe if the nuclei had stained properly. Then eosin (Sigma-Aldrich, UK) staining was applied to the sections by placing in eosin stain for 5 min and washed thoroughly with water. The slides were dehydrated by passing through several changes of alcohol from 70% and then 95% and then 100% ethanol each for 2 min. Then the slides were placed in xylene for 5 min twice to clear the tissue and to become more transparent. Finally, to mount the slides, a thin layer of DPX mountant (Sigma-Aldrich, UK) was applied and a glass cover slip was placed on each slide and kept overnight to dry. All slides were examined blind under the light microscope (Leica Microsystems, Germany).

2.9 Quantification of mRNA gene transcripts

2.9.1 RNA extraction

In order to quantify cytokine gene expression by HD11 cells and liver and spleen samples from chickens after they were infected with

different strains of *M. avium* at set time point post-inoculation, total RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel; Fisher Scientific, UK) according to the manufacturer's instructions. For HD11 cells, after each time point, supernatants were removed and the cells lysed directly in 350 µl RA1, lysis buffer containing 3.5 µl β-mercaptoethanol. The chicken liver and spleen samples were homogenized and lysed using a TissueLyser II (Qiagen, UK). The tubes containing liver and spleen samples that had previously been kept in RNA-later were taken out and placed on dry ice. 30 mg of each frozen sample was cut and placed in a 2 ml microcentrifuge tube containing 350 µl RA1, lysis buffer with 3.5 µl (10%) β-mercaptoethanol and one sterile stainless steel bead (5mm in diameter) on dry ice. The tubes were placed in the TissueLyser adaptor set and the tissue samples were disrupted by operating the TissueLyser for 2 min at 20 Hz. To ensure uniform disruption and homogenization, the tubes were rearranged in the TissueLyser adaptor set and the machine operated for another 2 min at 20 Hz. The lysates were filtered through a filter column, provided with the kit, and centrifuged for 1 min at 11,000 g and 350 µl of 70% ethanol added to homogenize the lysate. To bind RNA, the mixture was loaded onto the RNA column and centrifuged for 30 s at 11,000 g. Then, 350 µl of membrane desalting buffer were added and centrifuged at 11,000 g for 1 min to dry the membrane. To eliminate contaminating DNA, DNase treatment was applied to the isolations by addition of 95 µl of DNase reaction mixture to each tube which were incubated for 15 min at room temperature. To

wash and dry the RNA column, it was washed three times by RA2 and RA3 buffer provided by the kit. First, 200 µl RA2 were added and centrifuged for 30 s at 11,000 g, then 600 µl RA3 were added and centrifuged at 11,000 g for 30 s and then 250 µl RA3 were added to the RNA column and centrifuged at 11,000 g for 2 min. Finally, the RNA was eluted in 40 µl RNase-free water and centrifuged at 11,000 g for 1 min and the resulted RNA quantity and quality was tested by NanoDrop spectrophotometer ND-1000 (Labtech International Ltd, UK) before storing at -80°C until used.

2.9.2 cDNA synthesis

The RNA samples were reverse-transcribed to obtain cDNA using a Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, UK) according the manufacturer's instructions. For preparing each 20 µl RT-PCR reaction, 1 µg of total RNA per each sample was added to nuclease-free water to reach a total volume of 11 µl followed by the addition of 4 µl of reverse transcriptase reaction buffer, 0.5 µl of protector RNase inhibitor, 2 µl of deoxynucleotide mix, 2 µl of random hexamer primer and 0.5 µl of reverse transcriptase enzyme and the reagents were then mixed gently. For the control, a tube containing all reagents except reverse transcriptase enzyme was prepared alongside the samples. The following cycle profile was used using a Techne TC-512 thermal cycler (Bibby Scientific Ltd, UK): 10 min at 25°C followed by 30 min at 55°C and 5 min at 85°C. The resulting cDNA was stored at -20°C until used to perform the qRT-PCR.

2.9.3 Quantitative real-time PCR

Cytokine and chemokine gene expression were quantified by qRT-PCR using the Light Cycler 480 System (Roche Applied Science, UK). Sequences of primers and probes specific for the reference gene (28S), IL-6, CXCLi1 (K60), CXCLi2 (IL-8), iNOS, IL-1β and IFN-γ genes used in HD11 cells and chicken tissue samples have been described previously (Kaiser et al., 2000, Withanage et al., 2004, Avery et al., 2004, Vervelde et al., 2013). The primer and probe sequences used for chicken cells are shown in Table 2.2. The sequences of primers specific for TNF-α, IL-1β and β-actin used in THP-1 cells have also been described previously (Stordeur et al., 2002) and are shown in Table 2.3. All probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) and the quencher *N,N,N,N'*-tetramethyl-6-carboxyrhodamine (TAMRA) at the 5' and the 3' ends, respectively.

Table 2.2 Sequences of primers and probes for chicken cells.

Target RNA	Probe / primer*	Sequence	Accession number
28S	P	5'-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3'	X59733
	F	5'-GGCGAAGCCAGAGGAAACT-3'	
	R	5'-GACGACCGATTTGCACGTC-3'	
IL-6	P	5'-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3'	AJ250838
	F	5'-GCTCGCCGGCTTCGA-3'	
	R	5'-GGTAGGTCTGAAAGGCGAACAG-3'	
CXCLi1	P	5'-(FAM)-CCACATTCTTGCAGTGAGGTCCGCT-(TAMRA)-3'	AF277660
	F	5'-CCAGTGCATAGAGACTCATTCCAAA-3'	
	R	5'-TGCCATCTTTCAGAGTAGCTATGACT-3'	
CXCLi2	P	5'-(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)-3'	AJ009800
	F	5'-GCCCTCCTCCTGGTTTCAG-3'	
	R	5'-TGGCACCGCAGCTCATT-3'	
iNOS	P	5'-(FAM)-TCCACAGACATACAGATGCCCTTCCTCTTT-(TAMRA)-3'	U46504
	F	5'-TTGGAAACCAAAGTGTGTAATATCTTG-3'	
	R	5'-CCCTGGCCATGCGTACAT-3'	
IL-1β	P	5'-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3'	AJ245728
	F	5'-GCTCTACATGTCGTGTGTGATGAG-3'	
	R	5'-TGTCGATGTCCCGCATGA-3'	
IFN-γ	P	5'-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3'	Y07922
	F	5'-GTGAAGAAGGTGAAAGATATCATGGA-3'	
	R	5'-GCTTTGCGCTGGATTCTCA-3'	

*P, probe; F, forward primer; R, reverse primer

Table 2.3 Sequences of primers for THP-1 cells.

Target RNA	Sequences	Product size (bp)	Final concentration (nM)
TNF-α	F275:5'-CCCAGGGACCTCTCTCTAATC-3' R358:5'-TGGGCTACAGGCTTGTCACT-3'	84	F 300; R 300
IL-1β	F176: 5'-ACAGATGAAGTGCTCCTTCCA-3' R248: 5'-GTCGGAGATTCGTAGCTGGAT-3'	73	F 300; R 300
β-actin	F976: 5'-GGATGCAGAAGGAGATCACTG-3' R1065:5'-CGATCCACACGGAGTACTTG-3'	90	F 300; R 300

F, forward primer; R, reverse primer

The qRT-PCR was performed using the Light Cycler 480 Probes Master kit (Roche Applied Science, UK). For optimization, four different concentrations of forward and reverse primers were used in a qRT-PCR assay to select the best conditions. The following four conditions were examined; 300 nM forward primer and 300 nM reverse primer, 300 nM forward primer and 900 nM reverse primer, 900 nM forward primer and 300 nM reverse primer, and 900 nM forward primer and 900 nM reverse primer. As a result, the best primer concentrations were selected based on the earliest expression of Ct values and the strongest florescent signals. Thus, the following concentrations of forward and reverse primers of the tested genes were used in this study: 300 nM forward primer and 900 nM reverse primer for 28S, CXCLi1, CXCLi2, IL-6; 900 nM forward primer and 900 nM reverse primer for IL-1β; 900 nM forward primer and 300 nM reverse primer for iNOS and 300 nM forward primer and 300 nM reverse primer for IFN-γ. The qRT-PCR mixture consisted of 1x light cycler probe master, forward primer

and reverse primer (as mentioned previously), 100 nM probe, 2 µl (100 ng) template (cDNA), made up to 20 µl with RNase-free water. Three no-template controls were included in each qRT-PCR experiment. The following cycle profile was used to perform qRT-PCR to amplify and detection of specific products: one cycle at 95°C for 10 min, 45 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 s and one cycle at 40°C for 30 s.

In THP-1 cells, qRT-PCR was conducted on cDNA samples in triplicate using SYBR green master mix (Roche Applied Science, UK). The mixture for THP-1 samples consisted of 1x SYBR green, 300 nM forward primer, 300 nM reverse prime and 2 µl (100 ng) template (cDNA), made up to 20 µl with RNase-free water. The following cycle profile was used to perform qRT-PCR to amplify and detection of specific products: one cycle at 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s and 68°C for 3 s and one cycle at 40°C for 30 s. To generate standard curves for all tested genes, RNA extracted from LPS-stimulated HD11 cells or THP-1 cells were used as standard controls. Log_{10} dilutions series were made and the LC480 software was used to calculate the qRT-PCR slopes and efficiencies. The ideal efficiency of PCR should be 100% (E=2), which indicates that the amount of the product doubles each cycle.

2.9.4 qRT-PCR data analysis

In this study, 28S and β-actin were used as reference genes for normalisation of cytokine gene expression in chicken cells and THP-1 cells, respectively. The data were normalized with the reference

gene and expressed as corrected 40-Ct values as previously described (Kaiser et al., 2000, Hughes et al., 2007). The slopes were obtained from the standard curves in LC480 software by Ct value against $\log_{10}$ concentration to calculate differences in total RNA input and adjust Ct values of each cytokine. The normalized values calculated as: Corrected Ct value= Ct + (Nt-Ct') X S/S', whereby Ct is the mean sample Ct value of target gene, Nt is the mean experimental Ct value of reference gene, Ct' is the mean sample Ct value of reference gene and S and S' are slopes of target gene and reference gene, respectively (Hughes et al., 2007). The results were expressed as corrected 40-Ct and mRNA fold changes. The fold changes (F) between treatments were calculated as: $F=2^{(T-C)}$, T= expression level of treated samples and C= expression level of control samples (Wu et al., 2010).

2.10 Statistical analysis

By using Graph Pad Prism software, two-way ANOVA followed by Tukey's multiple comparisons test were used to assess significant differences between various mycobacterial strains with a negative control at each time point and also to detect significant differences at the indicated time points. In addition, one-way ANOVA followed by Tukey's multiple comparisons test were used to assess differences between chicken groups with regard to cytokine expression in the *in vivo* experiment. When $P<0.05$, the differences were considered as significant.

Values for TNF-α production were used to investigate normality. The difference between individual values and the arithmetic means for 2 h and 6 h were calculated. The differences were normalized against the means by dividing the differences by the appropriate means to ensure that difference in the mean values were taken into account. The difference/mean values were plotted demonstrating a good approximation to a normal distribution (Figure 2.1). A similar calculation was done with geometric means of the bacterial counts obtained from THP-1 cells. A good approximation to a normal distribution was again obtained.

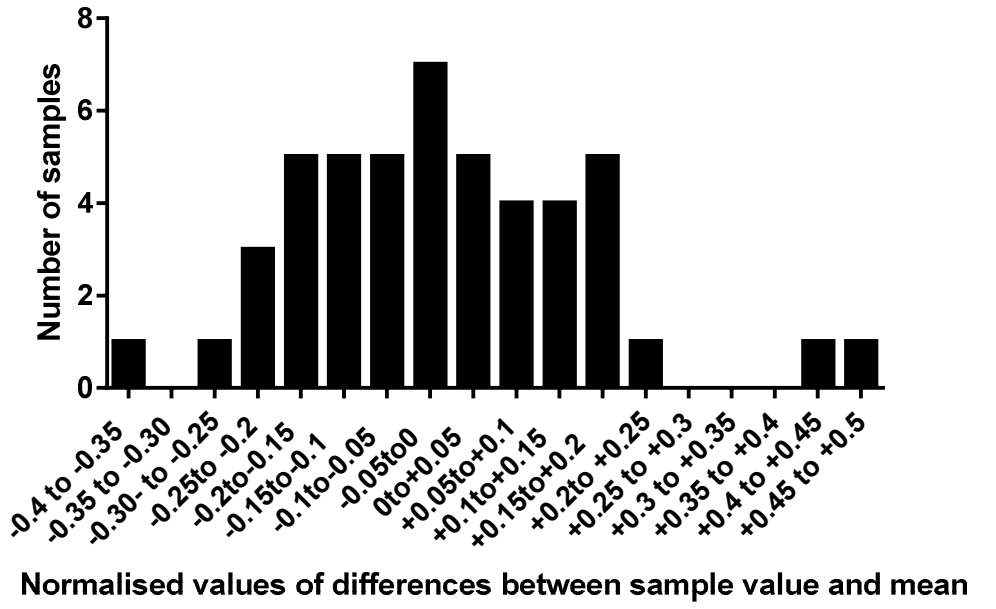


Figure 2.1: Distribution of TNF-α values at 2 h and 6 h post-infection

Values for TNF-α production were used to investigate normality. The difference between individual values and the arithmetic means for 2 h and 6 h were calculated. The differences were normalized against the means by dividing the differences by the appropriate means to ensure that difference in the mean values were taken into account.

3 Differential early immune responses to *M. avium* induced in avian and human macrophage-like cell lines

3.1 Introduction

The interaction between mycobacteria and host is a complex process and macrophages play a vital role in this process as a component of the innate immune response and initiator of the adaptive immune response. This interaction leads to release of several chemokines and cytokines (van Crevel et al., 2002). The cytokine network plays an important role in the immune response to mycobacteria and in determining the outcome of the infection (Cooper and Khader, 2008).

TNF-α is an essential pro-inflammatory cytokine that plays a role in phagocyte activation (Cooper, 2009), induction of apoptosis (Balcewicz-Sablinska et al., 1999) and formation and maintenance of granulomata in mycobacterial infections (Roach et al., 2002, Algood et al., 2005). Mice with a deficiency in TNF-α production or in the absence of its receptor p55, are more susceptible to mycobacterial infection than the wild type animal (Algood et al., 2005). As a result of oxidative deamination of L-arginine by NOS, NO is produced. Three isoforms of NOS have been identified, nNOS, which is found in neuronal tissue, iNOS that is found in a wide range of cells and eNOS which is available in vascular endothelial cells (Alderton et al., 2001). IL-1β has been reported to regulate iNOS production (Chan et al., 2001), in addition to phagosomal acidification and expression of adhesion molecules (Dinarello, 2009). IL-6 also plays a protective role in mycobacterial infection as mice deficient in IL-6 showed increased *M. tuberculosis* susceptibility (Ladel et al., 1997) .

The chicken macrophage-like HD11 cell was generated by transformation of chicken bone marrow cells by avian leukaemia virus (Beug et al., 1979). These cells have been widely used in immunity studies. HD11 cells are reported to produce NO (He et al., 2006), cytokines such as IL-6 and IL-1β (Smith et al., 2005) and chemokines including CXCLi1 and CXCLi2 (Setta et al., 2012) in response to *Salmonella* and *Campylobacter*.

The THP-1 cell line is a human promonocytic cell line that can be differentiated to macrophage-like cells using PMA. These cells have also been extensively used as a human macrophage model to investigate immune response to mycobacteria. The production of cytokines including TNF-α, IL-1β, IL-6 in THP-1 cells has been reported in response to *M. tuberculosis* (Chakraborty et al., 2013).

The pathogenicity of mycobacterial species and strains within species may be correlated with the nature of the host immune response. Therefore, it is important to evaluate differential immune responses to various mycobacteria strains within individual species. It is not surprising that the most detailed studies on the interactions between mycobacteria and host cells have been with *M. tuberculosis* and human cells. In this chapter we describe differential immune responses to the *M. avium* strains in human THP-1 cells and chicken HD11 cells.

3.2 Results

3.2.1 Bacterial susceptibility to gentamicin

The susceptibility of *M. avium* strains to gentamicin was detected by culture and a sensitivity test. After inoculating 7H10 agar plates with the different *M. avium* strains, gentamicin discs were placed on the surface of the plates. After 10 days, growth inhibition zones were detected around the gentamicin discs on all plates. Furthermore, the susceptibility of the bacteria to the tested concentration (100 µg/ml) of gentamicin was also examined. Gentamicin (100 µg/ml) was added to bacterial suspensions in 7H9 broth. After one hour of incubation, 100 µl of the suspension were inoculated and spread on 7H10 agar plates and incubated at 37°C. After 2 weeks no colonies were observed on these plates whereas full size colonies had appeared on plates inoculated with broth cultures with no added gentamicin.

3.2.2 Intracellular viability of *M. avium* in THP-1 cells at MOI 2:1

THP-1 cells were infected with five *M. avium* strains at a MOI of 2:1 for 1 h and the viable bacterial numbers were counted at 2, 6 and 24 h post-infection. No significant differences were observed between the bacterial uptakes by the cells infected with various *M. avium* strains (Figure 3.1).

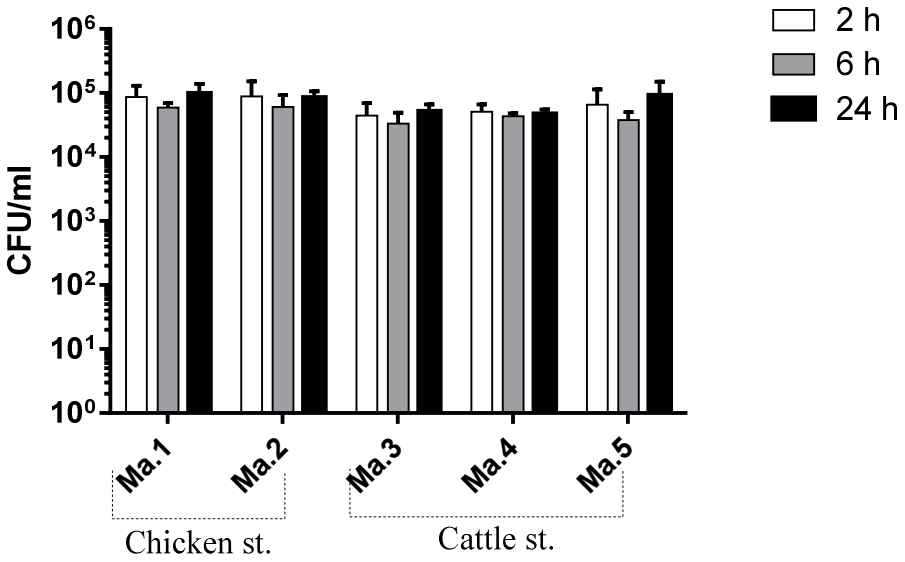


Figure 3.1: Intracellular kinetics of *M. avium* strains in THP-1 cells.

PMA-differentiated THP-1 cells infected with five *M. avium* strains at MOI of 2 for 1 h. Bacterial numbers enumerated at 2, 6 and 24 h post-infection Data presented as mean concentration ± SEM of one experiment performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623.05), Ma.4 (61.4627.08), Ma.5 (61.3728.08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains together and between different time points post-infection.

3.2.3 Intracellular viability of *M. avium* in THP-1 and HD11 cells at MOI 10:1

The viability of the *M. avium* strains inside THP-1 and HD11 cells was assessed using the gentamicin protection assay. The viable bacterial counts at different time point post-infection are shown in Figure 3.2 and Figure 3.3.

3.2.3.1 THP-1

In THP-1 cells, we observed that Ma.5 showed a significantly higher initial uptake compared to Ma.1 and Ma.4 ($P<0.05$) but not compared to the other strains. The number of intracellular mycobacteria reduced in all strains at 6 h post-infection which was

significant for Ma.1 and Ma.2 and not significant for the other strains (P<0.05) when compared to their corresponding CFU values at 2 h. The intracellular bacterial numbers then showed an increase for all strains at 24 h of infection in comparison to their CFU values at 6 h post-infection and which was significant for Ma.1, Ma.2 and Ma.3 (P<0.01) (Figure 3.2).

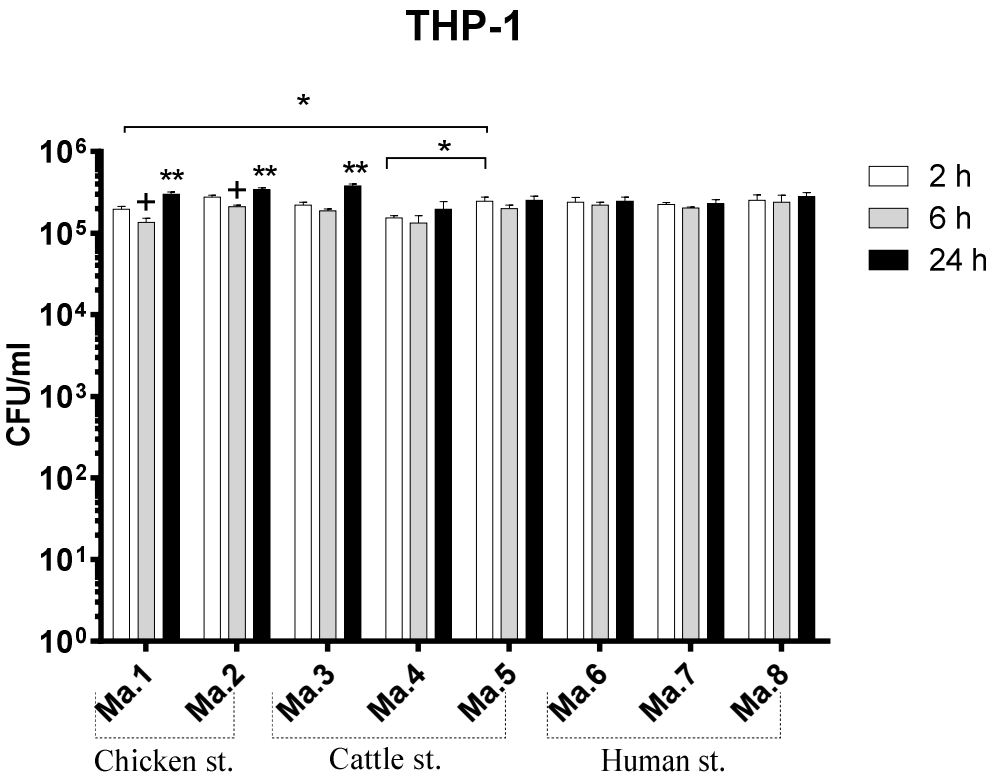


Figure 3.2: Intracellular kinetics of *M. avium* strains in THP-1 cells.

PMA-differentiated THP-1 cells infected with eight *M. avium* strains at MOI of 10 for 1 h. Bacterial numbers enumerated at 2, 6 and 24 h post-infection. * refers to significant differences (P<0.05) between CFU at 2 h post-infection, + refers to significant differences (*P*<0.05) between CFU at 2 h and 6 h post-infection, and ** denote significant differences (P<0.01), between CFU at 6 h and 24 h post-infection. Data presented as mean concentration ± SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623.05), Ma.4 (61.4627.08), Ma.5 (61.3728.08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains and between different time points post-infection.

3.2.3.2 HD11

The bacterial numbers in HD11 cells showed comparable values at 2 h post-infection for the different strains of *M. avium*. Ma.5 showed a significantly higher number of CFU at 2 h post-infection compared with that of Ma.3 and Ma.4 ($P<0.05$) but not significant with the other strains. The kinetics of intracellular survival of *M. avium* strains in HD11 cells was different from that in THP-1 cells. In HD11 cells, all strains showed an increase in the number of intracellular bacteria at 6 h of infection although this was not significant and there was no significant difference between any of the strains. After 24 h, the intracellular bacterial numbers decreased for all strains except Ma.1. This reduction in the number of intracellular mycobacteria was significant for Ma.4 and Ma.5 ($P<0.01$) but not significant for the other strains Figure 3.3.

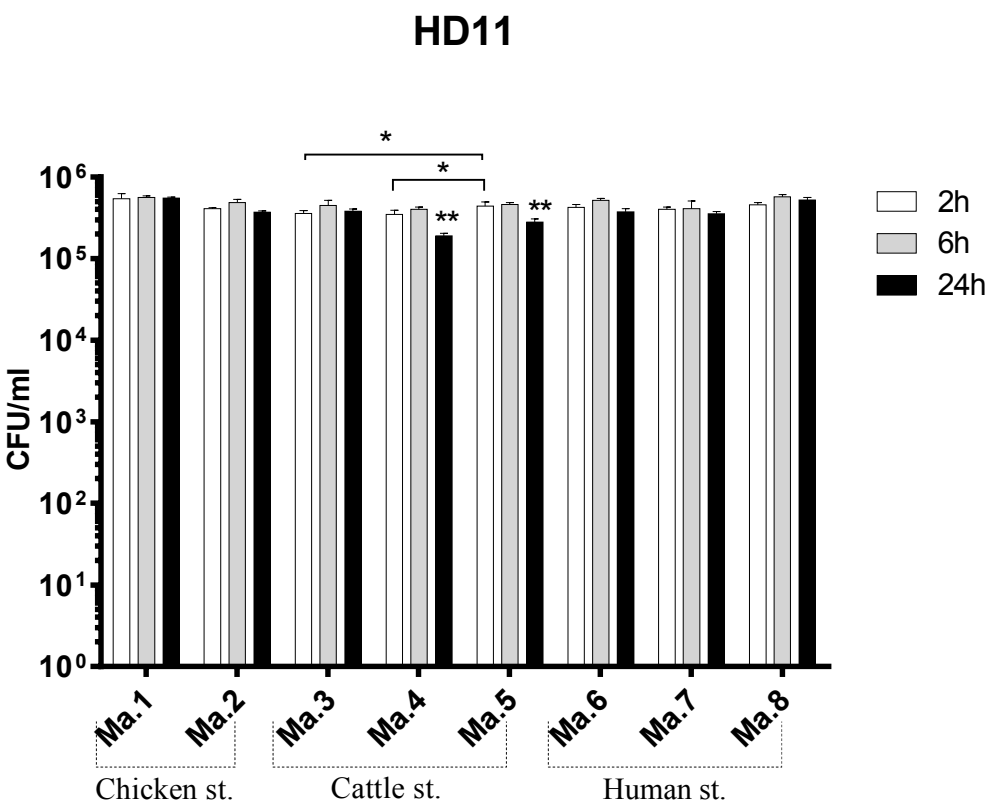


Figure 3.3: Intracellular kinetics of *M. avium* strains in HD11 cells.

HD11 cells infected with eight *M. avium* strains at MOI of 10 for 1 h. Bacterial numbers enumerated at 2, 6 and 24 h post-infection. * refers to significant differences (P<0.05) between CFU at 2 h post-infection, and ** denote significant differences (P<0.01), between CFU at 6 h and 24 h post-infection. Data presented as mean concentration ± SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623.05), Ma.4 (61.4627.08), Ma.5 (61.3728.08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains and between different time points post-infection.

3.2.4 Production of nitric oxide by THP-1 and HD-11 cells in response to infection with *M. avium*

Using the Griess assay, NO levels were measured in the supernatants of THP-1 and HD11 cells in response to *M. avium* infection.

3.2.4.1 THP-1

No significant levels of NO were detected in the supernatants of any *M. avium*-infected THP-1 cells at any time point post-infection (Figure 3.4) indicating that THP-1 cells do not produce NO at early time points post-infection.

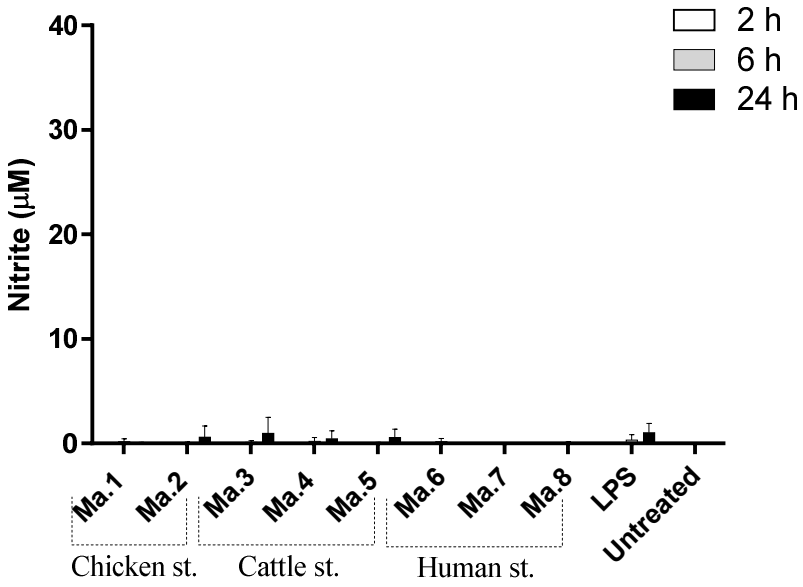


Figure 3.4: Nitric oxide production by THP-1 macrophages after challenge with *M. avium* strains.

THP-1 cells infected with various strains of *M. avium* and supernatants collected at different times after infection for measurement of NO by using the Griess assay. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08) and Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132).

3.2.4.2 HD11

In contrast, NO was produced by HD11 cells in response to *M. avium* infection with especially high levels detected at 24 h post-infection (Figure 3.5). At 2 and 6 h post-infection no significant differences were observed between the level of NO induced by various strains of *M. avium*. However, at 24 h post-infection statistically significant differences at the level $P<0.05$ were obtained

between the following pairs of data: Ma.2 and Ma.7; Ma.2 and Ma.8. Furthermore, statistically significant differences at the level P<0.01 were obtained between the following pairs of data: Ma.1 and Ma.4; Ma.1 and Ma.5; Ma.2 and Ma.4; Ma.2 and Ma.5; Ma.3 and Ma.4; Ma.3 and Ma.5; Ma.4 and Ma.6; Ma.4 and Ma.7; Ma.4 and Ma.8; Ma.5 and Ma.6; Ma.5 and Ma.7; Ma.5 and Ma.8. In addition, the concentration of NO was significantly higher at 24 h post-infection among all tested strains except Ma.7 and Ma.8 compared to the non-infected control samples (P<0.05).

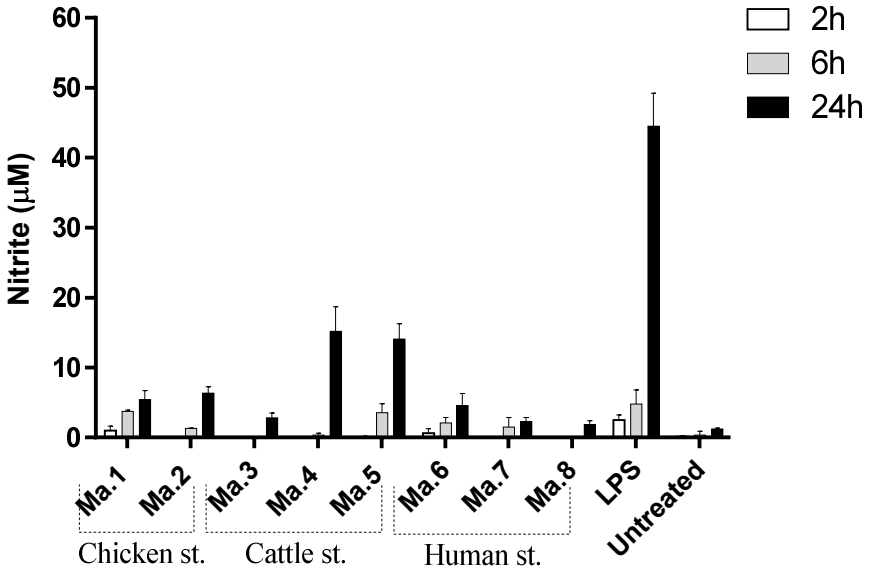


Figure 3.5: Nitric oxide production by HD11 macrophages after challenge with *M. avium* strains.

HD11 cells infected with various strains of *M. avium* and supernatants collected at different times after infection for measurement of NO by using the Griess assay. *=P<0.05, **=P<0.01 denote significant differences between NO levels induced by different strains at 24 h of infection. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08) and Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains at different time points post-infection.

Table 3.1: Comparison between NO levels induced by various *M. avium* strains at 24 h post-infection

	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	**	**	NS	NS	NS
Ma.2	Chicken			NS	**	**	NS	*	*
Ma.3	Cattle				**	**	NS	NS	NS
Ma.4	Cattle					NS	**	**	**
Ma.5	Cattle						**	**	**
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	**	**	**	**	**	**	**	**

* (P<0.05), ** (P<0.05), NS (not significant) 24 h post-infection

3.2.5 Cytokine production in THP-1 cells in response to infection with *M. avium* strains

Using commercial ELISA kits, the levels of cytokines TNF-α, IL-1β, IL-6, and CXCL8 production by macrophages were estimated at 2, 6 and 24 h post-infection.

3.2.5.1 TNF-α

There were significant increases in the levels of TNF-α at 6 and 24 h post-infection with all strains examined in this study compared to non-infected control samples (P<0.01). Although all the strains tested induced high levels of TNF-α production, these were not equal between the strains. The highest level of this cytokine was detected from macrophages infected with *M. avium* strain Ma.4, Ma.5 and human strains (Ma.6-Ma.8), and the lowest level was induced by Ma.3 at 24h post-infection (Figure 3.6). There was no significant difference between strains in induction of this cytokine at 2 h of infection. However, at 6 h post-infection statistically significant

differences at the level P<0.05 were obtained between the following pairs of data: Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, and Ma.3 and Ma.8. Furthermore, at 24 h post-infection statistically significant differences at P<0.05 were obtained between the following pairs of data: Ma.1 and Ma.2., Ma.1 and Ma.4, Ma.1 and Ma.5, Ma.1 and Ma.6, Ma.1 and Ma.7, Ma.1 and Ma.8, Ma.2 and Ma.4, Ma.2 and Ma.5, Ma.2 and Ma.6, Ma.2 and Ma.7, Ma.2 and Ma.8, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, Ma.3 and Ma.8, Ma.4 and Ma.8, Ma.6 and Ma.8.

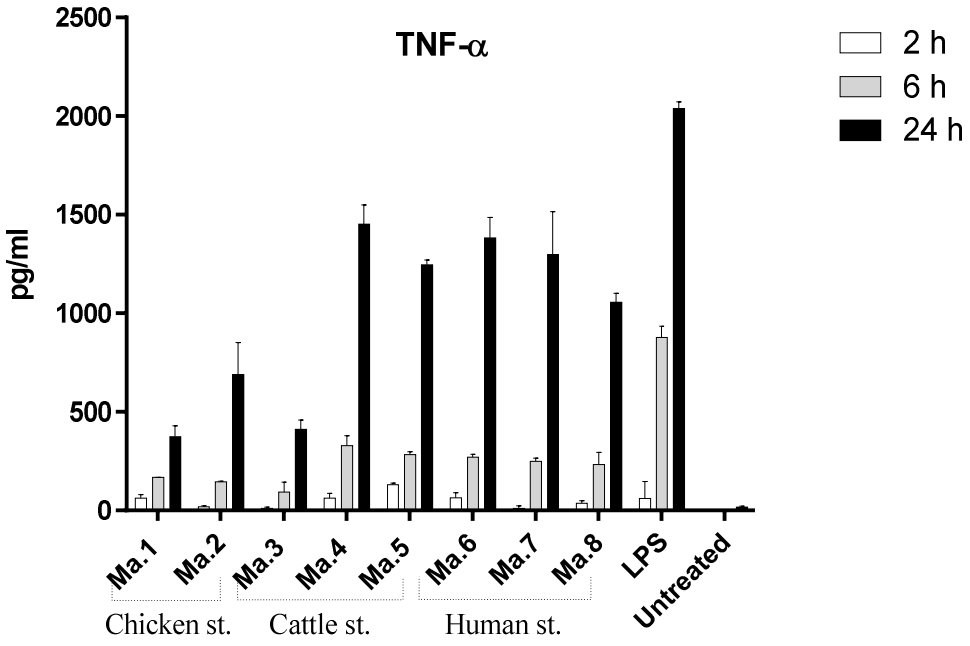


Figure 3.6: TNF-α production by differentiated THP-1 cells infected with various *M. avium* strains at different time points post-infection.

Production of TNF-α by PMA-differentiated THP-1 cells after challenge with different strains of *M. avium* is shown. *=P<0.05, **=P<0.01 indicate differences between levels of TNF-α induced by various strains at different time points post-infection. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to find differences between the groups.

Table 3.2: Comparison between TNF-α levels induced by various *M. avium* strains at 6 h post-infection

TNF-α	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	*	NS	NS	NS	NS
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	**	**	**	**	**	**	**	**

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

Table 3.3: Comparison between TNF-α levels induced by various *M. avium* strains at 24 h post-infection

TNF-α	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		*	NS	*	*	*	*	*
Ma.2	Chicken			NS	*	*	*	*	*
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	NS	NS	*
Ma.5	Cattle						NS	NS	
Ma.6	Human							NS	*
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	**	**	**	**	**	**	**	**

* (P<0.05), ** (P<0.05), NS (not significant) 24 h post-infection

3.2.5.2 IL-1β

IL-1β production by macrophages in response to various strains of *M. avium* is shown in Figure 3.7. All strains induced significantly higher level of IL-1β at 6 h and 24 h of infection compared to non-infected control samples (P<0.05) but there were some differences amongst the strains. At 2 h post infection, statistically significant

differences were obtained at the level P<0.05 between the following pairs of data: Ma.1 and untreated cells, Ma.2 and Ma.5, Ma.3 and Ma.5, Ma.4 and untreated cells, Ma.5 and untreated cells, Ma.6 and untreated cells, Ma.7 and untreated cells.

In addition, at 6 h post-infection statistically significant differences at P<0.05 were obtained between the following pairs of data: Ma.1 and Ma.4, Ma.1 and Ma.6, Ma.1 and Ma.7, Ma.2 and Ma.4, Ma.2 and Ma.6, Ma.2 and Ma.7, Ma.2 and Ma.8, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, Ma.3 and Ma.8.

Furthermore at 24 h post infection, significant differences at P<0.05 were obtained between the following pairs of data: Ma.1 and Ma.4, Ma.1 and Ma.5, Ma.1 and Ma.6, Ma.1 and Ma.7, Ma.1 and Ma.8, Ma.2 and Ma.4, Ma.2 and Ma.5, Ma.2 and Ma.6, Ma.2 and Ma.7, Ma.2 and Ma.8, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, Ma.3 and Ma.8.

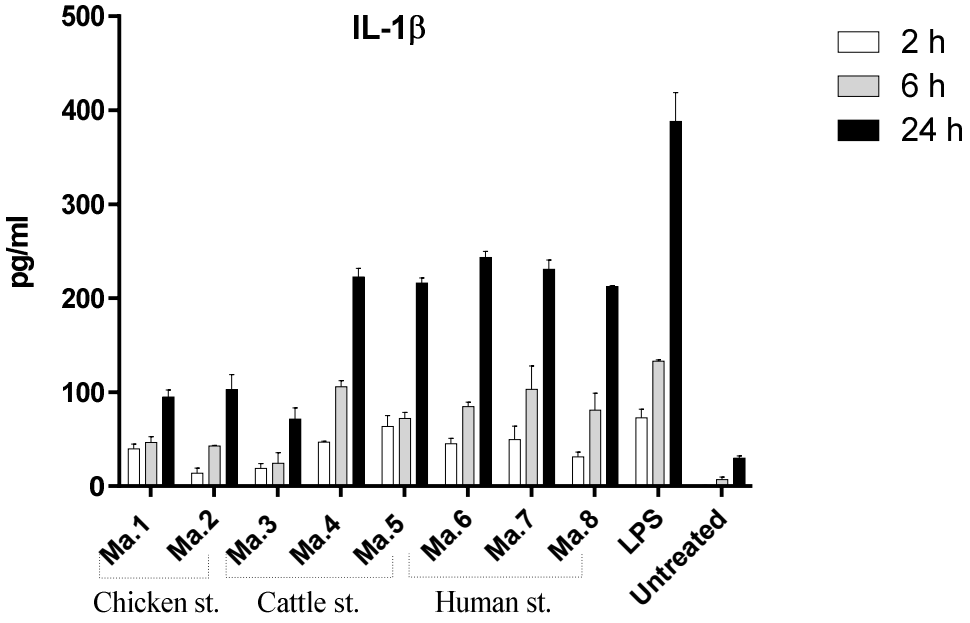


Figure 3.7: IL-1β production by differentiated THP-1 cells infected with various *M. avium* strains at different time points post-infection.

Production of IL-1β by PMA-differentiated THP-1 cells after challenge with different strains of *M. avium* is shown. *=P<0.05, **=P<0.01 indicate differences between levels of cytokines induced by various strains at different time points post-infection. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to find differences between the groups.

Table 3.4: Comparison between IL-1β levels induced by various *M. avium* strains at 2 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	*	NS	NS	NS
Ma.3	Cattle				NS	*	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	*	NS	NS	*	*	*	*	NS

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.5: Comparison between IL-1β levels induced by various *M. avium* strains at 6 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	*	NS	*	*	NS
Ma.2	Chicken			NS	*	NS	*	*	*
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	*	*	*	*	*	*	*	*

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

Table 3.6: Comparison between IL-1β levels induced by various *M. avium* strains at 24 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	*	*	*	*	*
Ma.2	Chicken			NS	*	*	*	*	*
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	*	*	*	*	*	*	*	*

* (P<0.05), ** (P<0.05), NS (not significant) 24 h post-infection

3.2.5.3 IL-6

IL-6 production was only detected in supernatant of cells after 24 h of infection with different strains of *M. avium* Figure 3.8. The levels of IL-6 induced by all strains by this time point were significantly higher than non-infected cells (P<0.05). Statistically significant differences at P<0.05 were obtained between the following pairs of data: Ma.1 and Ma.2, Ma.1 and Ma.3, Ma.1 and Ma.4, Ma.1 and Ma.5, Ma.1 and Ma.6, Ma.1 and Ma.8, Ma.2 and Ma.3, Ma.2 and Ma.4, Ma.2 and Ma.5, Ma.2 and Ma.7, Ma.2 and Ma.8, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, Ma.3 and Ma.8, Ma.4 and Ma.6, Ma.4 and Ma.7, Ma.4 and Ma.8, Ma.5 and Ma.6, Ma.5 and Ma.7, Ma.5 and Ma.8, Ma.6 and Ma.7, Ma.6 and Ma.8, Ma.7 and Ma.8.

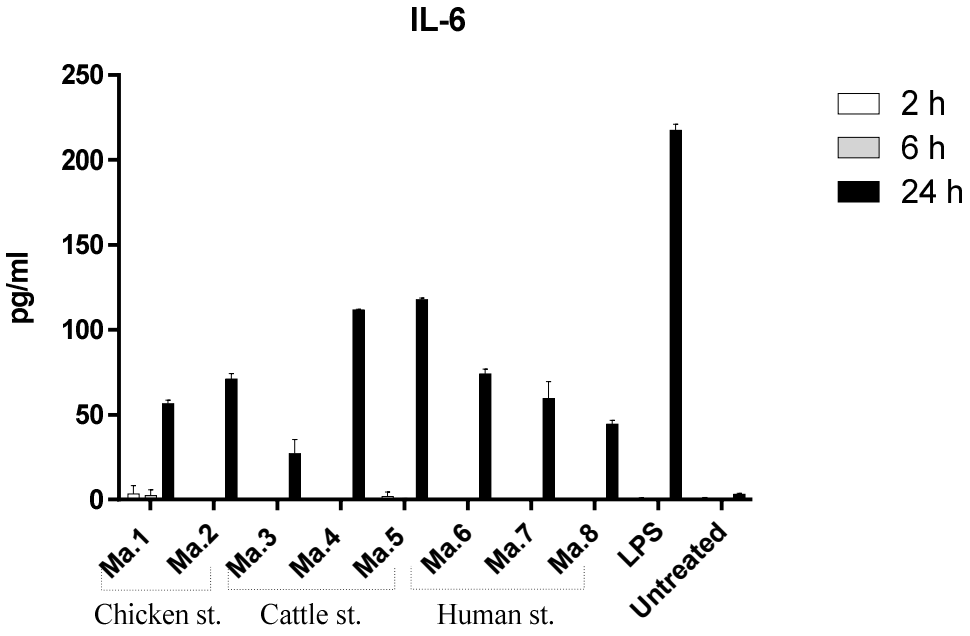


Figure 3.8: IL-6 production by differentiated THP-1 cells infected with various *M. avium* strains at different time points post-infection.

Production of IL-6 by PMA-differentiated THP-1 cells after challenge with different strains of *M. avium* is shown. *=P<0.05, **=P<0.01 indicate differences between levels of cytokines induced by various strains at different time points post-infection. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to find differences between the groups.

Table 3.7: Comparison between IL-6 levels induced by various *M. avium* strains at 6 h post-infection

IL-6	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		*	*	*	*	*	NS	*
Ma.2	Chicken			*	*	*	NS	*	*
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	*	*	*
Ma.5	Cattle						*	*	*
Ma.6	Human							*	*
Ma.7	Human								*
Ma.8	Human								
Untreated	THP-1	*	*	*	*	*	*	*	*

* (P<0.05), ** (P<0.05), NS (not significant) 24 h post-infection

3.2.5.4 CXCL8

The levels of CXCL8 released by THP-1 macrophages during 2, 6 and 24 h of infection with different strains of *M. avium* are shown in Figure 3.9. At 2 h of infection there was no significant difference between strains in induction of this cytokine. At 6 h post-infection, statistically significant differences at $P<0.05$ were obtained between the following pairs of data: Ma.4 and Ma.5, Ma.5 and Ma.6. In addition, statistically significant differences at $P<0.01$ were obtained between the following pairs of data: Ma.1 and Ma.5, Ma.2 and Ma.5, Ma.3 and Ma.5, Ma.5 and Ma.7, Ma.5 and Ma.8, Ma.5 and untreated cells.

At 24 h post-infection, statistically significant differences at the level $P<0.05$ were obtained between the following pairs of data: Ma.1 and Ma.3, Ma.1 and Ma.4, Ma.1 and Ma.6, Ma.1 and Ma.8, Ma.2 and Ma.6, Ma.2 and Ma.7, Ma.2 and Ma.8, Ma.3 and untreated cells, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.3 and Ma.6, Ma.3 and Ma.7, Ma.3 and Ma.8, Ma.4 and Ma.6, Ma.4 and Ma.7, Ma.4 and Ma.8, Ma.5 and Ma.6, Ma.5 and Ma.7, Ma.5 and Ma.8. Furthermore, statistically significant differences at $P<0.01$ were obtained between the following pairs of data: Ma.1 and Ma.5, Ma.1 and untreated cells, Ma.2 and Ma.4, Ma.2 and Ma.5, Ma.2 and untreated cells, Ma.4 and untreated cells, Ma.5 and untreated cells, Ma.6 and untreated cells, Ma.7 and untreated cells, Ma.8 and untreated cells.

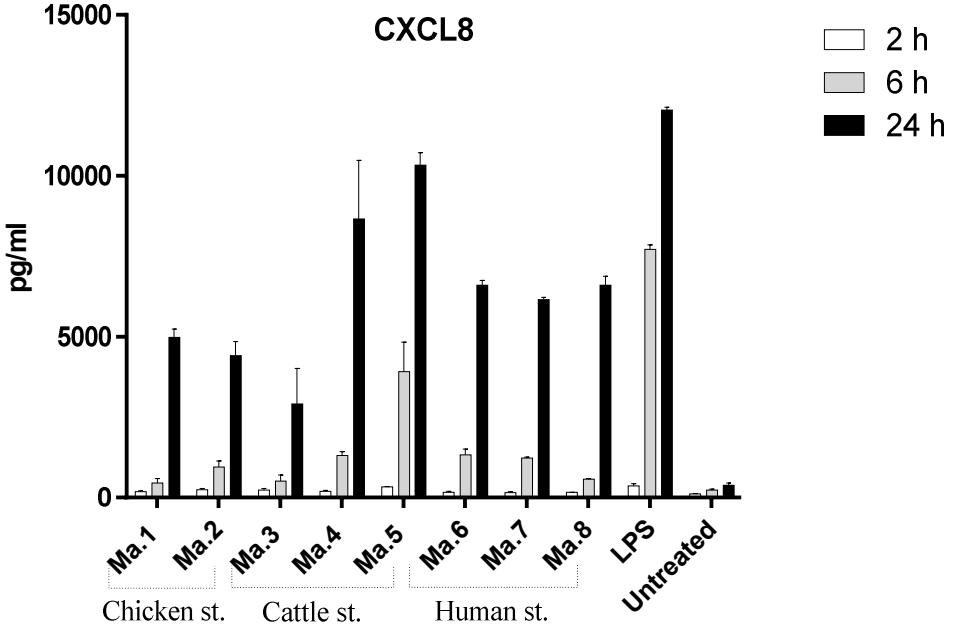


Figure 3.9: CXCL8 production by differentiated THP-1 cells infected with various *M. avium* strains at different time points post-infection.

Production of CXCL8 by PMA-differentiated THP-1 cells after challenge with different strains of *M. avium* is shown. *=P<0.05, **=P<0.01 indicate differences between levels of cytokines induced by various strains at different time points post-infection. Data presented as mean concentration ± SEM of three independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to find differences between the groups.

Table 3.8: Comparison between CXCL8 levels induced by various *M. avium* strains at 6 h post-infection

CXCL8	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	**	NS	NS	NS
Ma.2	Chicken			NS	NS	**	NS	NS	NS
Ma.3	Cattle				NS	**	NS	NS	NS
Ma.4	Cattle					*	NS	NS	NS
Ma.5	Cattle						*	**	**
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	NS	NS	NS	NS	**	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

Table 3.9: Comparison between CXCL8 levels induced by various *M. avium* strains at 24 h post-infection

	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken			*	*	**	*	*	*
Ma.2	Chicken				**	**	*	*	*
Ma.3	Cattle				*	*	*	*	*
Ma.4	Cattle					NS	*	*	*
Ma.5	Cattle						*	*	*
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	**	**	*	**	**	**	**	**

* (P<0.05), ** (P<0.05), NS (not significant) 24 h post-infection

3.2.6 Cytokine gene expression in THP-1 cells following infection with *M. avium* strains

To further evaluate the differential ability of *M. avium* strains to induce cytokine production, the mRNA levels of TNF-α and IL-1β were quantified in THP-1 cells at 6 h post-infection using RT-qPCR.

3.2.6.1 TNF-α

Figure 3.10 shows statistically significant differences in TNF-α expression between the following pairs of data at the level P<0.05: Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.4 and untreated cells, Ma.5 and untreated cells.

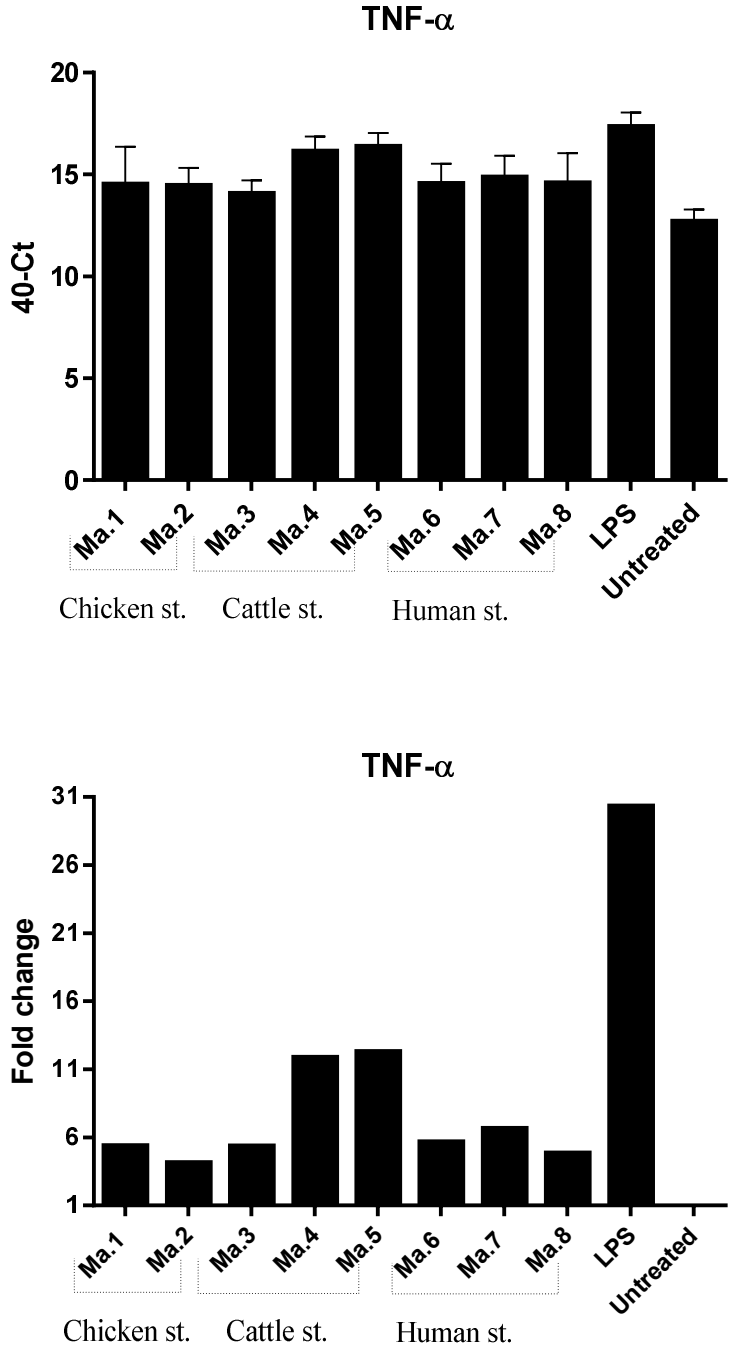


Figure 3.10: TNF-α mRNA expression by differentiated THP-1 cells infected with various *M. avium* strains at 6 h post-infection.

TNF-α expressed by PMA-differentiated THP-1 cells after challenges with different strains of *M. avium* were quantified by RT-qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The corrected Ct values shown as fold changes in the mRNA level of cytokines in comparison to those from uninfected control cells at 6 h post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *=P<0.05, indicates

differences between levels of cytokines induced by various strains. Data obtained from two independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). One-way ANOVA was used to detect the differences between the strains.

Table 3.10: Comparison between TNF-α mRNA levels induced by various *M. avium* strains at 6 h post-infection

TNF-α	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				*	*	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	NS	NS	NS	*	*	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.6.2 IL-1β

Similarly, statistically significant differences in IL-1β expression were observed between the following pairs of data at the level P<0.05: Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.4 and untreated cells, Ma.5 and untreated cells (Figure 3.11).

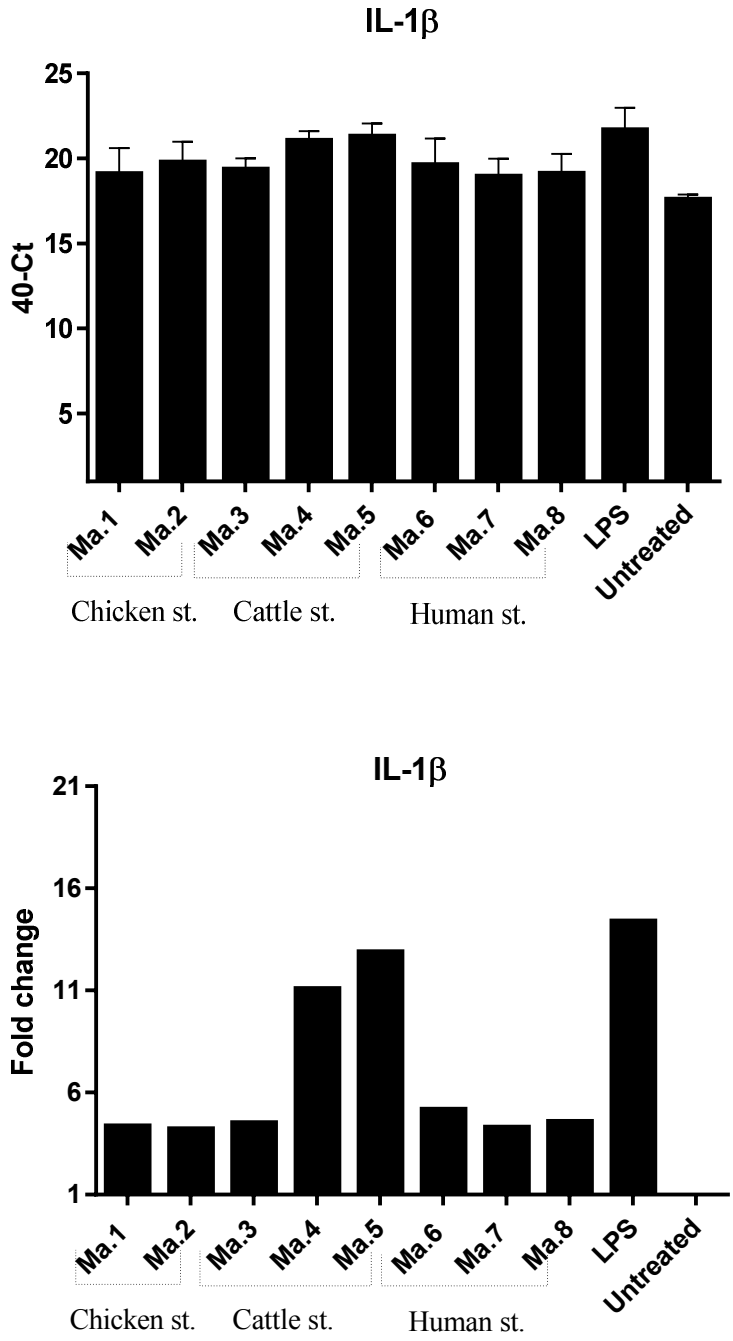


Figure 3.11: IL-1β mRNA expression by differentiated THP-1 cells infected with various *M. avium* strains at 6h post-infection.

IL-1β expressed by PMA-differentiated THP-1 cells after challenges with different strains of *M. avium* were quantified by RT-qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The corrected Ct values shown as fold changes in the mRNA level of cytokines in comparison to those from uninfected control cells at 6h post infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *=P<0.05, indicates differences between levels of cytokines induced by various strains. Data

obtained from two independent experiments. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). One-way ANOVA was used to detect the differences between the strains.

Table 3.11: Comparison between IL-1β mRNA levels induced by various *M. avium* strains at 6 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				*	*	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	THP-1	NS	NS	NS	*	*	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.7 Optimization of RT-qPCR reaction in HD11 cells

For optimization of RT-qPCR reactions, four different concentrations of forward and reverse primers were used in a qRT-PCR assay to select the optimal conditions. The following four conditions were examined; 300 nM forward primer and 300 nM reverse primer, 300 nM forward primer and 900 nM reverse primer, 900 nM forward primer and 300 nM reverse primer, and 900 nM forward primer and 900 nM reverse primer. The concentration of probes in all conditions was 100 nM. As a result, the best primer concentrations were selected based on the earliest expression of Ct values and the strongest florescent signals. As a result of this, the following concentrations of forward and reverse primers of the tested genes were used in this study: 300 nM forward primer and 900 nM reverse

primer for 28S, CXCLi1, CXCLi2, IL-6; 900 nM forward primer and 900 nM reverse primer for IL-1β; 900 nM forward primer and 300 nM reverse primer for iNOS.

Table 3.12: The Ct values of cytokine and chemokine gene expression from four different concentrations of forward and reverse primers for each gene

	F 300 nM R 300 nM	F 300 nM R 900 nM	F 900 nM F 300 nM	F 900 nM R 900 nM
28S	11.73667±0.025166	11.38±0.09609	11.81333±0.04242	11.42667±0.11015
iNOS	24.15333±0.240901	24.90666±0.15534	24.14±0.03	24.91±0.02
IL-1β	28.35±0.037859389	28.46±0.364280112	28.65667±0.121655	28.18666±0.0814452
CXCLi1	29.24666±1.31058	28.545±0.12	29.12±0.03511884	29.01333±0.0503322
CXCLi2	26.4±0.080208063	25.87333±0.060827	26.08±0.04509249	26.59±0.09165151
IL-6	35.44333±0.394250	34.82±0.036055513	35.13333±0.541510	35.19±0.36692415

Four different concentrations of forward and reverse primers were used in a qRT-PCR assay to select the optimal conditions. The best primer concentrations were selected based on the earliest expression of Ct values and the strongest florescent signals.

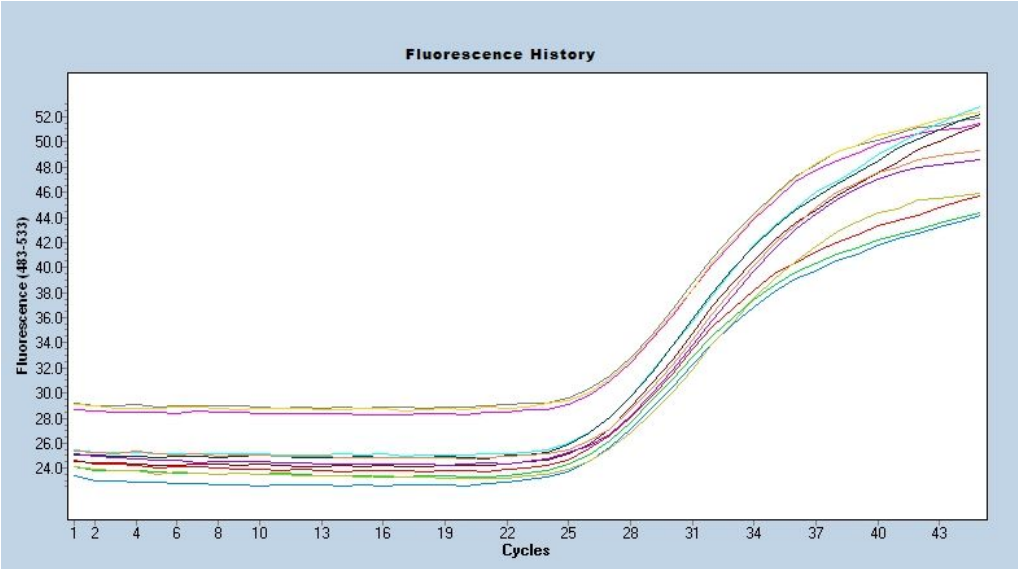


Figure 3.12: Different concentrations of forward and reverse primers used for CXCLi2 expression

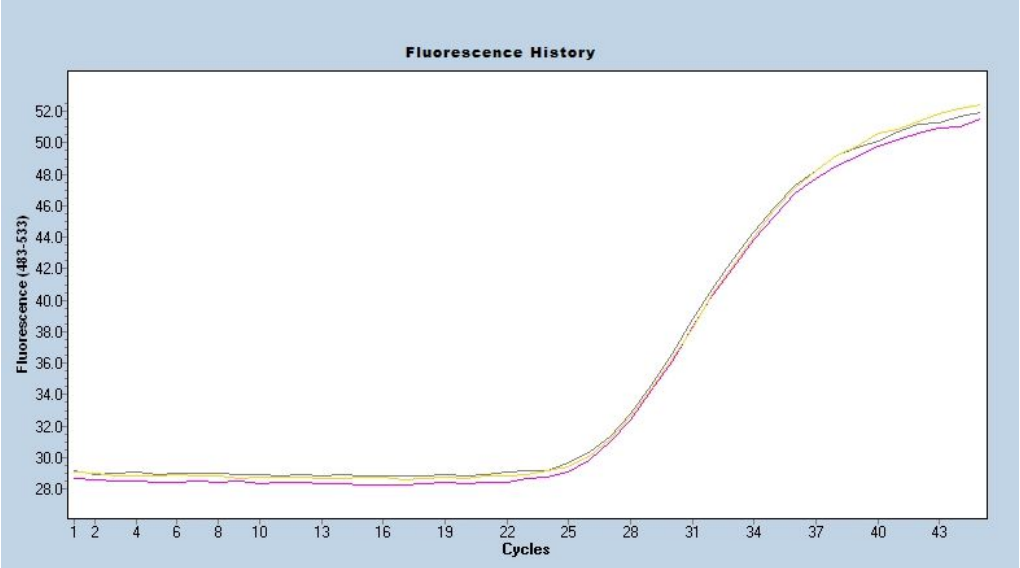


Figure 3.13: The concentration of 300 nM forward primer and 900 nM reverse primer was selected for CXCLi2 expression

3.2.8 Cytokine and chemokine gene expression in HD11 cells in response to infection with *M. avium* strains

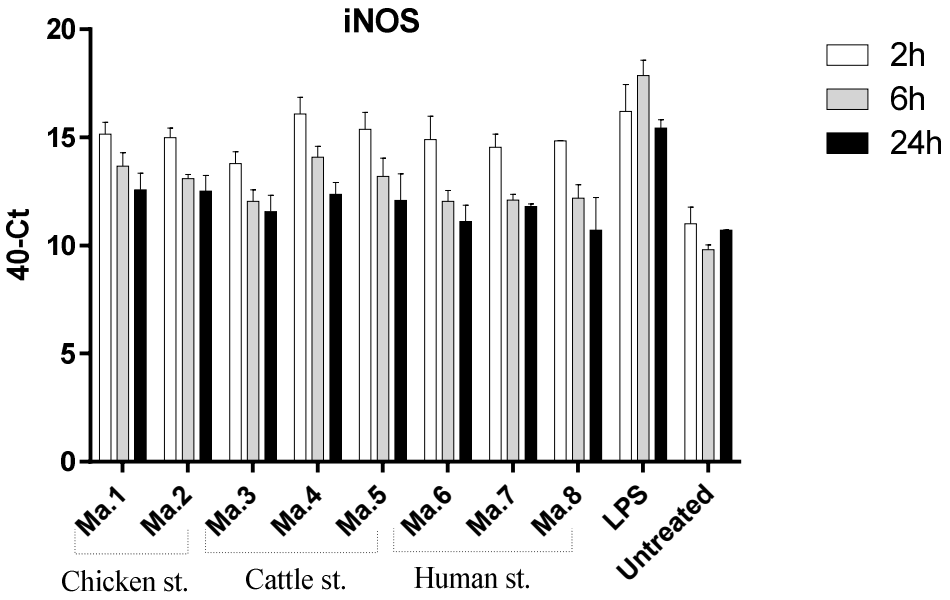
The induction of mRNA expression of iNOS gene and pro-inflammatory cytokines IL-1β, IL-6, and chemokines CXCLi1 and CXCLi2 were quantified in HD11 following infection with various *M. avium* strains. High levels of mRNA expression of IL-1β, iNOS, IL-6, CXCLi1 and CXCLi2 were observed. Variable patterns of cytokine mRNA expression were observed between different strains of *M. avium*. The corrected 40-Ct values of all cytokines examined at various time points post-infection and the fold changes of cytokine gene expression are shown for each of the cytokine and chemokines.

3.2.8.1 iNOS

At 2 h post-infection, statically significant differences were obtained in the mRNA expression level of iNOS between the following pairs of data: Ma.1 and untreated, Ma.2 and Ma.4, Ma.2 and untreated,

Ma.3 and Ma.5, Ma.5 and untreated, Ma.8 and untreated ($P<0.05$) and Ma.3 and Ma.4, Ma.4 untreated ($P<0.01$) (Figure 3.14).

At 6 h post-infection statistically significant differences at $P<0.05$ were obtained between the following pairs of data: Ma.1 and Ma.3, Ma.2 and untreated cells, Ma.4 and untreated cells, Ma.5 and untreated cells, Ma.6 and untreated cells. Furthermore, statistically significant differences at 6 h post-infection at $P<0.01$ were obtained between the following pairs of data: Ma.3 and Ma.4, Ma.7 and untreated cells. At 24 h post-infection, no significant differences were observed between the strains.



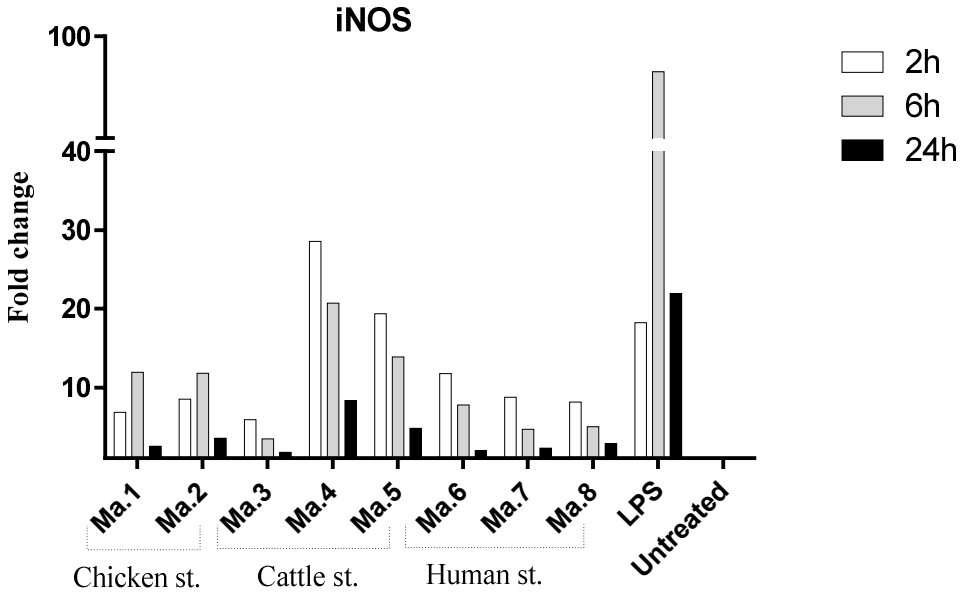


Figure 3.14: iNOS mRNA expression by HD11 cells infected with *M. avium* strains at different time points post-infection.

The corrected Ct values were subtracted from 40 (the negative end-point) after standardization for input RNA. Higher values represent higher levels of cytokine mRNA. The data are corrected Ct values of iNOS gene expression by HD11 cells after challenge with different strains of *M. avium*. Data presented as mean concentration ± SEM of three independent experiments performed in triplicates. **=P<0.01 and *=P<0.05, indicate differences between levels of cytokines induced by various strains. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected cells at different time points post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains.

Table 3.13: Comparison between iNOS mRNA levels induced by various *M. avium* strains at 2 h post-infection

iNOS	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	*	NS	NS	NS	NS
Ma.3	Cattle				**	*	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	**	*	**	**	**	*	**

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.14: Comparison between mRNA iNOS levels induced by various *M. avium* strains at 6 h post-infection

iNOS	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	*	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				**	*	NS	NS	NS
Ma.4	Cattle					NS	*	*	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	**	*	**	**	*	*	*

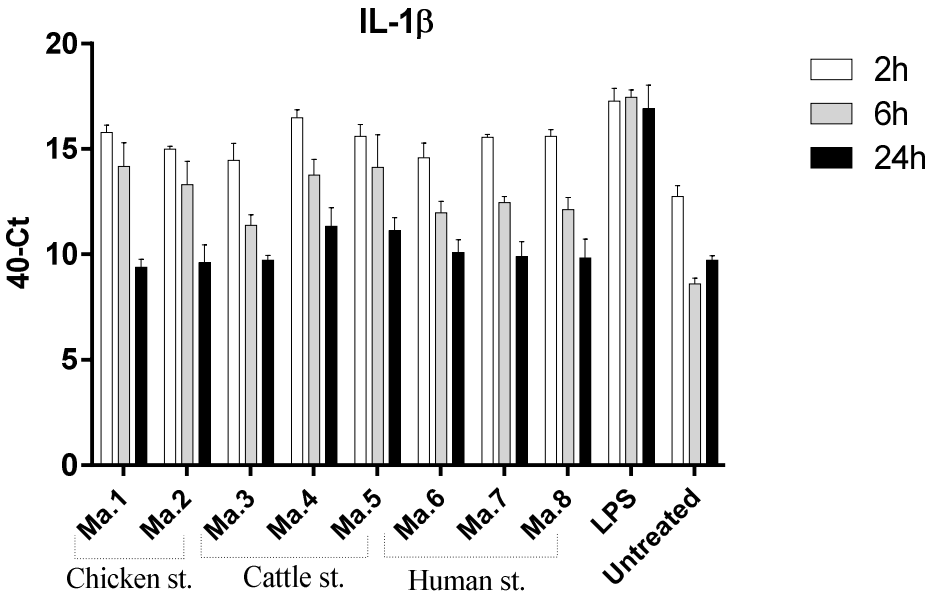
* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.8.2 IL-1β

Statistically significant differences in mRNA expression of IL-1β at 2 h post-infection at P<0.05 were obtained between Ma.1 and Ma.4, Ma.2 and Ma.7, Ma.4 and Ma.6. In addition, at P<0.01 statistically significant differences were obtained at 2 h post-infection between

the following data: Ma.1 and untreated cells Ma.2 and untreated cells, Ma.3 and Ma.4, Ma.4 and untreated, Ma.5 and untreated cells, Ma.7 and untreated cells, Ma.8 and untreated cells (Figure 3.15).

At 6 h post-infection, statistically significant differences were obtained between the following pairs of data at $P<0.05$: Ma.1 and Ma.3, Ma.1 and Ma.6, Ma.1 and Ma.7, Ma.1 and Ma.8, Ma.2 and untreated cells, Ma.3 and Ma.7, Ma.3 and Ma.8, Ma.7 and untreated cells. Furthermore, statistically significant differences at $P<0.01$ were obtained between the following pairs of data: Ma.1 and untreated cells, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.4 and untreated cells, Ma.5 and untreated cells. At 24 h post-infection, no significant differences were observed between the strains.



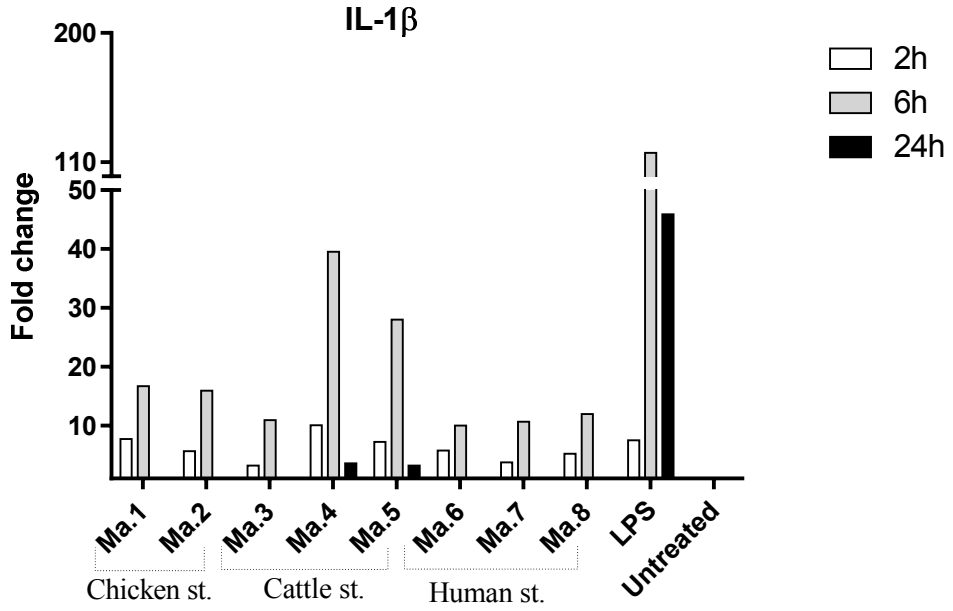


Figure 3.15: IL-1β mRNA expression by HD11 cells infected with *M. avium* strains at different time points post-infection.

The corrected Ct values were subtracted from 40 (the negative end-point) after standardization for input RNA. Higher values represent higher levels of cytokine mRNA. The data are corrected Ct values of IL-1β expression by HD11 cells after challenge with different strains of *M. avium*. Data presented as mean concentration ± SEM of three independent experiments performed in triplicates. **=P<0.01 and *=P<0.05, indicate differences between levels of cytokines induced by various strains. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected cells at different time points post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains.

Table 3.15: Comparison between IL-1β mRNA levels induced by various *M. avium* strains at 2 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	*	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	*	NS
Ma.3	Cattle				**	*	NS	NS	NS
Ma.4	Cattle					NS	*	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	**	NS	**	**	NS	**	NS

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.16: Comparison between IL-1β mRNA levels induced by various *M. avium* strains at 6 h post-infection

IL-1β	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	*	NS	NS	*	*	*
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				**	**	NS	*	*
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	*	NS	**	**	NS	*	NS

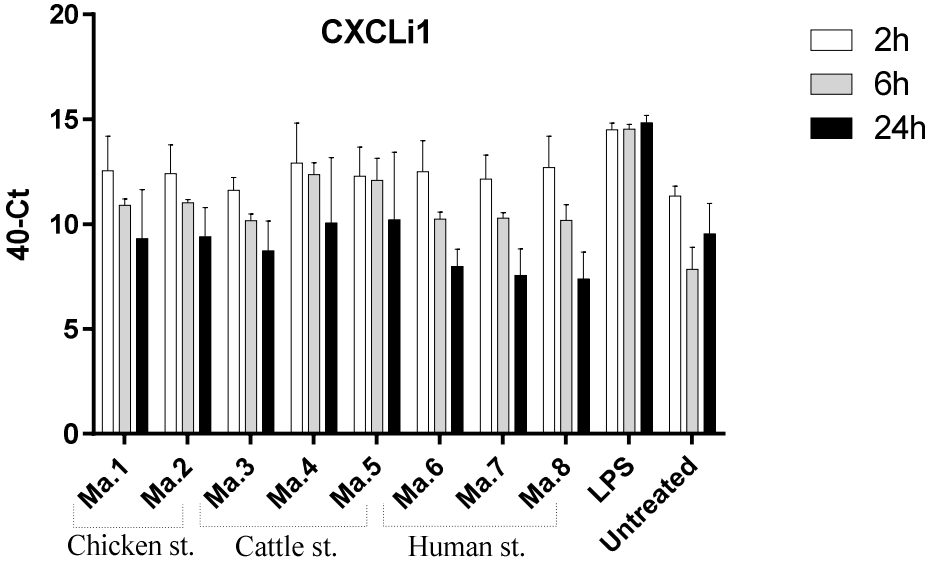
* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.8.3 CXCLi1

At 2 h post-infection, no significant differences were observed in mRNA expression of CXCLi1 between the *M. avium* strains (Figure 3.16).

Statistically significant differences were obtained at P<0.05 between the following pair of data: Ma.1 and Ma.6, Ma.1 and Ma.7, Ma.2 and untreated cells, Ma.3 and untreated cells, Ma.6 and untreated cells, Ma.7 and untreated cells. At P<0.01 significant differences were obtained between the following pairs of data: Ma.1 and untreated cells, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.4 and untreated cells, Ma.5 and untreated cells.

At 24 h post-infection, no significant differences were observed between the strains in induction of CXCLi1.



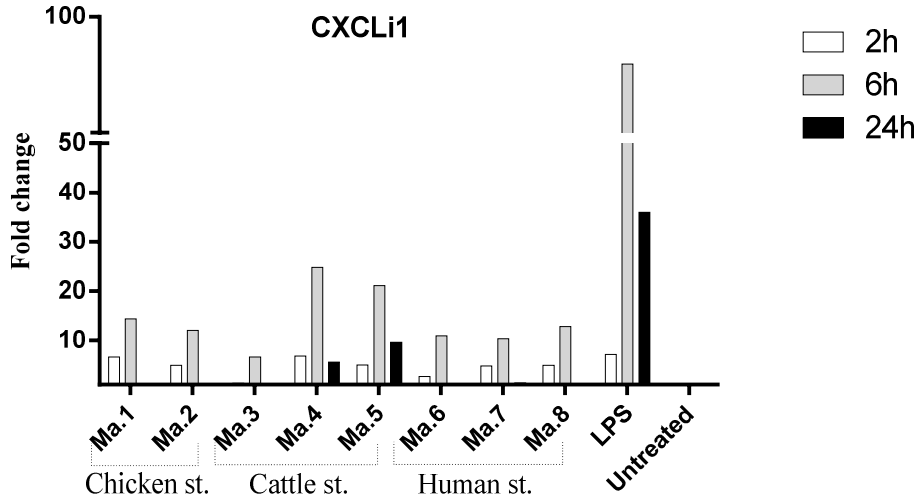


Figure 3.16: CXCLi1 mRNA expression by HD11 cells infected with *M. avium* strains at different time points post-infection.

The corrected Ct values were subtracted from 40 (the negative end-point) after standardization for input RNA. Higher values represent higher levels of cytokine mRNA. The data are corrected Ct values of CXCLi1 expression by HD11 cells after challenge with different strains of *M. avium*. Data presented as mean concentration ± SEM of three independent experiments performed in triplicates. **=P<0.01 and *=P<0.05, indicate differences between levels of cytokines induced by various strains. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected cells at different time points post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains.

Table 3.17: Comparison between CXCLi1 mRNA levels induced by various *M. avium* strains at 2 h post-infection

CXCLi1	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				NS	NS	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	NS	NS	NS	NS	NS	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.18: Comparison between CXCLi1 mRNA levels induced by various *M. avium* strains at 6 h post-infection

CXCLi1	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	*	*	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				**	**	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	*	*	**	**	*	*	NS

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.8.4 CXCLi2

At 2 h post-infection, statistically significant differences were obtained at P<0.05 between the following pair of data: Ma.2 and Ma.3, Ma.4 and untreated cells (Figure 3.17).

At 6 h post-infection statistically significant differences were obtained at P<0.05 between the following pair of data: Ma.3 and untreated cells, Ma.4 and untreated cells, Ma.6 and untreated cells, Ma.7 and untreated cells, Ma.8 and untreated cells. At the level P<0.01 significant differences were obtained between the following pairs of data: Ma.1 and untreated cells, Ma.2 and untreated cells, Ma.3 and Ma.4, Ma.3 and Ma.5, Ma.5 and untreated cells.

At 24 h post-infection, no significant differences were observed between the strains.

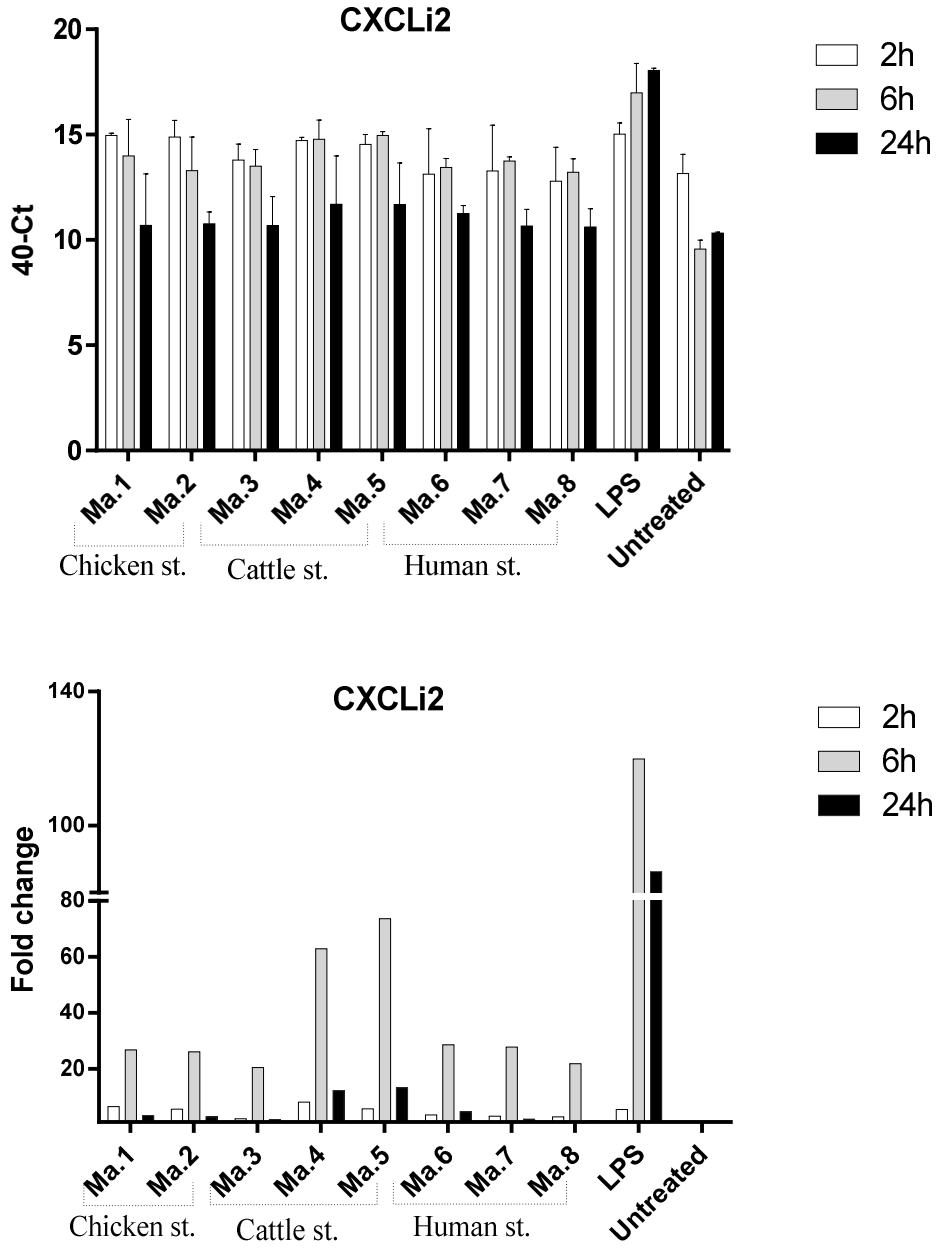


Figure 3.17: CXCLi2 mRNA expression by HD11 cells infected with *M. avium* strains at different time points post-infection.

The corrected Ct values were subtracted from 40 (the negative end-point) after standardization for input RNA. Higher values represent higher levels of cytokine mRNA. The data are corrected Ct values of CXCLi2 expression by HD11 cells after challenge with different strains of *M. avium*. Data presented as mean concentration ± SEM of three independent experiments performed in triplicates. **=P<0.01 and *=P<0.05, indicate differences between levels of cytokines induced by various strains. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected cells at different time points post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-

08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains.

Table 3.19: Comparison between CXCLi2 mRNA levels induced by various *M. avium* strains at 2 h post-infection

CXCLi2	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			*	NS	NS	NS	NS	NS
Ma.3	Cattle				NS	NS	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	NS	NS	NS	*	NS	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.20: Comparison between CXCLi2 mRNA levels induced by various *M. avium* strains at 6 h post-infection

CXCLi2	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				**	**	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	**	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	**	*	*	**	*	*	NS

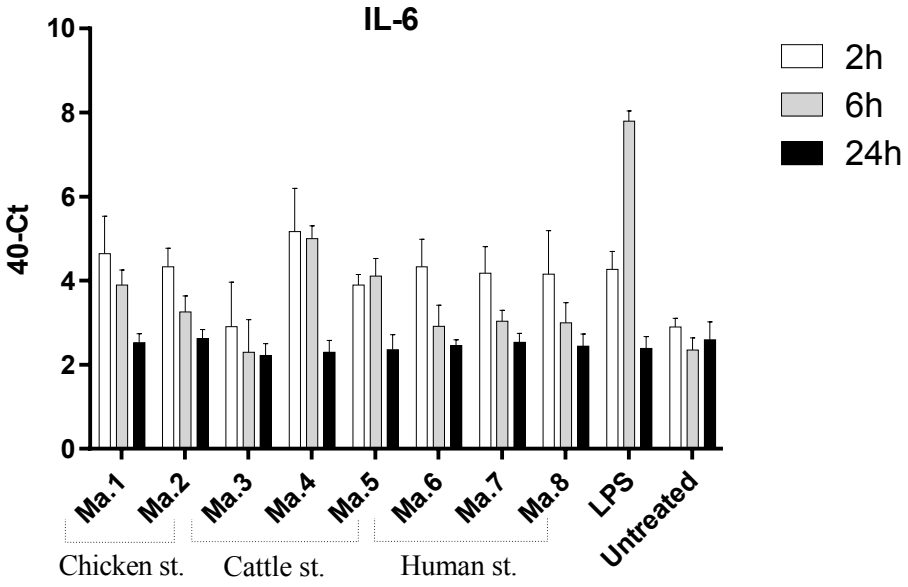
* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.2.8.5 IL-6

At 2 h post-infection, significant differences were obtained at P<0.05 between the following pairs of data: Ma.3 and Ma.4, Ma.4 and Ma.8 (Figure 3.18).

At 6 h post-infection, significant differences ($P<0.05$) were obtained between the following pairs of data: Ma.1 and Ma.2, Ma.1 and Ma.3. In addition, significant differences at the $P<0.01$ were obtained between the following pairs of data: Ma.1 and untreated cells, Ma.3 and Ma.4, Ma.4 and untreated cells, Ma.5 and untreated cells.

At 24 h post-infection, no significant differences were observed between the strains.



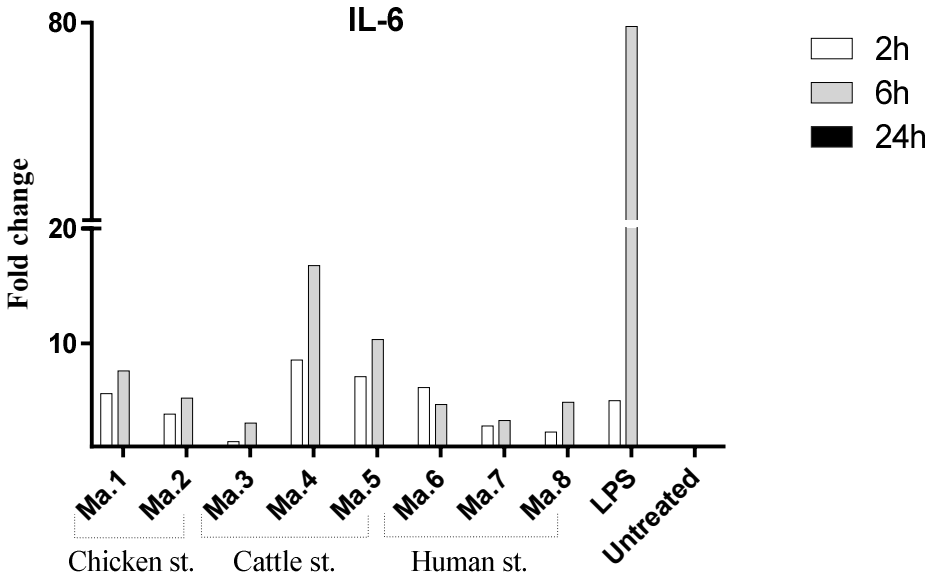


Figure 3.18: IL-6 mRNA expression by HD11 cells infected with *M. avium* strains at different time points post-infection.

The corrected Ct values were subtracted from 40 (the negative end-point) after standardization for input RNA. Higher values represent higher levels of cytokine mRNA. The data are corrected Ct values of IL-6 gene expression by HD11 cells after challenge with different strains of *M. avium*. Data presented as mean concentration ± SEM of three independent experiments performed in triplicates. **=P<0.01 and *=P<0.05, indicate differences between levels of cytokines induced by various strains. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected cells at different time points post-infection. For this, the levels of cytokine mRNA in uninfected control samples were set as 1. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between the strains.

Table 3.21: Comparison between IL-6 mRNA levels induced by various *M. avium* strains at 2 h post-infection

IL-6	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		NS	NS	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				NS	NS	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	*
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	NS	NS	NS	*	NS	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 2 h post-infection

Table 3.22: Comparison between IL-6 mRNA levels induced by various *M. avium* strains at 6 h post-infection

IL-6	Source	Ma.1	Ma.2	Ma.3	Ma.4	Ma.5	Ma.6	Ma.7	Ma.8
Ma.1	Chicken		*	*	NS	NS	NS	NS	NS
Ma.2	Chicken			NS	NS	NS	NS	NS	NS
Ma.3	Cattle				**	*	NS	NS	NS
Ma.4	Cattle					NS	NS	NS	NS
Ma.5	Cattle						NS	NS	NS
Ma.6	Human							NS	NS
Ma.7	Human								NS
Ma.8	Human								
Untreated	HD11	**	NS	NS	**	**	NS	NS	NS

* (P<0.05), ** (P<0.05), NS (not significant) 6 h post-infection

3.3 Discussion

The cellular response to mycobacterial infection is influenced by the virulence of the infecting species. The outcome of infection is critically dependent upon early immune responses. The detailed interaction between these bacteria and early immune responses are not fully understood, and very little is known about the early immune responses to *M. avium.* This study compared the eight strains of *M. avium* for their invasion, induction of NO production, ability to induce pro-inflammatory cytokines release and induction of cytokine gene expression in human and chicken macrophages.

The results in this study clearly show that all strains of *M. avium* were able to survive inside both human and chicken macrophages over the period of infection which was 24 h in this study. The kinetics of intracellular survival and multiplication of *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* have been reported for J774 murine macrophages (Kuehnel et al., 2001). They found that, in contrast to pathogenic mycobacteria, including *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis,* other non-pathogenic species such as *M. smegmatis* and *M. gordonae,* were not able to resist the intracellular killing mechanism of macrophages for longer than 24 h (Kuehnel et al., 2001). They also found that intracellular survival of *M. avium* in J774 macrophages decreased at 24 h post-infection and increased at 48 h post-infection, whereas we observed an increase in intracellular bacterial numbers at 24 h post-infection in THP-1 cells and a reduction in HD11 cells at the same time of infection. This difference might be

related to the different cell models used in these two studies. Some studies with THP-1 have indicated that mycobacteria may have a tendency to clump with each other during infections and this could conceivably lead to an underrepresentation in intracellular counts for either or both cell lines (Manganelli et al., 2001, Dubnau et al., 2002, Walters et al., 2006, Fontan et al., 2008).

A recent study showed that, in HD11, various *Salmonella* serovars showed a reduction in CFU, although the extent of this reduction was different between serovars (He et al., 2012). Our data showed a similar reduction in the intracellular bacillary numbers at 24 h of infection in chicken cells. However, the reduction was not similar amongst all strains, as strains Ma.4 and Ma.5 showed higher reductions than observed in other strains. Differences in intracellular survival could relate to differences in pathogenicity between various *M. avium* strains and we assessed this using *in vivo* studies described in chapter 5.

NO production with the respiratory burst is the most characteristic mechanism by which the macrophages exert their antimicrobial function through oxidative damage (Appelberg, 2006). Although it is argued that NO production does not seem to play an effective role in growth restriction of bacteria (Bermudez, 1993, Doherty and Sher, 1997), several studies have reported an effective role of NO in killing a wide range of bacteria, including mycobacteria (Flesch and Kaufmann, 1991, Nathan and Xie, 1994, Chan et al., 1995, MacMicking et al., 1997, Garcia et al., 2000, Shiloh and Nathan, 2000). Furthermore, mycobacterial tolerance to reactive nitrogen

intermediates *in vitro* depends on many factors including strain, time and dose, the non-pathogenic strains clearly appearing to be more sensitive than pathogenic ones (Obrien et al., 1994, Rhoades and Orme, 1997, Long et al., 1999, Long et al., 2005, Jordao et al., 2008). In order to test the susceptibility of *M. bovis* and BCG to NO *in vitro* Jordao et al. (2008) used NOC-18 (a diazeniumdiolate compound) as a source of NO, and they found that *M. bovis* is more resistant to this compound compared with BCG, again reflecting a difference in virulence.

NO was not detected in the supernatants of THP-1 cell cultures and this is in agreement with previous published work indicating that THP-1 does not produce significant level of NO at early time points post-stimulation (Perezperez et al., 1995). Wang et al. (2002) found that 1α-25$(OH)_2D_3$-differentiated THP-1 cells are able to produce NO following stimulation with PMA but at a low level. However, it has been reported that the production of NO in THP-1 cells begins usually at 4 days following stimulation with mycobacteria (Ferrer et al., 2010). Production of NO by human macrophages *in vitro*, is a controversial subject. It has been reported that *M. tuberculosis* induced the production of NO in human alveolar macrophages at days 4 and 7 post-infection, but failed to induce its production in human monocytes (Rich et al., 1997). There are several lines of evidence suggesting that formation of NO is not detectable in the supernatant of human macrophages and monocytes during *in vitro* infection (Clemens and Horwitz, 1995, Jordao et al., 2008). Furthermore, negative results have been obtained by several

researchers in assessment of NO synthesis from human macrophages after stimulation with lymphokines and/or other bacterial products (Murray and Teitelbaum, 1992, Padgett and Pruett, 1992). Inability to detect NO production in human macrophages in this study could be due to the absence of tetrahydrobiopterin, which is an essential cofactor for iNOS activation. Supplementation of an *in vitro* cell culture with this cofactor is necessary to induce NO production in the human monocytic cell line U937 (Bertholet et al., 1999). It might also be due to measuring NO at early time point post-infection, up to 24 h, as it has been reported that THP-1 cells can produce NO at a very late time, 4 days following infection (Ferrer et al., 2010). However, in our study we aimed to evaluate the early immune response to the bacteria in these cells.

In contrast to THP-1 cells, NO production was detected in HD11 cells at 24 h following challenge with *M. avium* strains. The observed variation between the cell lines has been observed before among cells from different species in a different context. For instance, LPS and IFN-γ stimulation induced high levels of NO in murine macrophages (Stuehr et al., 1989). However, there was a failure to elicit production of NO in peripheral blood mononuclear cells of humans (Schneemann et al., 1993). Furthermore, different cell models show variable responses to mycobacteria with regard to NO production. Whereas the rodent macrophage cell line J774, produces high levels of NO in response to infection with *M. smegmatis* after 2 h (Anes et al., 2006), NO production has been detected in cattle

macrophages infected with either *M. avium* subspecies *avium* or *M. avium* subspecies *paratuberculosis* after 12 and 24 h post-infection (Weiss et al., 2002). In a study by Piercy et al., (2007), cell-specific differences in the production of NO in response to mycobacterial species have also been investigated in bovine macrophages. Bovine blood-derived macrophages produced more NO than bovine alveolar macrophages at 24 h post-infection (Piercy et al., 2007). Induction of NO by *M. tuberculosis* has also been reported in PMA-primed THP-1 cells at a later time of infection, day 4 (Ferrer et al., 2010).

Our data showed that NO production does play an effective role in killing mycobacteria, at least *in vitro*. Higher induction levels of NO by strains Ma.4 and Ma.5 in HD11 cells could be the reason for the reduction in their intracellular numbers at 24 h of infection. It would be interesting to know how this correlates with their behaviour *in vivo*. It has been suggested that although, for control of salmonellosis by murine macrophages, production of both NADPH and iNOS are required, the dynamic process of killing changes over time. For killing intracellular *Salmonella* by macrophages in the early stages of the infection, macrophages require NADPH oxidase, whereas iNOS appears to be necessary in the later stages to control the bacterial growth (Vazquez-Torres et al., 2000). Similarly in the murine *in vivo* model of infection, NADPH and iNOS perform their essential role to control *Salmonella* infection at different stages of the experiment in the early and later stages of the infection, respectively (Mastroeni et al., 2000). Thus NO plays an essential role to control the infection in later stages of salmonellosis where it

becomes more chronic and, to some extent resembles mycobacterial infection. So, it would be worth demonstrating the stage that NO operates its controlling mechanism in mycobacterial infection *in vivo*.

iNOS is expressed in response to stimuli including pro-inflammatory cytokines; however, iNOS is not found in resting cells (Widdison et al., 2007). In the current study, as with NO production pattern in HD11 cells, high expression of iNOS was detected in macrophages infected with two cattle strains, Ma.4 and Ma.5. This high expression might lead to a reduction in the intracellular number of these two strains at 24 h of infection. It is accepted that reactive nitrogen intermediates are not the only bacterial killing mechanism, as although human macrophages do not show iNOS activity and therefore do not produce NO, they are still capable of killing mycobacteria. Here, although undetectable NO levels in THP-1 have been reported, these macrophages seem to be able to kill mycobacteria in the first 6 h of infection. It will be worth investigating what other mechanisms are used in killing intracellular mycobacteria at this time point of infection.

The role of ROS in restriction of mycobacterial growth is controversial. Some studies have found reactive oxygen species ineffective in restriction of mycobacterial growth (Jones et al., 1990, Chan et al., 1992) and the beneficial effect of ROS for mycobacterial growth has also been observed by our research group (N. Issa, M. Jones and P. Barrow, unpublished data). In contrast, it has been reported that generation of ROS can perform antimicrobial activity in

mycobacterial infection (Walker and Lowrie, 1981). The effect of these antimicrobial mechanisms on the restriction of intracellular growth of *M. avium* thus remains unclear.

The current study measured production and expression of several cytokines and chemokines at the protein level in THP-1 cells and at the transcript level in HD11 cells. It is known that control of mycobacterial infection is greatly dependent upon relevant cytokine production. Macrophage activation and subsequent production of cytokines such as TNF-α and IL-1β, and chemokines including CCL2 and CCL5 are required for the successful control of intracellular growth of *M. tuberculosis* (Flynn and Chan, 2001).

Differences between the pro-inflammatory cytokines induced by virulent and avirulent mycobacterial species have been reported (Beltan et al., 2000, Chakraborty et al., 2013). The induction of the protective pro-inflammatory cytokine TNF-α can be suppressed by some strains of *M. tuberculosis* such as the Beijing genotype, and this ability could be a key to identify them as more virulent strains (Theus et al., 2005, Parwati et al., 2010). Higher induction of TNF-α production by *M. bovis* BCG has also been reported compared with virulent *M. leprae* (Suzuki et al., 1993, Oliveira et al., 2001). It has been reported that more virulent strains of *M. tuberculosis* are able to produce a specific glycolipid (phthiocerol dimycocerosate, PDIM) which can abolish the ability of the host to control the infection (Domenech and Reed, 2009). The existence of this cell-associated compound was essential for *M. tuberculosis* to be fully virulent in the mouse model (Camacho et al., 1999, Cox et al., 1999) and *M.*

tuberculosis mutants deficient in the synthesis of PDIM or its translocation are highly attenuated *in vivo* (Cox et al., 1999, Camacho et al., 1999, Domenech et al., 2005). The exact role of PDIM is not fully understood, although a range of roles have been suggested for it, such as modification of host plasma membrane (Astarie-Dequeker et al., 2009), modulation of innate immune system and bacterial protection from the toxic effect of reactive nitrogen intermediates derived from host cells (Rousseau et al., 2004). In the case of *M. avium* as a pathogenic mycobacterial species in birds and immune compromised people, it would be interesting to know if there is a role for PDIM specifically in relation to infection in birds.

The differential induction of cytokines could also be linked to mycobacterial components such as LAM which is a major cell wall molecule found in both pathogenic and non-pathogenic mycobacteria. This glycolipid has two forms, Man-LAM and Ara-LAM. The former is usually associated with pathogenic mycobacteria and the later commonly found on non-pathogenic mycobacteria (Chatterjee et al., 1992). LAM variation could be a possible reason for differential behaviour of *M. avium* strains used in this study and their LAM forms could be assessed in further studies.

We found that inoculation of differentiated THP-1 macrophages with various strains of *M. avium* induced the production of TNF-α. Mice with a deficiency in TNF production or with absence of its receptor p55 are shown to be more susceptible to mycobacterial infection than wild type (Algood et al., 2005).

The association between the capability of the *M. avium* strains to induce cytokine secretion and their virulence has previously been investigated (Furney et al., 1992, Appelberg et al., 1995). Different variants of *M. avium* showed various levels of TNF-α induction in murine macrophages. Virulent smooth transparent variants induced lower level of TNF-α compared with less virulent smooth opaque variants (Appelberg et al., 1995). Similarly, despite induction of TNF-α to some extent by all *M. avium* strains, those that grew well in host cells showed delayed induction of this cytokine (Furney et al., 1992). In accordance with a study carried out by Blumenthal and co-workers (Blumenthal et al., 2005) which investigated differential activation by various strains of *M. avium*, differences in the pathogenicity among *M. avium* strains has been observed in our investigation. We observed that the number of CFU at 24 h post-infection in macrophages infected with cattle strains Ma.4 and Ma.5, and human strains Ma.6, Ma.7 and Ma.8 were significantly lower than those in cells infected with Ma.3. On the other hand the TNF-α levels produced by cells infected with Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8 were significantly higher than those by cells infected with Ma.3. This could indicate that there is a correlation between induction of TNF-α and the virulence of mycobacterial strains. So it might be suggested that higher levels of TNF-α production in cells infected with Ma.4 and Ma.5, for example, could be the reason for the lower CFU in these cells. This correlation between production of TNF-α and pathogenicity of *M. avium* strains in murine macrophages has also been confirmed by other researchers (Sarmento and

Appelberg, 1995). Although *M. tuberculosis* and *M. bovis* are closely related pathogens, they show different ability to induce infection in cattle (Widdison et al., 2008). To investigate the possible reason for the greater virulence of *M. bovis* for cattle compared with *M. tuberculosis*, Widdison and colleagues (2008) found several differentially expressed genes between these two pathogens in bovine alveolar macrophages. Chemokines including CXCL8 and several other chemokines were found to be significantly more highly expressed in macrophages infected with *M. tuberculosis* than those with *M. bovis* (Widdison et al., 2008). So, the differences in expression in cytokines observed in this study may correlate with differences in their virulence potential. *In vivo* studies would be required to demonstrate if this is the case.

The mRNA level of TNF-α and IL-1β were not corroborated with their protein levels in THP-1 cells. The protein level of TNF-α and IL-1β were significantly higher in cells infected with human strains; Ma.6-Ma.8 in comparison to those infected with Ma.3. However, there were no significant differences between these strains at mRNA expression levels of these two cytokines. It is known that the processes following mRNA expression play an essential role in regulation of protein production. These include post-transcriptional, and post-translational regulation and degradation of protein that can affect the abundance of the protein (Vogel and Marcotte, 2012). Protein abundance could only be partially predictable from the abundance of steady-state of transcript (de Sousa Abreu et al., 2009). In addition, *M. tuberculosis* have been reported to be able to

destabilize mRNA through the effect of their microbial lipoprotein (Rajaram et al., 2011).

In the current study, differences in the induction of IL-6, IL-1β and CXCL8 production were also observed between various strains of *M. avium.* There are several lines of evidence to indicate that more virulent strains induce much lower level of these cytokines compared to less virulent strains (Wang et al., 2010, Sarkar et al., 2012, Chakraborty et al., 2013). The *M. tuberculosis* Beijing genotype has been reported to induce significantly lower levels of TNF-α, IL-1β, IL-6 and IL-12 in human THP-1 cells compared to other less pathogenic strains (Chakraborty et al., 2013). Similar to data from infection of THP-1 cells with *M. avium,* diversity in induction of cytokine gene expression in HD11 was also observed between different strains of *M. avium* in this study. The existence of differences at the level of cytokine secretion triggered by different strains of *M. avium* in our data suggests a further hypothesis of this being the basis for differences in virulence. It seems that the original host species from which the strains were isolated had not obvious effect on these differences, as both Ma.3 and Ma.4 were isolated from cattle but behaved differently in terms of cytokine induction.

Consistent with previously published studies, our results suggested that induction of pro-inflammatory cytokines can be strain dependent. This may relate to virulence, in which case we might predict that Ma.3 would be the most pathogenic strain and the Ma.4

and Ma.5 would be the least virulent strains in this study. All three strains have been isolated from cattle, although it is not clear whether the source of these strains for cattle was originally wild birds with possible transfer to cattle. Although some similarities have been reported between *M. avium* isolates recovered from animals and human, closer behavioural relationships have been observed between human isolates and those recovered from pigs rather than those isolated from birds (Fulton and Sanchez, 2013).

4 The role of MAPK signalling pathways in signal transduction of cytokine induction by *M. avium* strains

4.1 Introduction

It is known that several cell signaling pathways are involved in the regulation of expression of a number of cytokines induced by mycobacterial infection. It has been suggested that the MAPK pathway plays a crucial role in the pathogenesis of mycobacterial infection (Roach and Schorey, 2002). Regulation of cytokine biosynthesis is one of the important roles of the MAPK pathway. Mycobacterial components (Chan et al., 2001, Jones et al., 2001b) and viable mycobacteria (Reiling et al., 2001) are able to activate the MAPK signaling cascades. Activation of this pathway by mycobacteria and their microbial components can lead to production of immune effector molecules including NO, TNF-α and IL-1β (Roach and Schorey, 2002, Yadav et al., 2004). However, activation of this pathway is not induced equally by pathogenic and non-pathogenic mycobacteria (Roach and Schorey, 2002). It has long been suggested that specific inhibitors of MAPK could be used in the treatment of inflammatory diseases (Ajizian et al., 1999). So research on this important superfamily of signal transductions remains an interesting area of study.

PD98059 is a specific and potent inhibitor of ERK pathway. This inhibitor is known to block ERK activation via upstream MAPK kinases, MEK1 and MEK2 (Dudley et al., 1995). U0126 is also a selective inhibitor of ERK with higher affinity to MEK1 (Favata et al., 1998). SB203580 specifically inhibits p38 MAPK pathway and has no effect on ERK or JNK (Cuenda et al., 1995). Finally, SP600125 is known to inhibit the activity of JNK pathway (Bennett et al., 2001).

These inhibitors have been used widely to examine the role of MAPK signaling pathway subfamilies in regulation of cytokine production in both human (Reiling et al., 2001) and chicken cells (He and Kogut, 2003, Kogut et al., 2008). In human cells, it has been reported that treatment of the cells with PD98059 reduced the level of TNF-α in response of *M. avium* (Reiling et al., 2001). Kogut et al. (2008) found that the expression of CXCli2 and IL-6 reduced significantly in response to LPS and flagellin following treatment of chicken heterophils with PD98059 or U0126. This indicates that the ERK pathway plays an important role in regulation of these cytokine and chemokine in chicken heterophils. However, the precise signaling pathways that lead to induction of cytokines by *M. avium* are not fully understood. In addition, very little is known about the role of each pathway in signal transduction in cells infected with *M. avium* strains especially in avian cells. So in this study, we aimed to evaluate the role of each MAPK pathway superfamily member, ERK, p38 and JNK, in the regulation of cytokine production in THP-1 and HD11 cells in response to infection with *M. avium* strains.

4.2 Results

4.2.1 The cytotoxic effect of MAPK inhibitors on THP-1 cells and HD11 cells

To evaluate the cytotoxic effect of selected concentrations of the inhibitors on the viability of the cells used in this study, the LDH liberation assay was performed as explained in detail in chapter 2. Using the chemicals PD98059, U0126, SB203580 and SP600125 at the concentrations of 50μM we did not observe any cytotoxic effect on human THP-1 (Figure 4.1) and chicken HD11 cells (Figure 4.2). This was expected as these chemicals have been used widely to inhibit MAPK pathways and no cytotoxic effects have been reported so far.

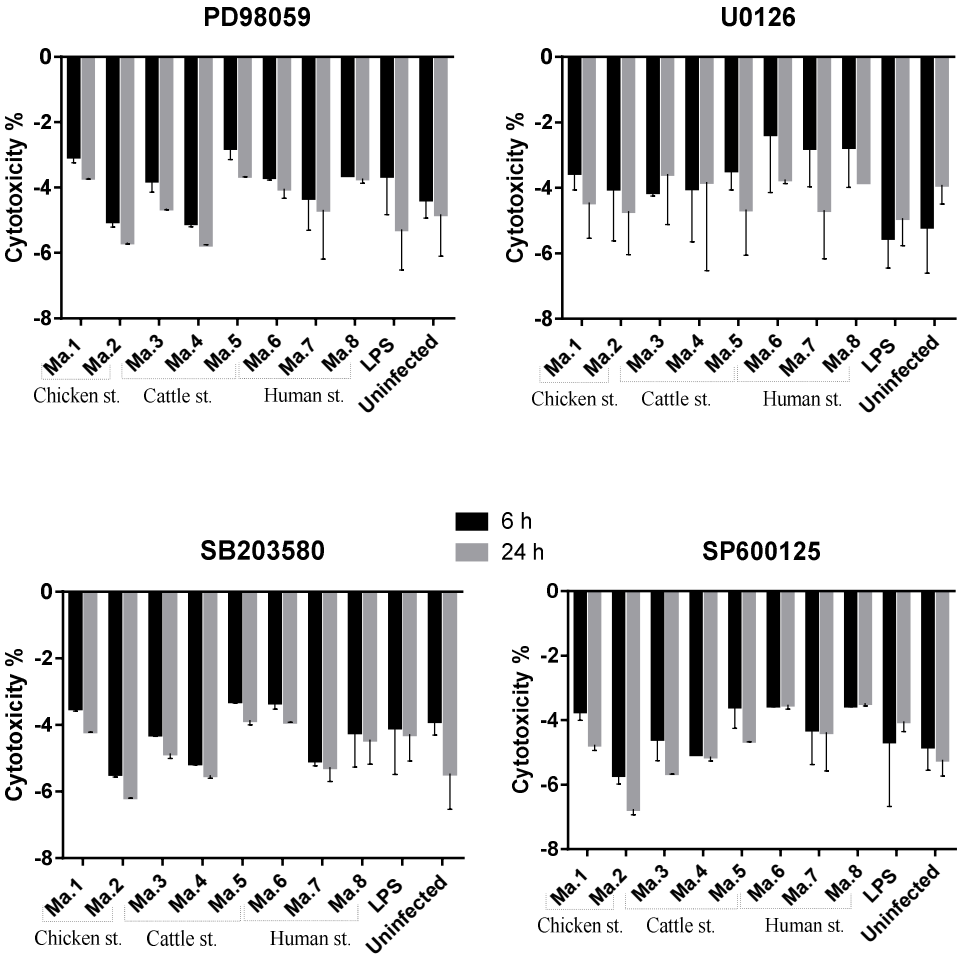


Figure 4.1: Effect of MAPK inhibitors on THP-1 cells

The inhibitors (PD98059, U0126, SB203580 and SP600125) were added to the cells 30 min prior to *M. avium* infection. LDH liberation assay was performed on the supernatants from all samples at different time point post infection (6 and 24 h) to detect the percentage of cytotoxicity of each inhibitor (PD98059, U0126, SB203580 and SP600125) on HD11 and THP-1 cells. The test was performed once in triplicate.

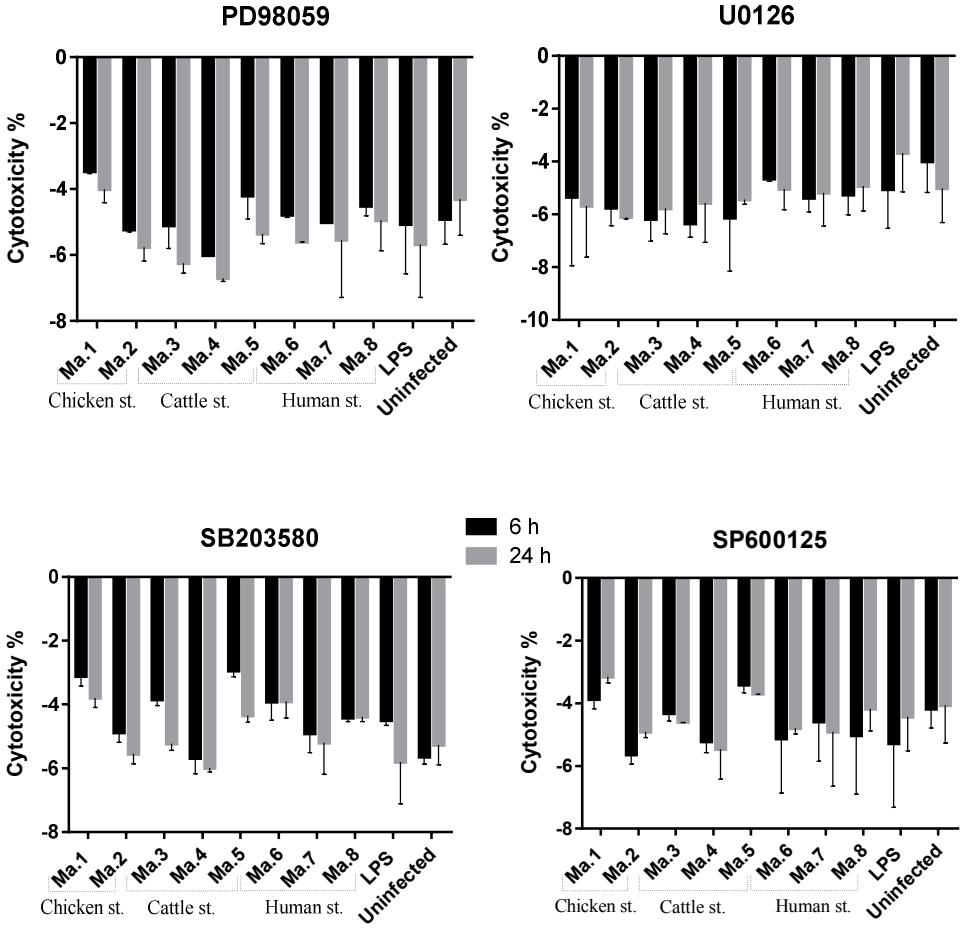


Figure 4.2: Effect of MAPK inhibitors on HD11 cells

The inhibitors (PD98059, U0126, SB203580 and SP600125) were added to the cells 30 min prior to *M. avium* infection. LDH liberation assay was performed on the supernatants from all samples at different time point post infection (6 and 24 h) to detect the percentage of cytotoxicity of each inhibitor (PD98059, U0126, SB203580 and SP600125) on HD11 and THP-1 cells. The test was performed once in triplicate.

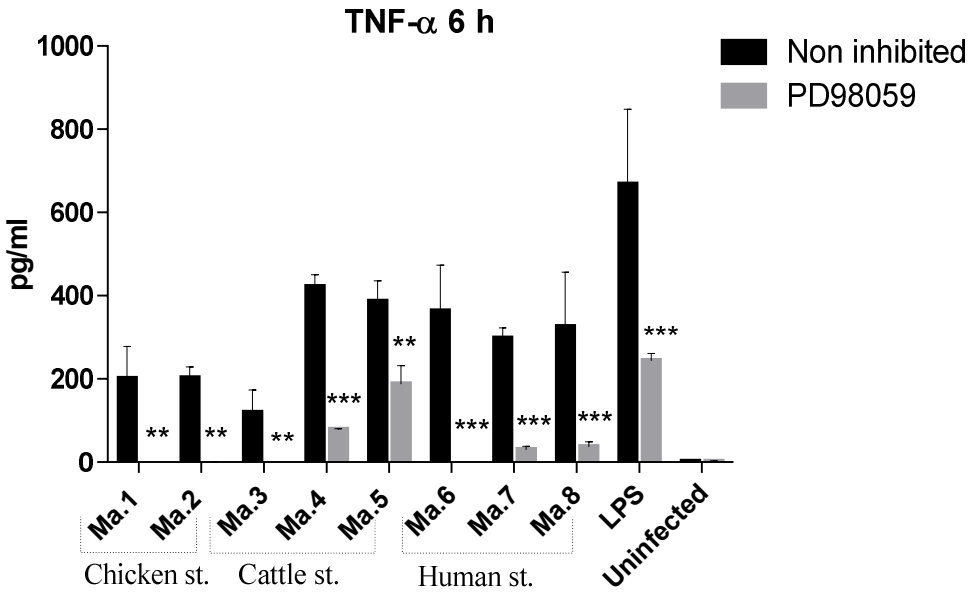
4.2.2 The effect of ERK inhibition by PD98059 on *M. avium*-induced cytokine and chemokine production in THP-1 cells

To evaluate the effect of PD98059 on the production of cytokines by THP-1 and HD11 cells in response to *M. avium* infection, these cells were pretreated with the ERK inhibitor, PD98059, 30 min prior to infection with the different *M. avium* strains. For THP-1 cells the level of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 and chemokine

CXCL8 induced were assessed by ELISA. Cytokine production was greatly affected.

4.2.2.1 TNF-α

Macrophages treated with the ERK inhibitor and infected with *M. avium* strains showed significantly lower levels of TNF-α at 6 h post-infection compared to macrophages infected with *M. avium* alone ($P<0.01$ for Ma.1, Ma2, Ma.3 and Ma.5 and $P<0.001$ for Ma.4, Ma.6, Ma.7 and Ma.8, respectively). TNF-α production at 24 h post-infection also markedly decreased in macrophages infected with Ma.1, Ma.3 ($P<0.01$), Ma.2, Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8 ($P<0.001$) following PD98059 treatment compared to those not treated with the inhibitor (Figure 4.3).



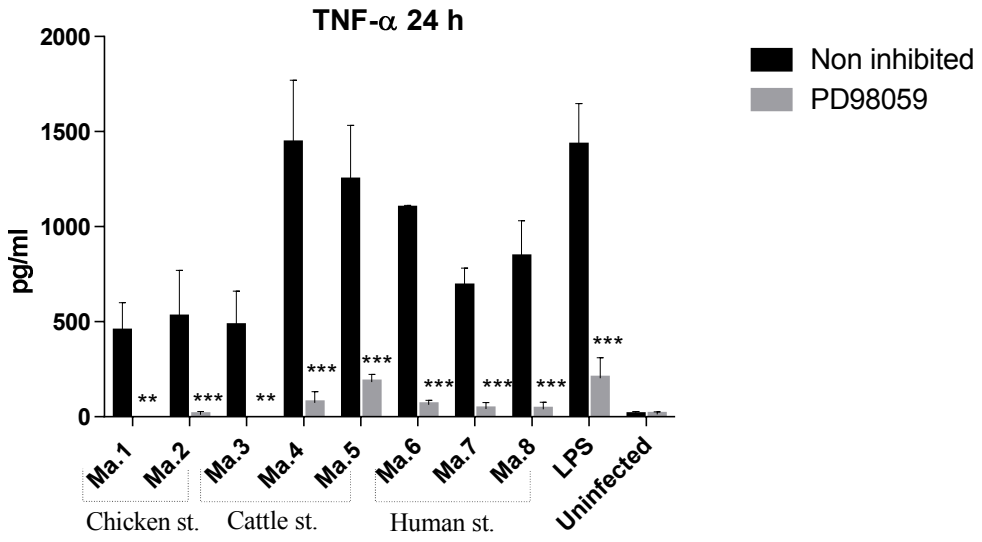


Figure 4.3: Effect of ERK pathway inhibitor, PD98059, on TNF-α induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM PD98059, 30 min prior to infection with *M. avium* strains. TNF-α concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001 respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.2.2 IL-1β

Similarly, PD98059 significantly reduced production of IL-1β at 6 h post-infection in these cells in response to infection with Ma.8 (P<0.05), Ma.2, Ma.4 (P<0.01) Ma.6 and Ma.7 (P<0.001). At 24 h post-infection, in the absence of PD98059, the level of IL-1β was significantly higher in cells infected with Ma.1 (P<0.05), Ma.2 (P<0.01) and Ma.3, Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8 (P<0.001) in comparison to those treated with the inhibitor prior to the infection (Figure 4.4).

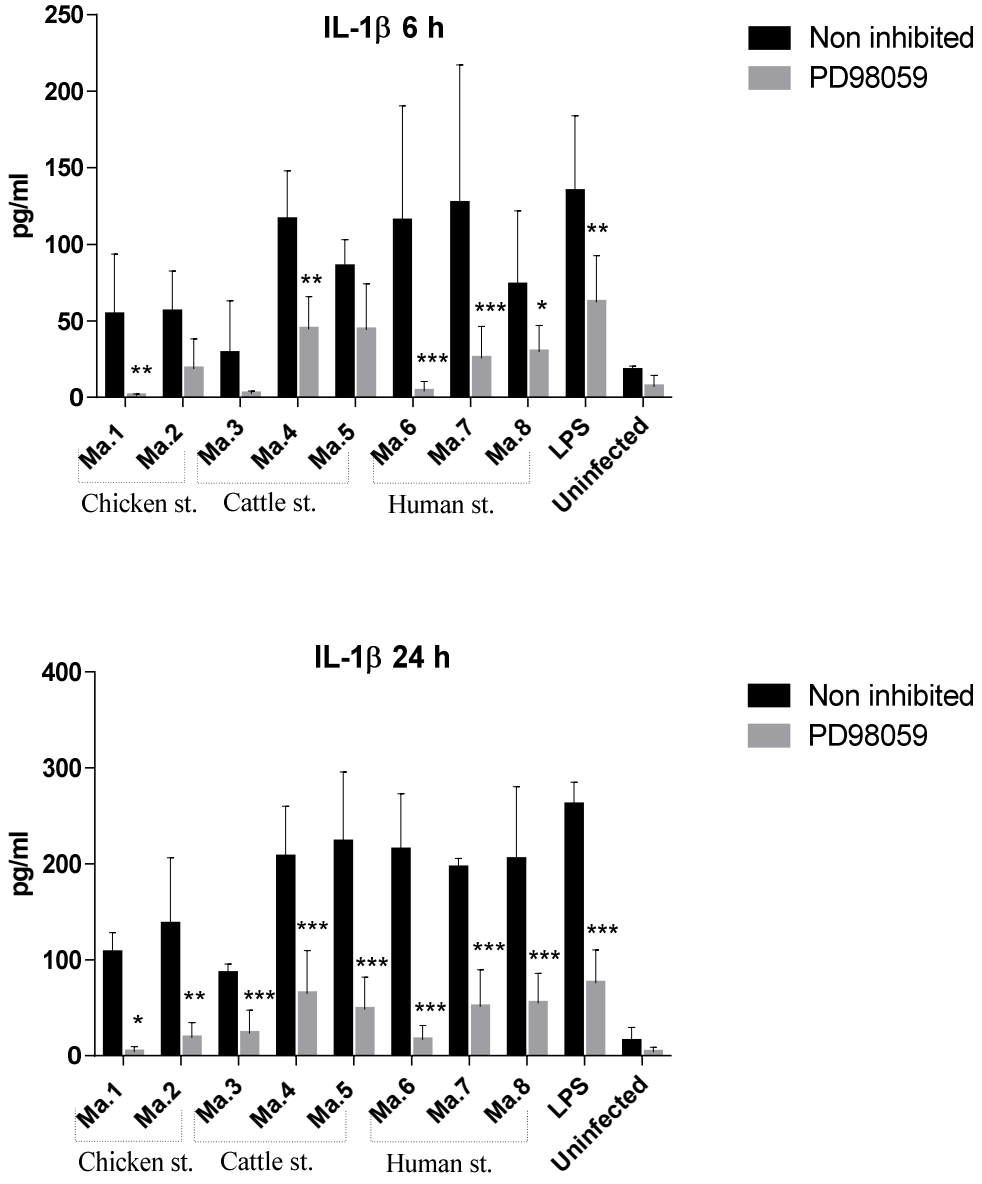


Figure 4.4: Effect of ERK pathway inhibitor, PD98059, on IL-1β induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM PD98059, 30 min prior to infection with *M. avium* strains. IL-1β concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.2.3 CXCL8

PD98059 also reduced the level of CXCL8 chemokine at 6 h post-infection in cells infected with Ma.1, Ma.7 ($P<0.05$) and Ma.3 ($P<0.01$). Likewise, at 24 h post-infection the level of CXCL8 was significantly reduced in cells pretreated with the inhibitor prior to infection with Ma.4, Ma.5, Ma.8 ($P<0.01$) and Ma.6 ($P<0.05$) strains compared with those infected with strains alone (Figure 4.5).

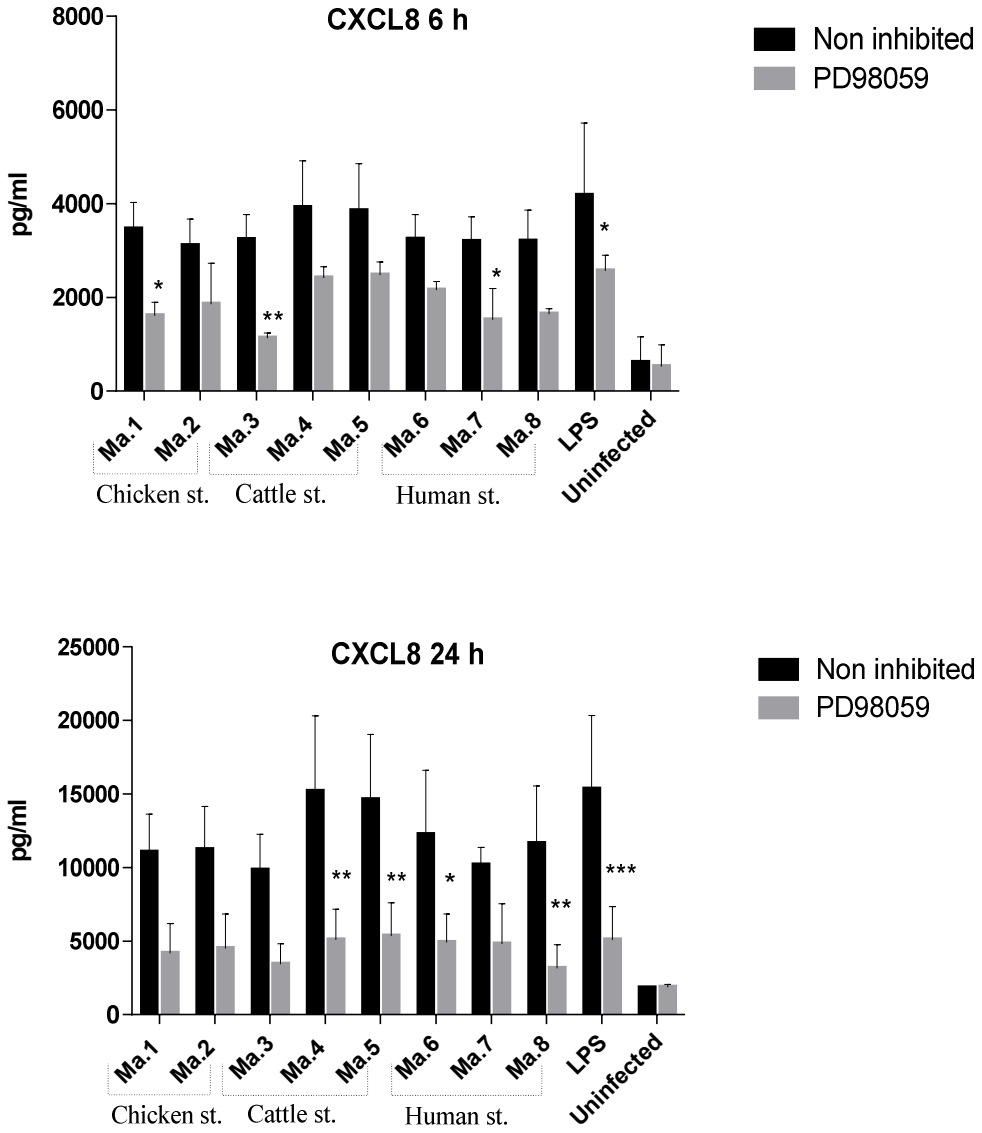


Figure 4.5: Effect of ERK pathway inhibitor, PD98059, on CXCL8 induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 µM PD98059, 30 min prior to infection with *M. avium* strains. CXCL8 concentrations in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001 respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.2.4 IL-6

Moreover, pretreatment of the macrophages with this inhibitor showed marked reduction in IL-6 production at 24 h post-infection with Ma.1, Ma.2, Ma3, Ma.6, Ma.7, Ma.8 (P<0.05), Ma.4 (P<0.01) and Ma.5 (P<0.001) compared to those without inhibitor (Figure 4.6).

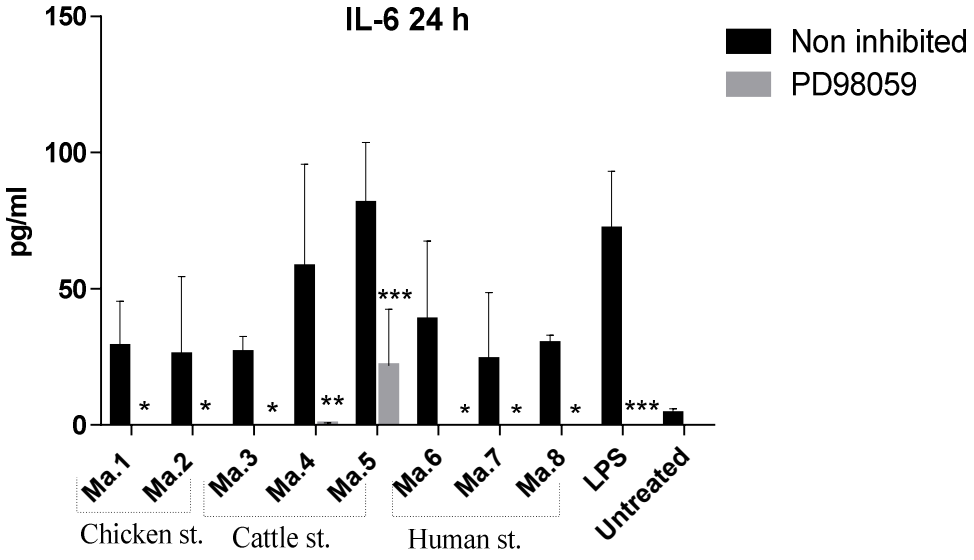


Figure 4.6: Effect of ERK pathway inhibitor, PD98059, on IL-6 induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM PD98059, 30 min prior to infection with *M. avium* strains. IL-6 concentrations in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3 Cytokine and chemokine gene expression in HD11 cells following inhibition of ERK by PD98059

To understand the role of ERK in the regulation of cytokine production in HD11 cells following challenge with *M. avium* strains, the cells were pretreated with 50 μM PD98059 prior to infection and the mRNA levels of iNOS, IL-1β, CXCLi1, CXCLi2 and IL-6 genes were quantified at 6 h post-infection using qRT-pCR.

4.2.3.1 iNOS

PD98059 treatment prior to infection with *M. avium* led to significant inhibition of iNOS gene expression induced by Ma.1, Ma.2, Ma.6 ($P<0.05$), Ma.4 and Ma.5 ($P<0.01$) compared to the cells infected with the strains alone (Figure 4.7).

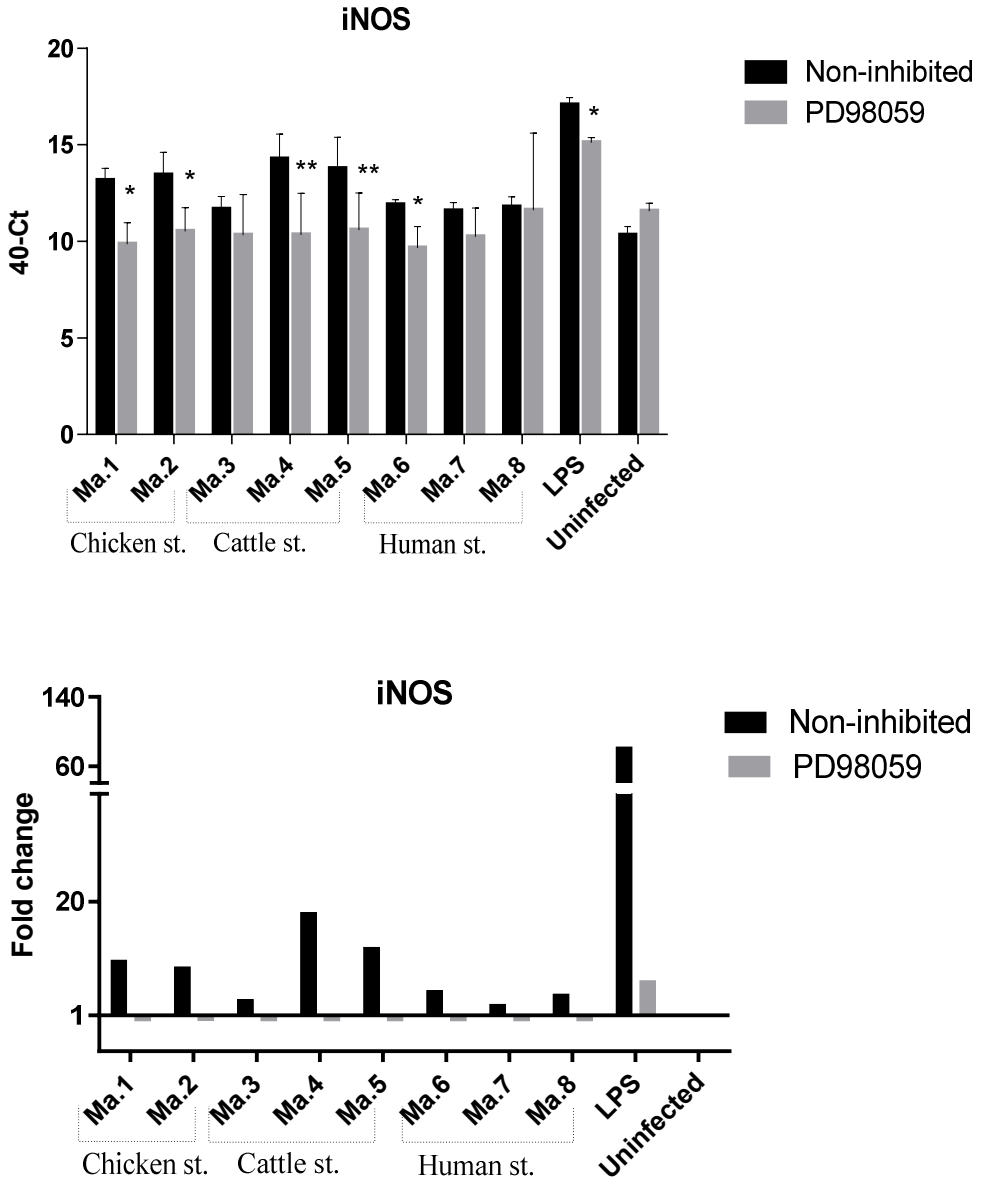


Figure 4.7: Effect of the ERK pathway inhibitor, PD98059, on iNOS induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 μM PD98059, 30 min prior to infection with *M. avium* strains. Expression of iNOS mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (* and **) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of iNOS from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7

(446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3.2 IL-1β

In addition, expression of IL-1β showed significant reduction in cells infected with Ma.1, Ma.2, Ma.4, Ma.5 ($P<0.01$) and Ma.3, Ma.7 and Ma.8 ($P<0.05$) (Figure 4.8).

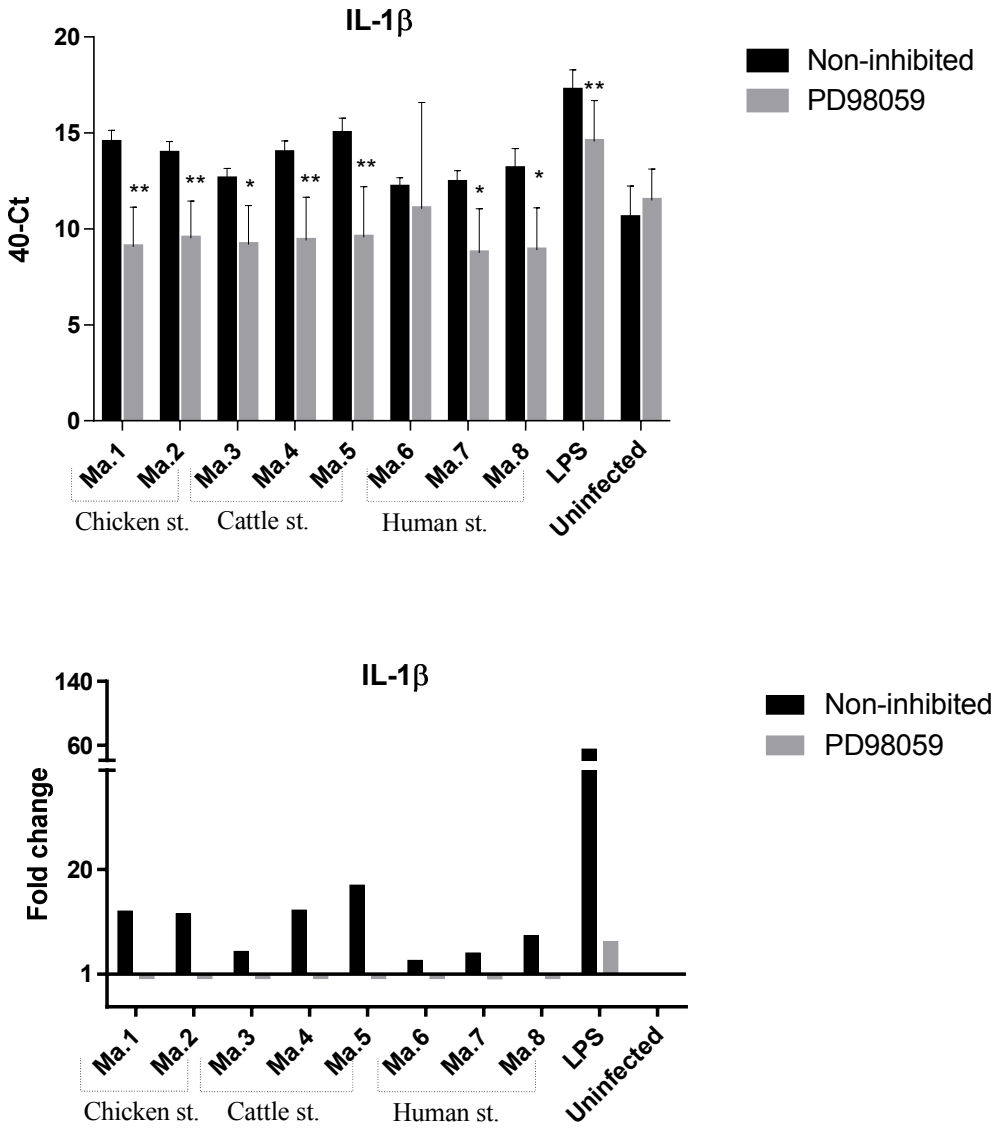
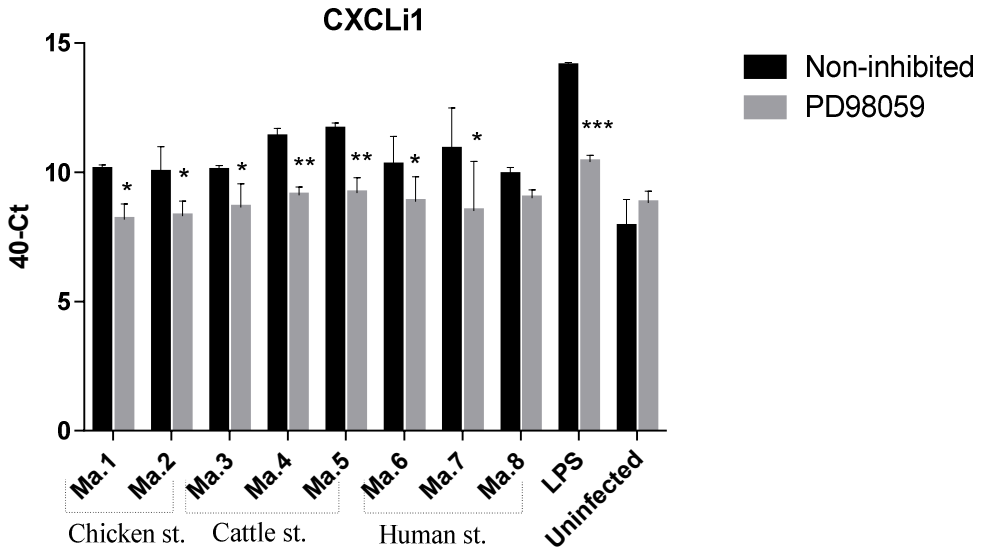


Figure 4.8: Effect of the ERK pathway inhibitor, PD98059, on IL-1β induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 μM PD98059, 30 min prior to infection with *M. avium* strains. Expression of IL-1β mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (* and **) refer to significant differences (P<0.05 and P<0.01 respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-1β from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3.3 CXCLi1

Similarly, ERK inhibition resulted in reduction of the CXCLi1 mRNA expression in cells infected with Ma.1, Ma.2, Ma.3, Ma.6, Ma.7 (P<0.05), Ma.4 and Ma.5 (P<0.01) (Figure 4.9).



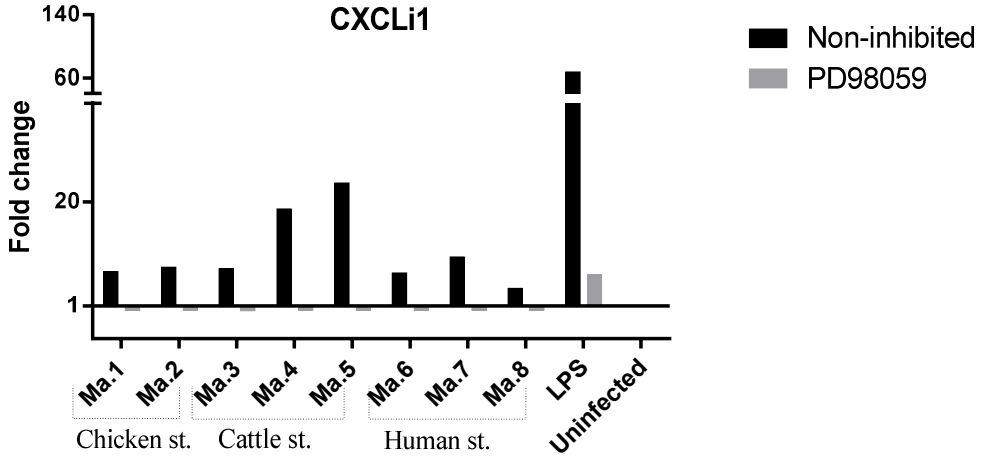


Figure 4.9: Effect of the ERK pathway inhibitor, PD98059, on CXCLi1 induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM PD98059, 30 min prior to infection with *M. avium* strains. Expression of CXCLi1 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi1 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3.4 CXCLi2

In the presence of PD98059, the infected cells all showed a significantly reduced mRNA level of CXCLi2 compared to the absence of this inhibitor ($P<0.01$) (Figure 4.10).

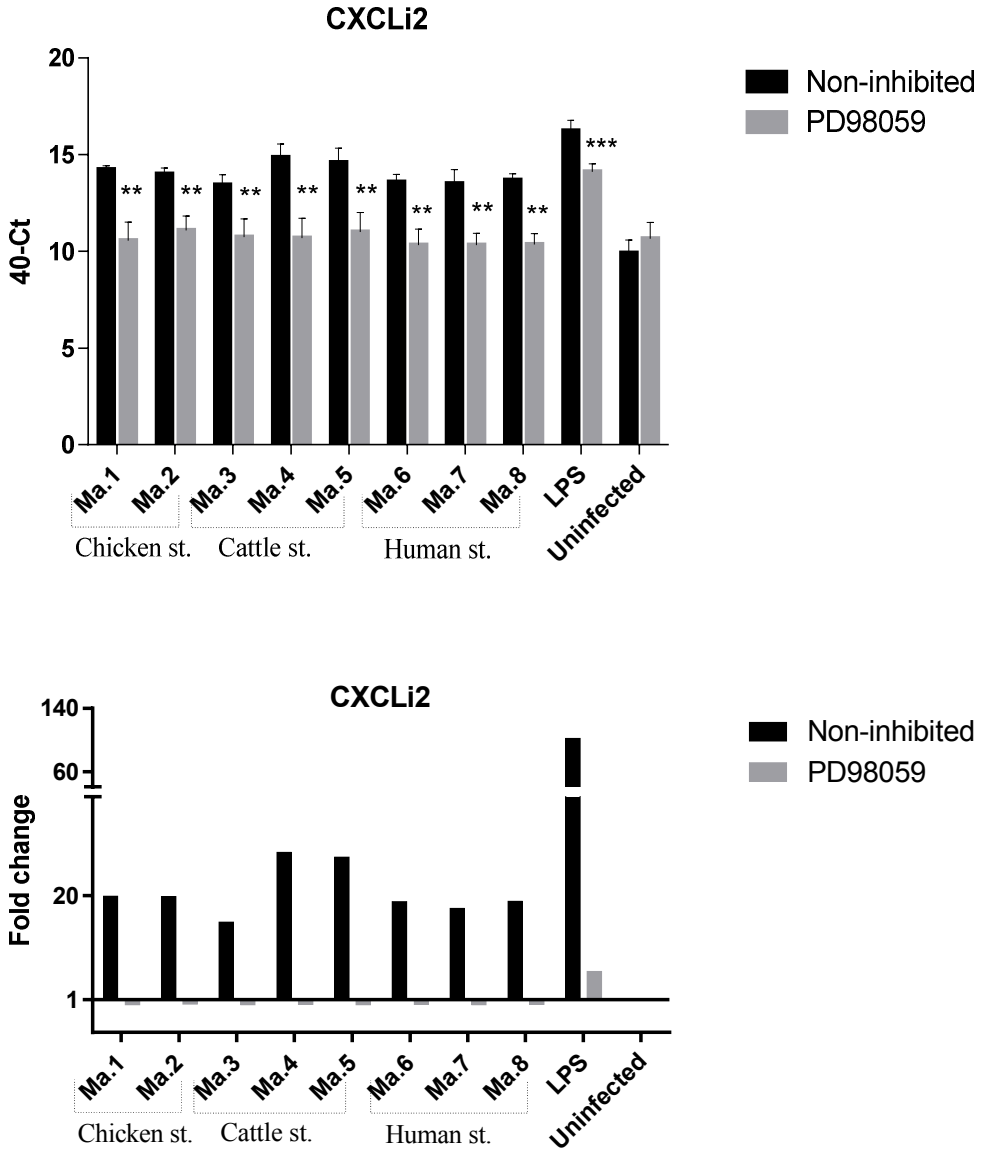


Figure 4.10: Effect of the ERK pathway inhibitor, PD98059, on CXCLi2 induction by *M. avium* strains in HD11 cells.

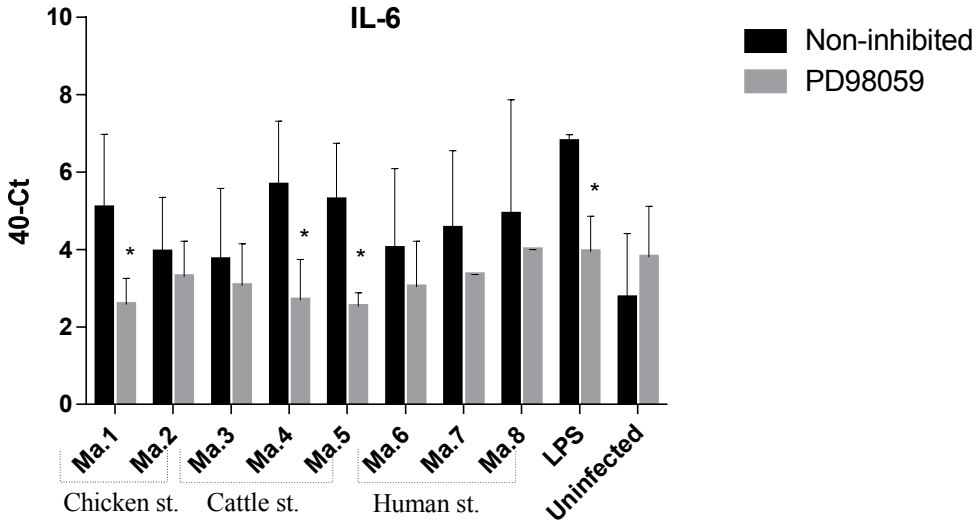
The ERK pathway was inhibited in HD11 cells by 50 µM PD98059, 30 min prior to infection with *M. avium* strains. Expression of CXCLi2 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi2 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330),

Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3.5 IL-6

IL-6 expression significantly decreased following the treatment of the cells in response to Ma.1, Ma.4, Ma.5 and Ma.7 ($P<0.05$) compared with cells only infected with *M. avium* strains (Figure 4.11).

In contrast to the cells infected with *M. avium* strains, in the presence of PD98059, there was significant up-regulation of all the cytokines tested in the cells stimulated with LPS compared to untreated cells except for the level of IL-6 gene expression. However, significantly lower mRNA levels of all cytokines tested in response to stimulation with LPS were noted following treatment of the cells with PD98059 compared with those not treated with the inhibitor.



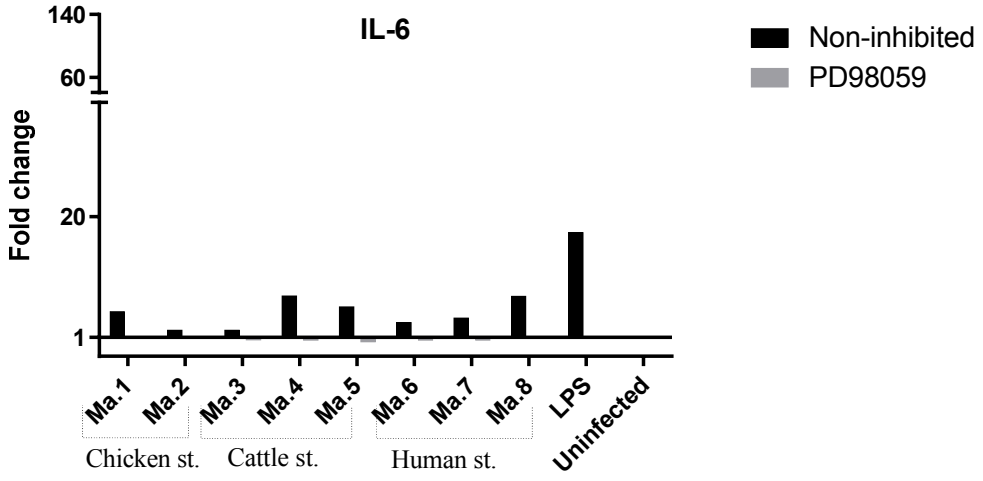


Figure 4.11: Effect of the ERK pathway inhibitor, PD98059, on IL-6 induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM PD98059, 30 min prior to infection with *M. avium* strains. Expression of IL-6 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*) refer to significant differences ($P<0.05$) between *M. avium*-infected cells treated with PD98059 (grey bars) and those not treated with PD98059 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-6 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.3.6 NO production

We also measured the level of NO production 24 h post-infection in HD11 cells following treatment. Nitrite concentrations below 5 µM were regarded as not significant as measurements are inaccurate at this level (Figure 4.12).

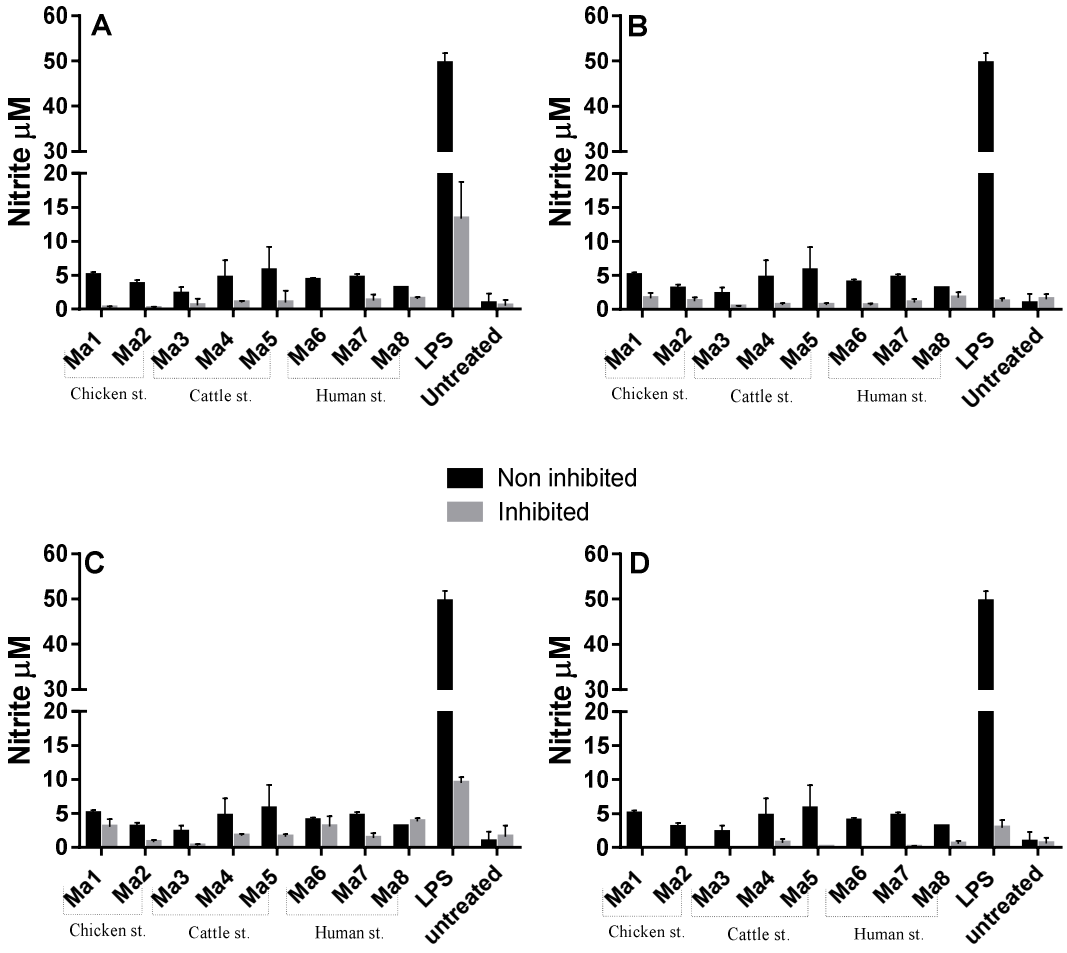


Figure 4.12: Effect of MAPK inhibitors on nitric oxide production by HD11 macrophages after challenge with various *M. avium* strains.

HD11 cells were pre-treated with MAPK inhibitors PD98059 (A), U0126 (B), SB203580 (C) and SP600125 (D), 30 min prior to infection with *M. avium* and the concentration of nitrite were measured at 24 h post-infection using Griess assay. Data shows *M. avium*-infected cells treated with the inhibitors (grey bars) and those not treated with the inhibitors (black bars). Data obtained from three independent experiments each performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132).

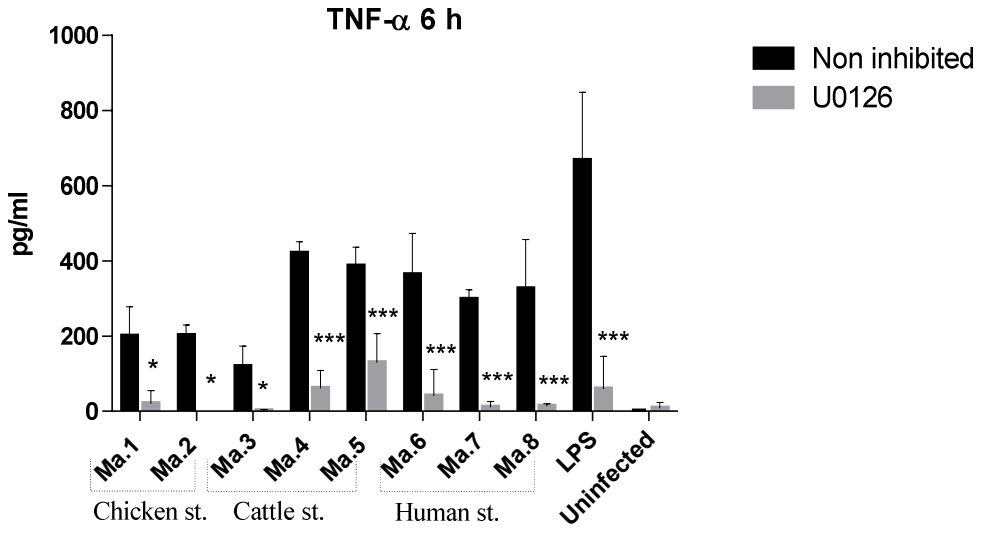
4.2.4 Inhibition of ERK pathway with U0126 in THP-1 cells

To further evaluate the role of the ERK pathway in regulation of cytokine production in response to *M. avium* infection in THP-1 cells,

this pathway was inhibited by another specific inhibitor, U0126, 30 min prior to the infection and the level of the cytokines were measured in the supernatant of the cell culture by ELISA.

4.2.4.1 TNF-α

Figure 4.16 shows that *M. avium*-stimulated release of TNF-α at 6 h post-infection was reduced significantly by treatment of the cells with 50 μM U0126 compared with cells infected with *M. avium* alone ($P<0.05$ for Ma.1, Ma.2 and Ma.3 and $P<0.001$ for Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8). The cells infected with Ma.1 ($P<0.05$), Ma.3 ($P<0.01$), Ma.2, Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8 ($P<0.001$) showed significant reduction in the level of TNF-α production at 24 h post-infection following treatment of the cells with U0126 compared with those without inhibitor (Figure 4.13).



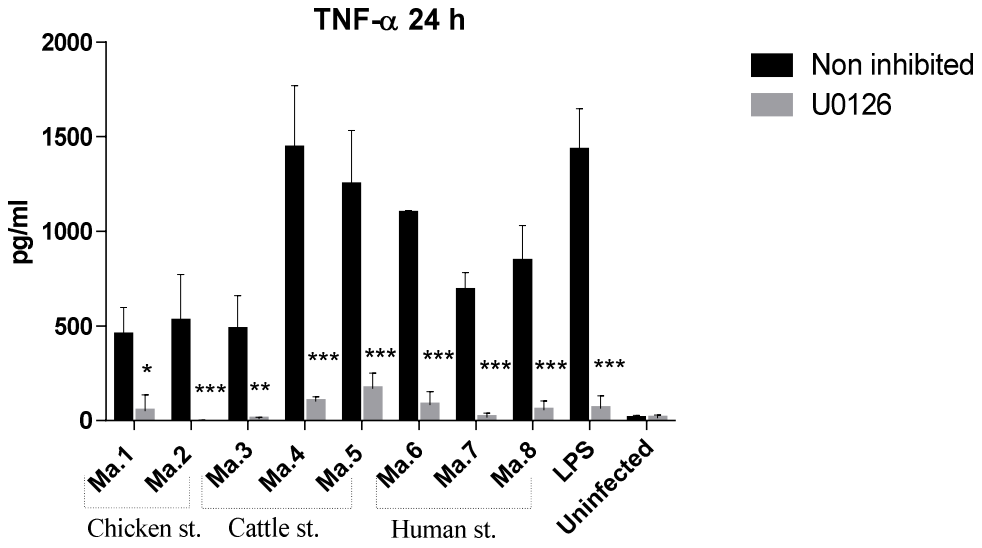


Figure 4.13: Effect of ERK pathway inhibitor, U0126, on TNF-α induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM U0126, 30 min prior to infection with *M. avium* strains. TNF-α concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, ** and ***) refer to significant differences (P<0.05, P<0.01 and P<0.001 respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.4.2 IL-1β

In contrast, the production of IL-1β at 6 h post-infection in response *to M. avium* infection was not affected by 50 μM U0126 and there was no significant difference between the cells treated with inhibitor and those not treated. Similarly, at 24 h post-infection the only significant differences were observed in the level of this cytokine in cells treated with U0126 in response to infection with Ma.6 (P<0.05) and Ma.7 (P<0.01) compared to the cells were not treated with the inhibitor (Figure 4.14).

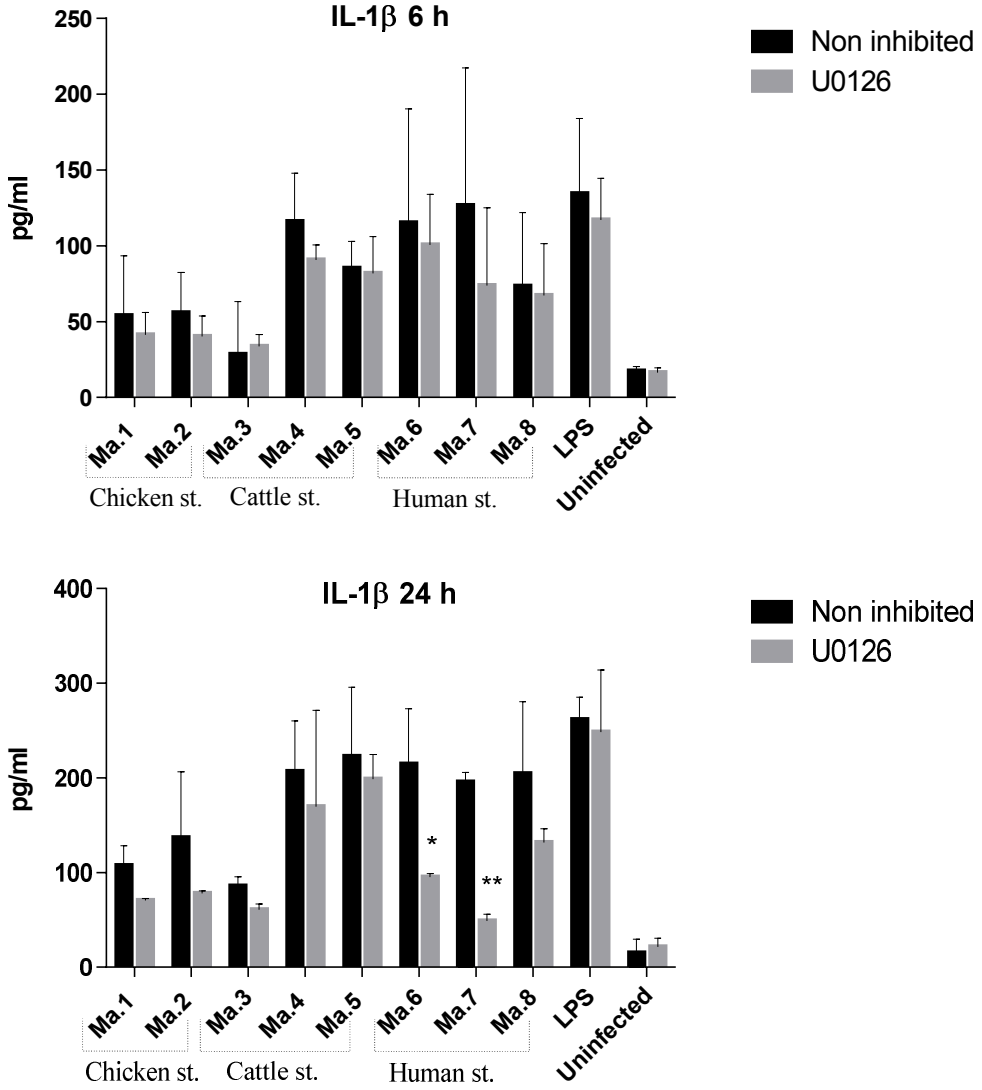
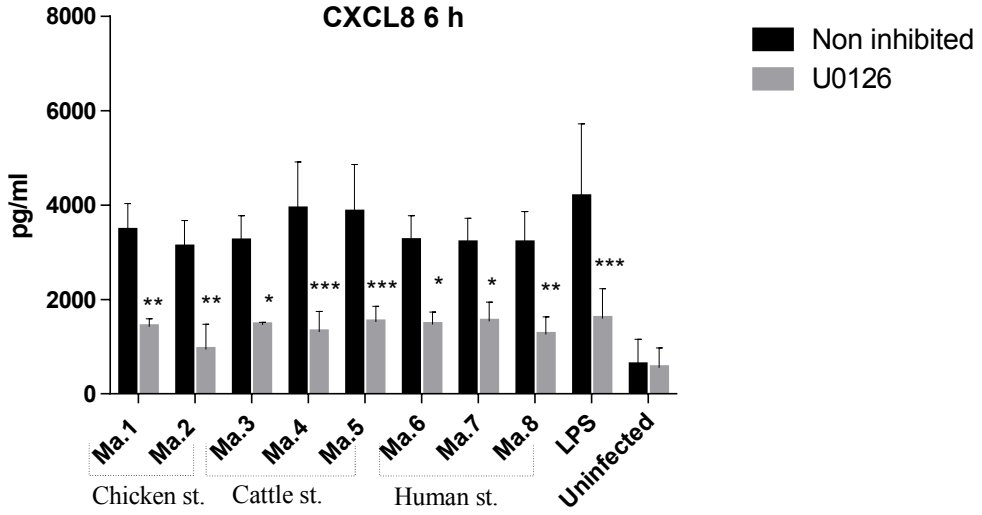


Figure 4.14: Effect of ERK pathway inhibitor, U0126, on IL-1β induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM U0126, 30 min prior to infection with *M. avium* strains. IL-1β concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (* and **) refer to significant differences ($P<0.05$ and $P<0.01$, respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.4.3 CXCL8

Inhibition of ERK by U0126 significantly reduced induction of CXCL8 at 6 h post infection in THP-1 cells by Ma.1, Ma.2, Ma.8 ($P<0.01$), Ma.3, Ma.6, Ma.7 ($P<0.05$), and Ma.4, Ma.5 ($P<0.001$) compared to the cells not treated with this inhibitor. Also, at 24 h post-infection the level of CXCL8 markedly reduced in response to infection with Ma.1, Ma.2, Ma.4, Ma.5, Ma.6, Ma.8 ($P<0.001$), Ma.3 and Ma.7 ($P<0.01$) following treatment of the cells with U0126 (Figure 4.15).



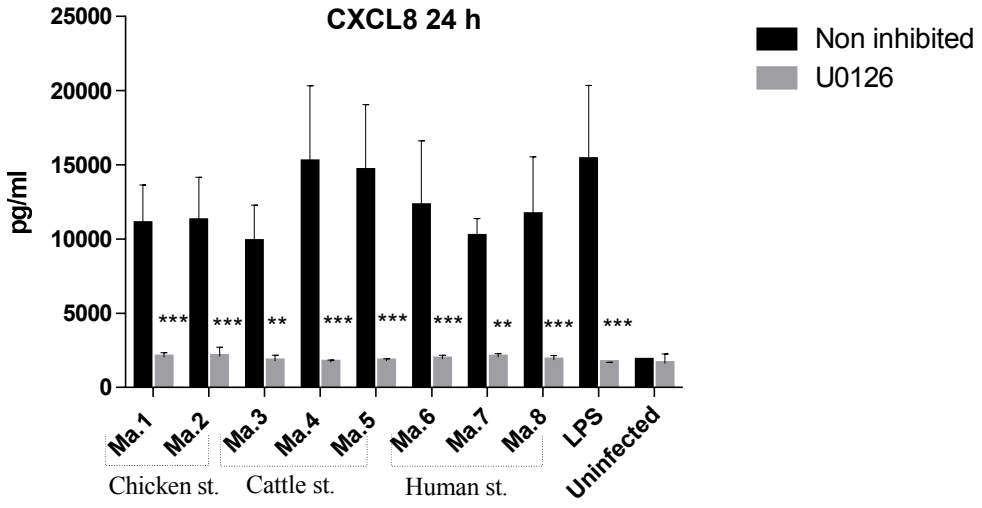


Figure 4.15: Effect of ERK pathway inhibitor, U0126, on CXCL8 induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM U0126, 30 min prior to infection with *M. avium* strains. CXCL8 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, ** and ***) refer to significant differences (P<0.05, P<0.01 and P<0.001 respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.4.4 IL-6

Moreover, pre-treatment of the cells with this inhibitor decreased *M. avium*-induced IL-6 production at 24 h post-infection in cells infected with Ma.1, Ma.2, Ma.3, Ma.6, Ma.7, Ma.8 (P<0.05) and Ma.4, Ma.5 (P<0.001) (Figure 4.16).

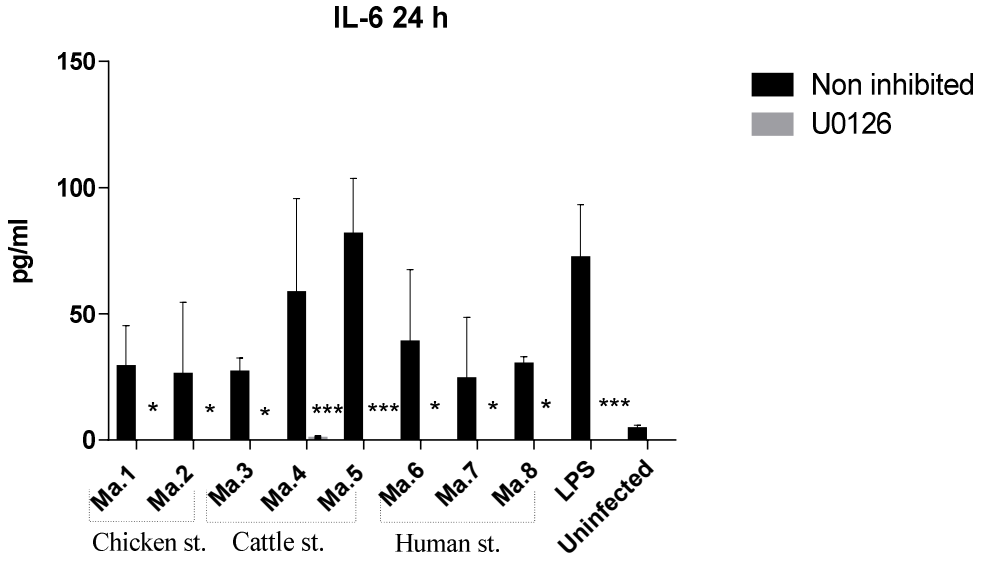


Figure 4.16: Effect of ERK pathway inhibitor, U0126, on IL-6 induction by *M. avium* strains in THP-1 cells.

The ERK pathway was inhibited in THP-1 macrophages by 50 μM U0126, 30 min prior to infection with *M. avium* strains. IL-6 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, ** and ***) refer to significant differences (P<0.05, P<0.01

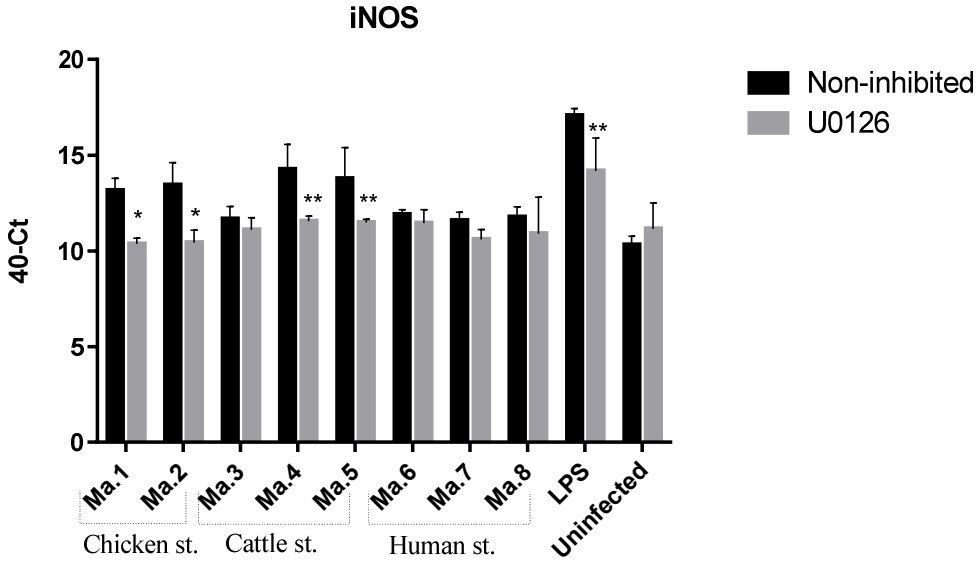
and P<0.001 respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.5 Inhibition of ERK pathway with U0126 in HD11 cells

The expression of iNOS, IL-1β, CXCLi1, CXCLi2 and IL-6 genes were all reduced in HD11 cells following treatment of these cells with 50 μM U0126 at 6 h post-infection with the *M. avium* strains.

4.2.5.1 iNOS

We observed that in the presence of U0126, expression of iNOS in HD11 cells challenged with Ma.1, Ma.2 (P<0.05), Ma.4 and Ma.5 (P<0.01) significantly reduced compared with those infected with the *M. avium* strains alone (Figure 4.17).



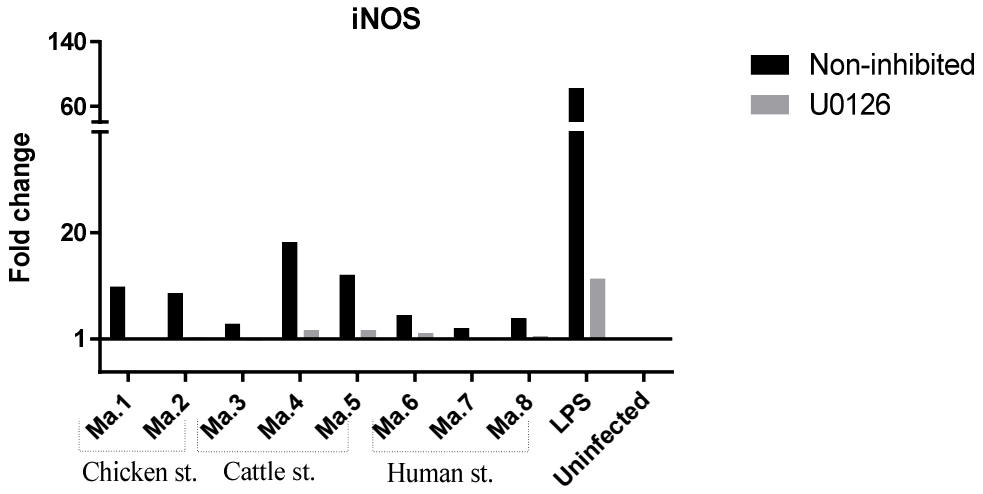


Figure 4.17: Effect of the ERK pathway inhibitor, U0126, on iNOS induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM U0126, 30 min prior to infection with *M. avium* strains. Expression of iNOS mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, and **) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of iNOS from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.5.2 IL-1β

The mRNA level of IL-1β also decreased in cells infected with Ma.1, Ma.2, Ma.3, Ma.4 and Ma.5 ($P<0.05$) following treatment of the cells with this inhibitor (Figure 4.18).

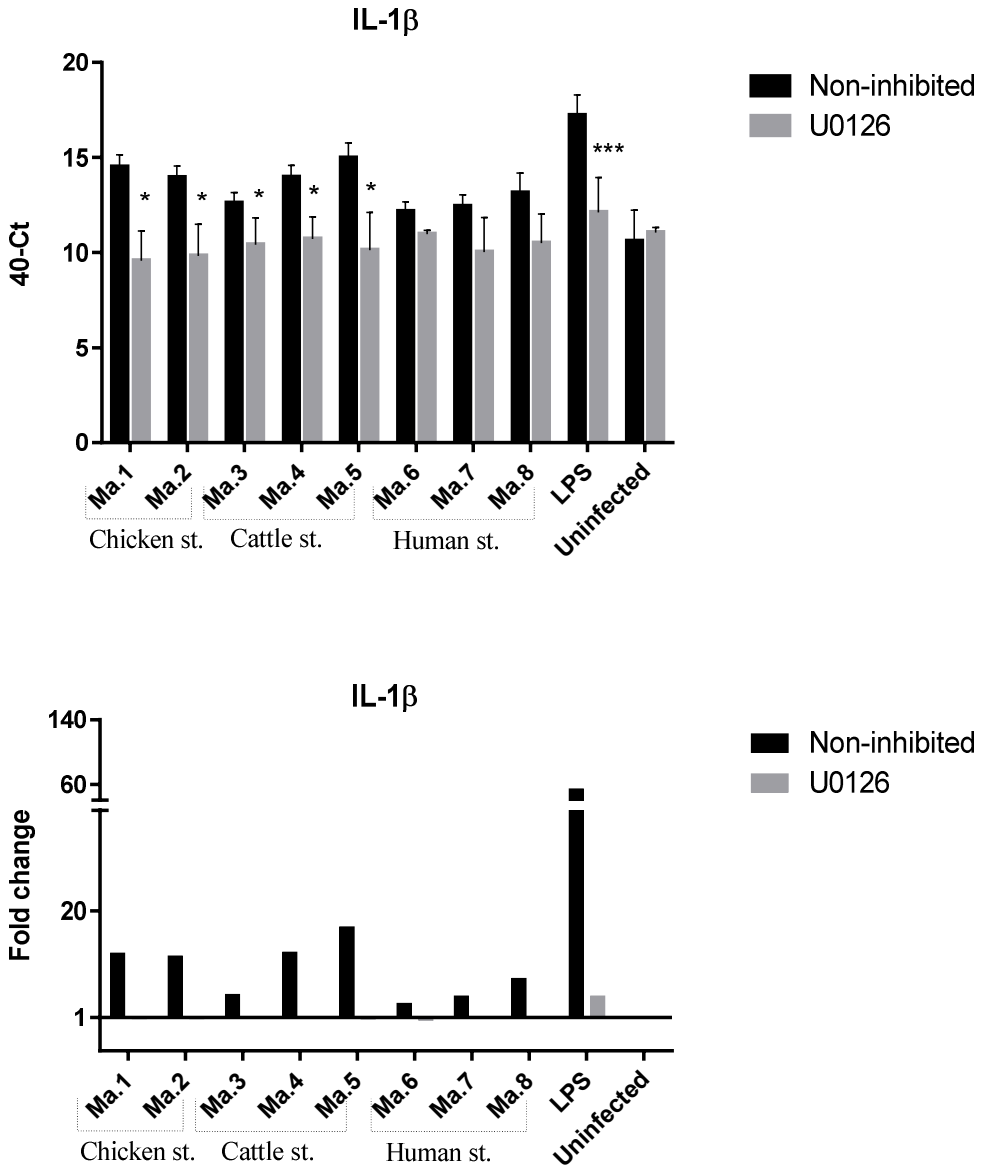


Figure 4.18: Effect of the ERK pathway inhibitor, U0126, on IL-1β induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM U0126, 30 min prior to infection with *M. avium* strains. Expression of IL-β mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-1β from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330),

Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.5.3 CXCLi1

Likewise, U0126 reduced the level of CXCLi1 in these cells in response to infection with Ma.1, Ma.2 ($P<0.05$), Ma.4 and Ma.5 ($P<0.01$) (Figure 4.19).

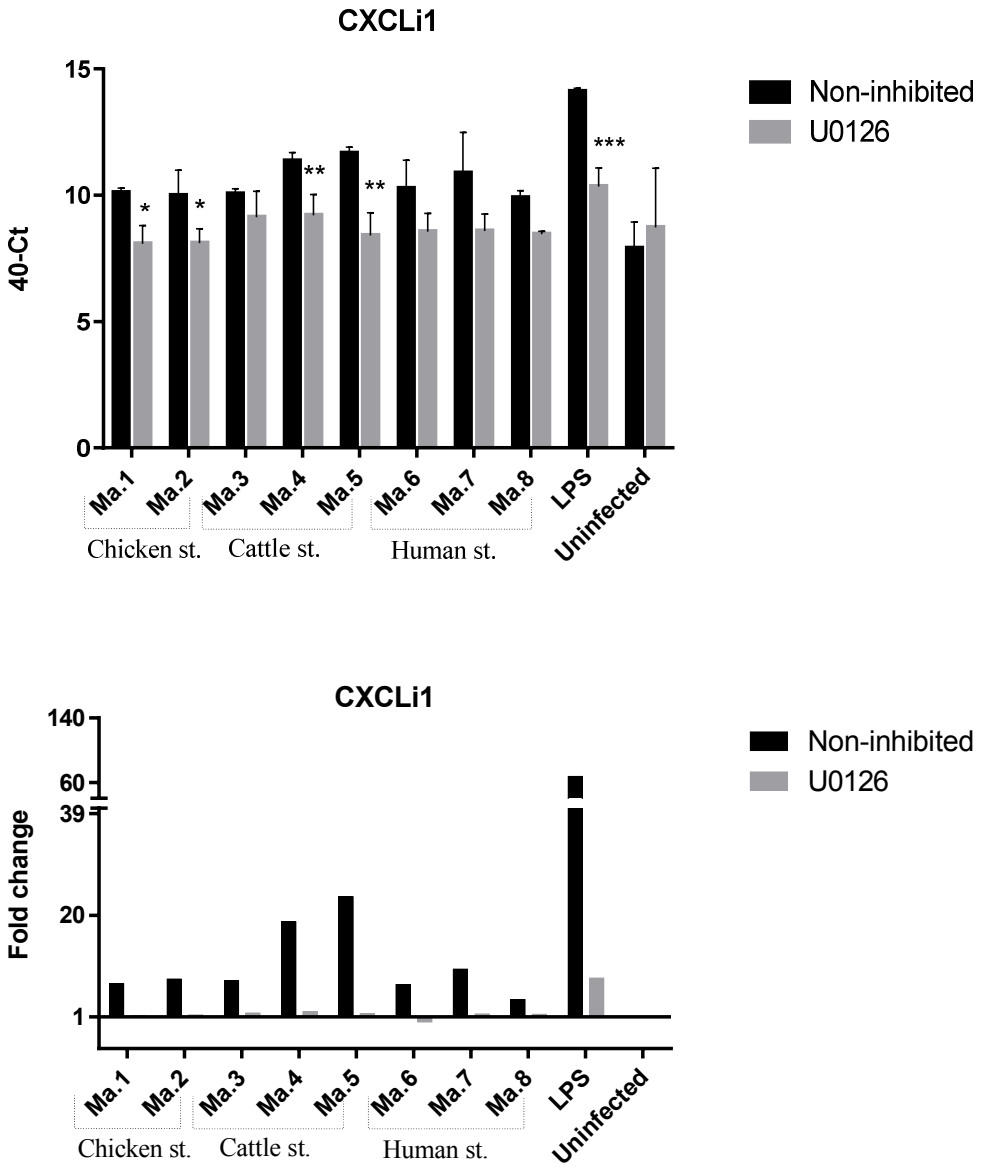
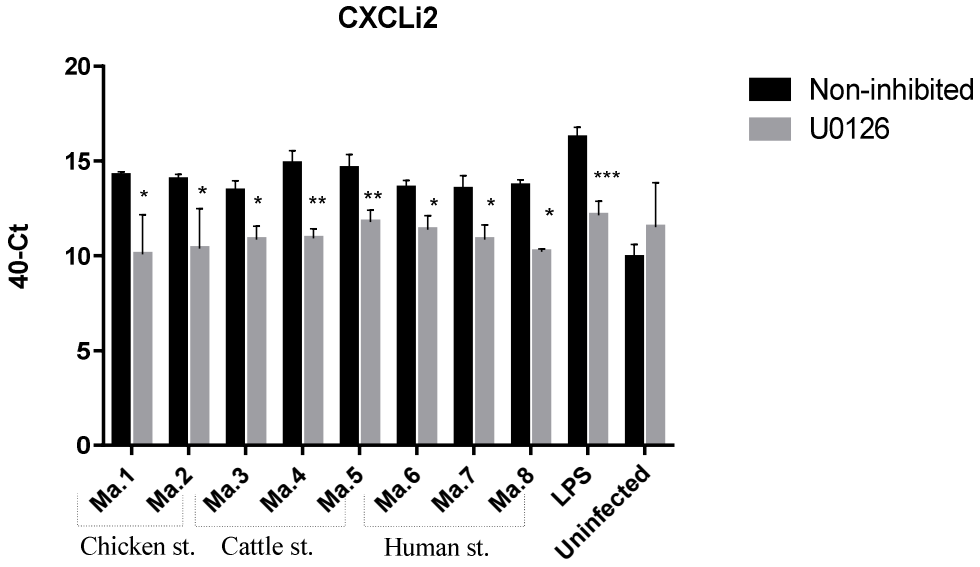


Figure 4.19: Effect of the ERK pathway inhibitor, U0126, on CXCLi1 induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM U0126, 30 min prior to infection with *M. avium* strains. Expression of CXCLi1 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001 respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi1 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.5.4 CXCLi2

Moreover, CXCLi2 significantly reduced in cells infected with Ma.1, Ma.2, Ma.3, Ma.6, Ma.7, Ma.8 (P<0.05), Ma.4 and Ma.5 (P<0.01) following treatment of the cells with U0126 (Figure 4.20).



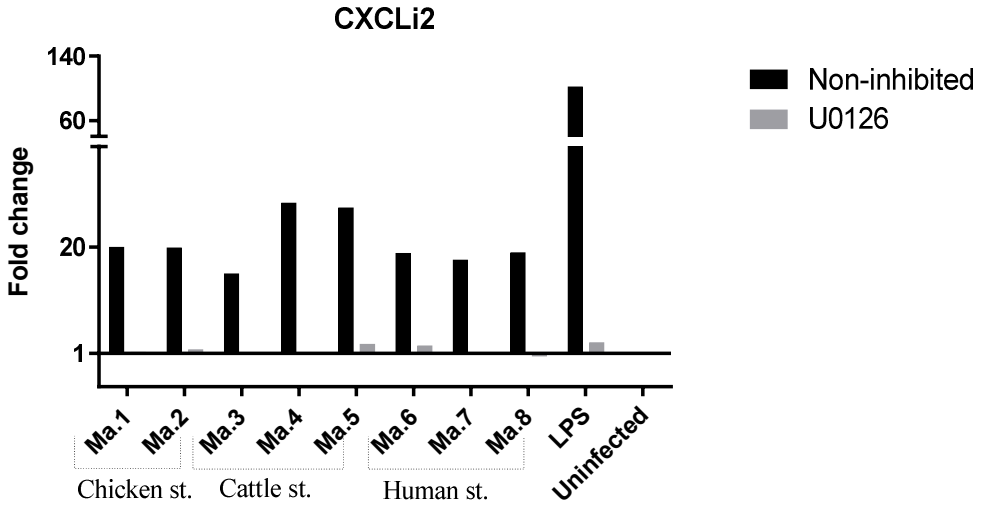


Figure 4.20: Effect of the ERK pathway inhibitor, U0126, on CXCLi2 induction by *M. avium* strains in HD11 cells.

The ERK pathway was inhibited in HD11 cells by 50 µM U0126, 30 min prior to infection with *M. avium* strains. Expression of CXCLi2 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi2 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.5.5 IL-6

In addition, following inhibition of the ERK pathway by U0126, IL-6 expression showed a significant reduction in cells infected with Ma.4 and Ma.5 ($P<0.05$) compared with *M. avium*-infected cells in the absence of the inhibitor (Figure 4.21).

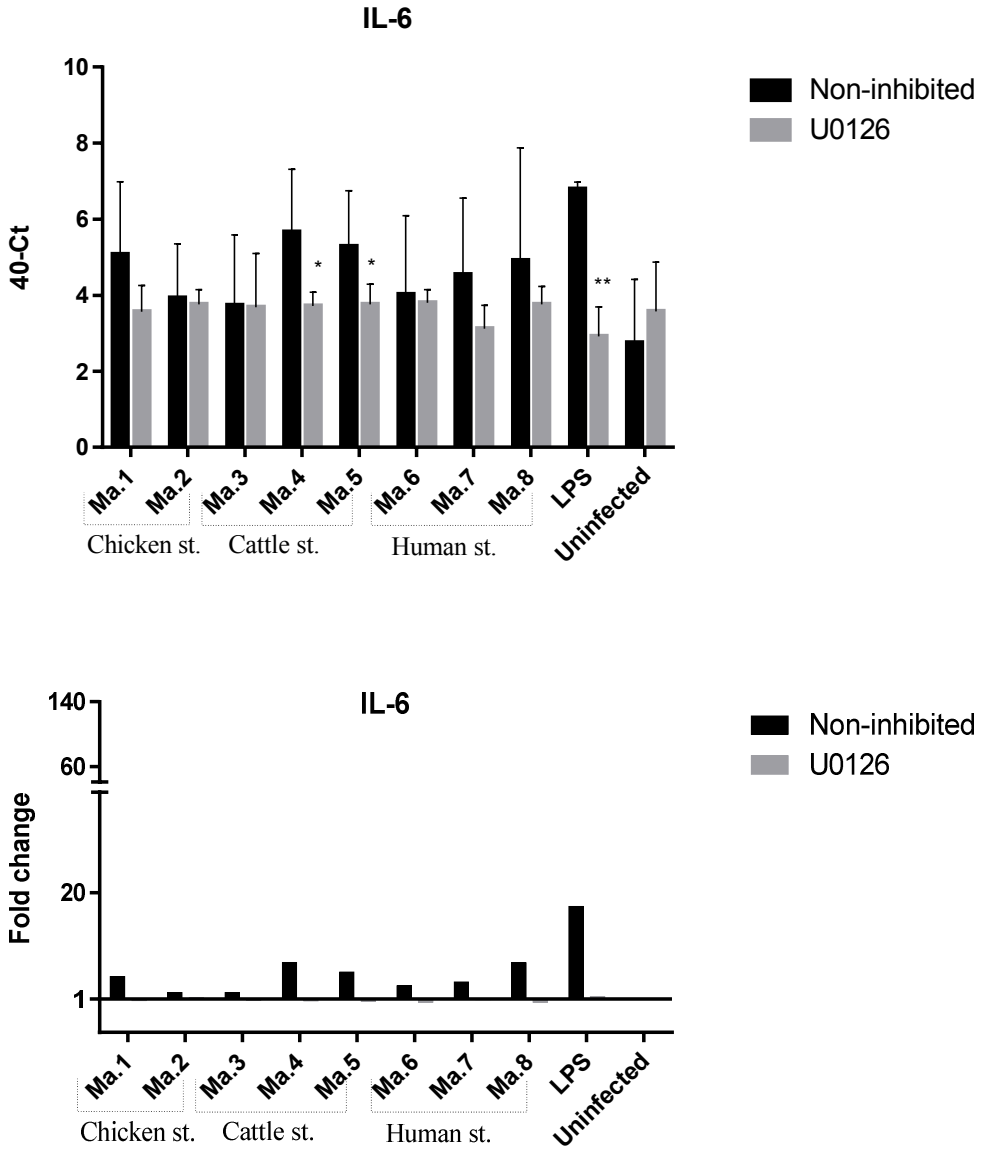


Figure 4.21: Effect of the ERK pathway inhibitor, U0126, on IL-6 induction by *M. avium* strains in HD11 cells.

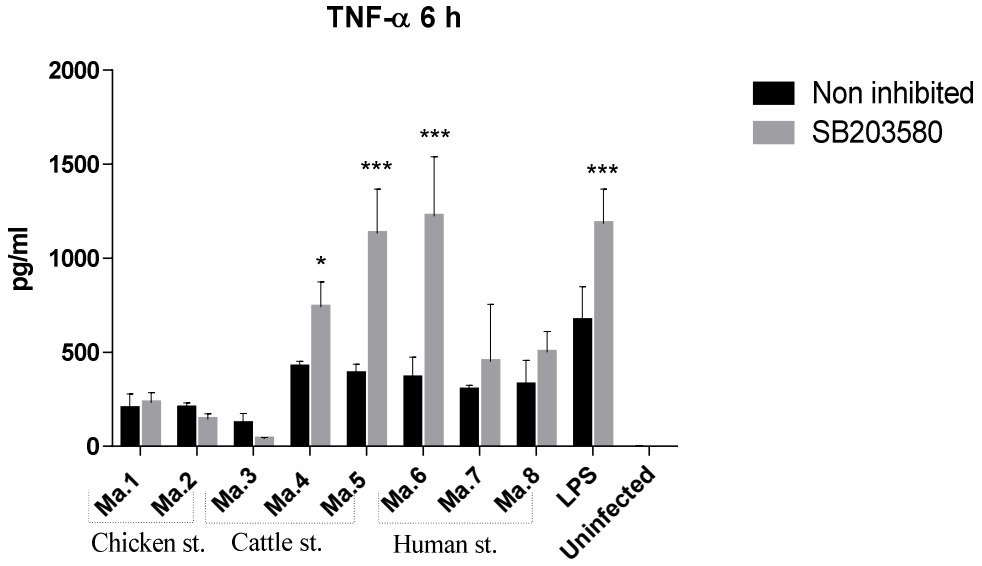
The ERK pathway was inhibited in HD11 cells by 50 μM U0126, 30 min prior to infection with *M. avium* strains. Expression of IL-6 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (* and **) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with U0126 (grey bars) and those not treated with U0126 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-6 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.6 The effect of p38 MAPK in regulating cytokine production in THP-1 cells

To determine the role of the p38 MAPK pathway in regulation of cytokine production in THP-1 cells as a response to infection with *M. avium*, this pathway was inhibited by a selective inhibitor, SB203580, 30 min prior to infection.

4.2.6.1 TNF-α

Pretreatment of THP-1 cells with 50 μM SB203580 resulted in significant increases in the level of TNF-α at 6 h post infection in response to challenge with Ma.4 ($P<0.05$), Ma.5 and Ma.6 ($P<0.001$) compared to the cells infected with *M. avium* strains without pretreatment with the inhibitor. This increase was not significant in cells infected with the other *M. avium* strains. Following treatment with SB203580, at 24 h post-infection the level of this cytokine significantly increased in cells infected with Ma.4 and Ma.5 ($P<0.05$) compared with those infected with the strains alone (Figure 4.22).



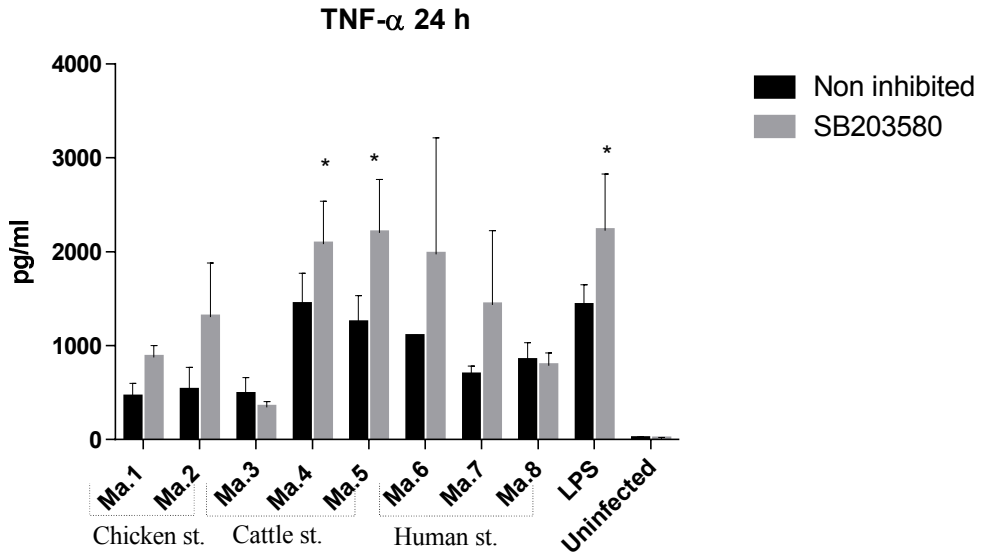


Figure 4.22: Effect of the p38 pathway inhibitor, SB203580, on TNF-α induction by *M. avium* strains in THP-1 cells.

The p38 pathway was inhibited in THP-1 macrophages by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. TNF-α concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.6.2 IL-1β

Similarly, a significant increase in the level of IL-1β was observed in cells challenged with Ma.4, Ma.5 (P<0.05), Ma.6 (P<0.001) at 6 h post-infection following treatment with SB203580. This significant increase in the level of IL-1β was also noted at 24 h post-infection in cells treated with SB203580 and infected with Ma.2 (P<0.05), Ma.4 and Ma.5 (P<0.00) (Figure 4.23).

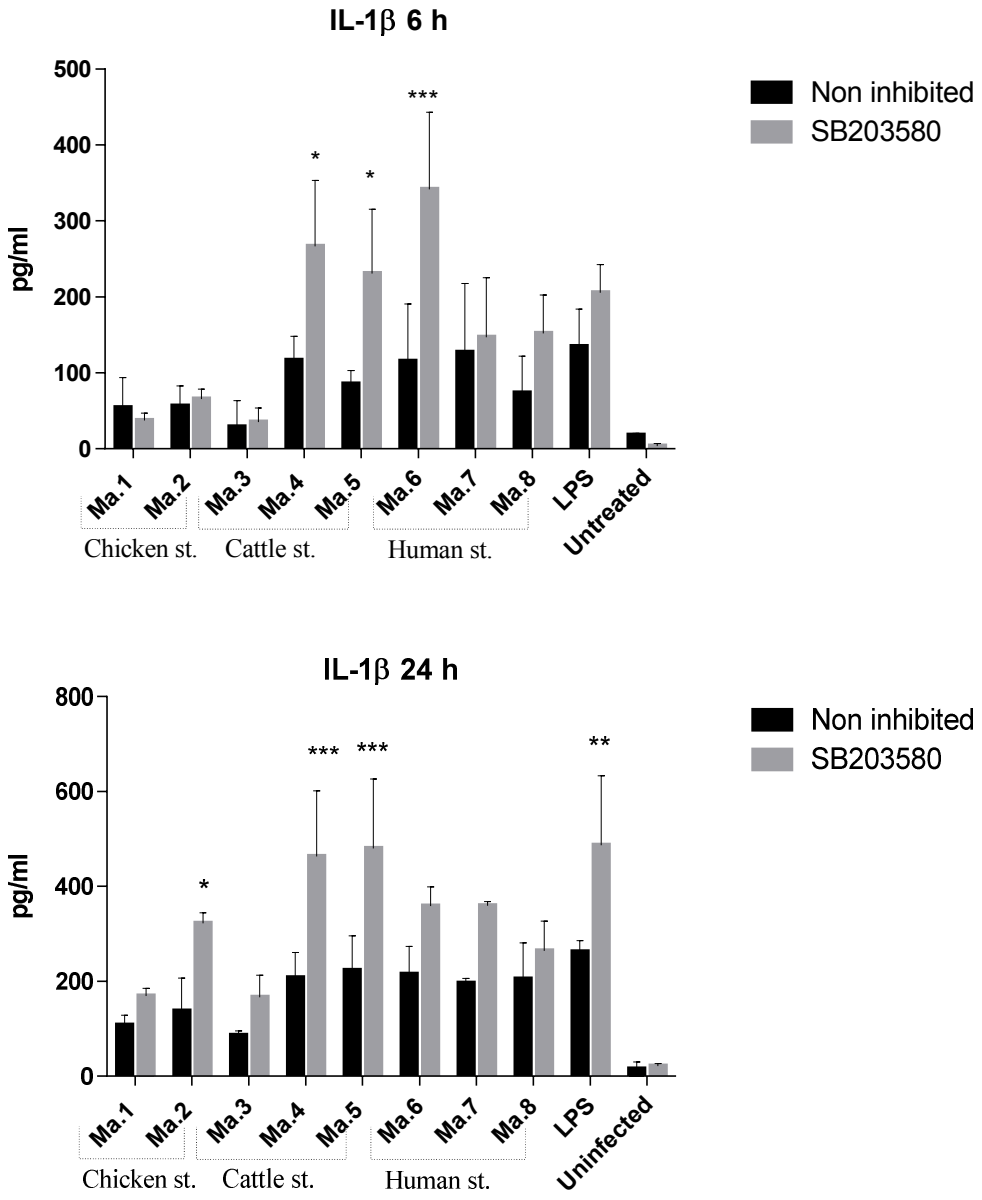
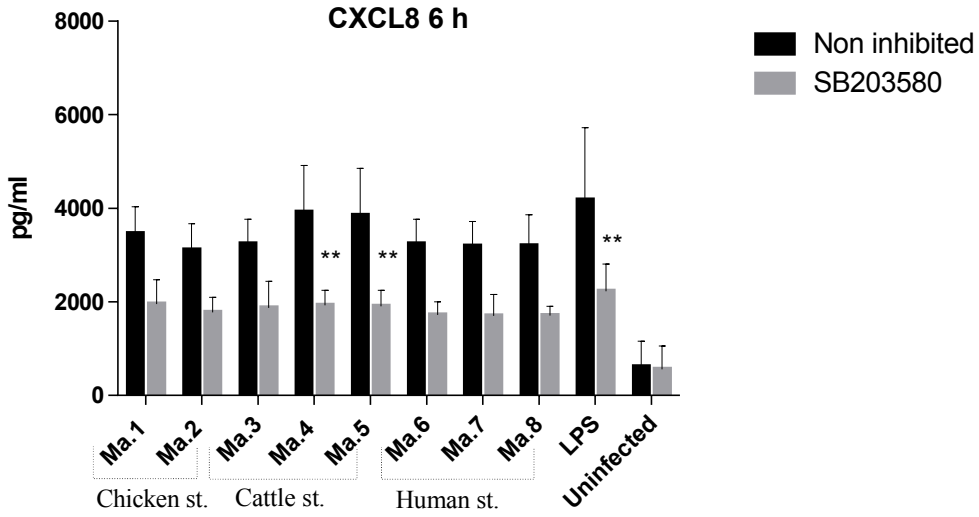


Figure 4.23: Effect of the p38 pathway inhibitor, SB203580, on IL-1β induction by *M. avium* strains in THP-1 cells.

The p38 pathway was inhibited in THP-1 macrophages by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. IL-β concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.6.3 CXCL8

Moreover, the CXCL8 chemokine level at 6 h post-infection was significantly reduced in SB203580-treated cells in response to infection with Ma.4 and Ma.5 ($P<0.01$) compared to those not treated with this inhibitor. Similarly, the level of this chemokine was significantly reduced at 24 h post-infection in the cells pretreated with SB203580 and infected with Ma.1, Ma.2, Ma.4, Ma.5, Ma.6, Ma.8 ($P<0.001$), Ma.3 and Ma.7 ($P<0.01$) (Figure 4.24).



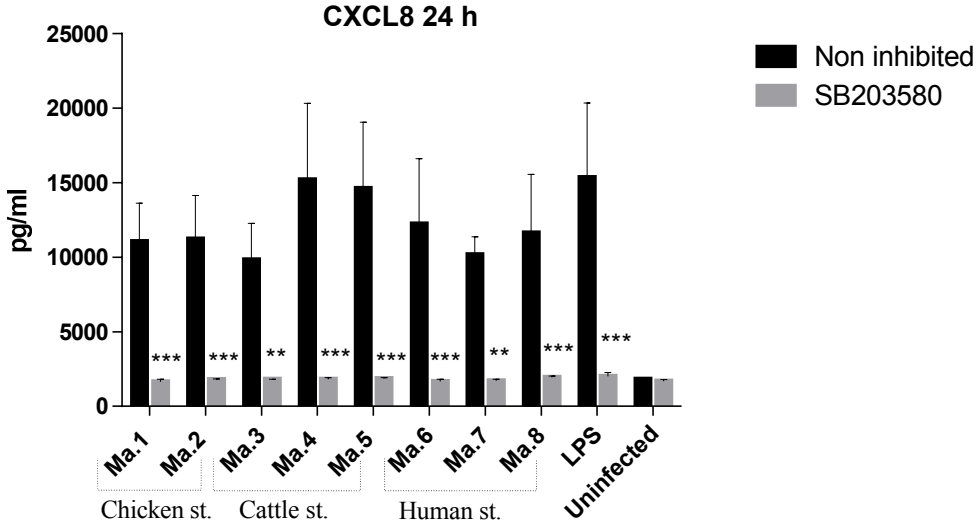


Figure 4.24: Effect of the p38 pathway inhibitor, SB203580, on CXCL8 induction by *M. avium* strains in THP-1 cells.

The p38 pathway was inhibited in THP-1 macrophages by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. CXCL8 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.6.4 IL-6

In contrast, production of IL-6 was significantly reduced following challenge with Ma.4 and Ma.5 (P<0.001) in THP-1 cells after inhibition of the p38 pathway in these cells (Figure 4.25).

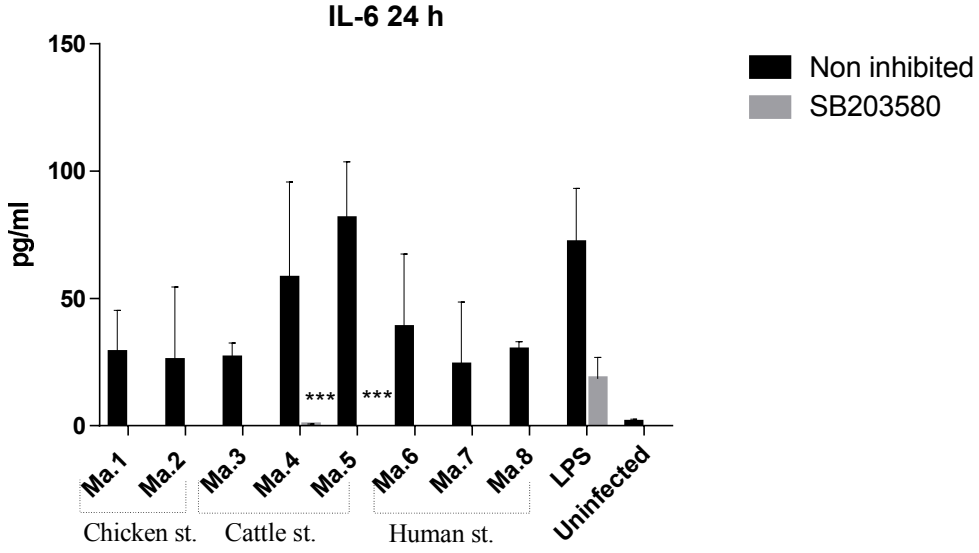


Figure 4.25: Effect of the p38 pathway inhibitor, SB203580, on IL-6 induction by *M. avium* strains in THP-1 cells.

The p38 pathway was inhibited in THP-1 macrophages by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. IL-6 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences (P<0.05, P<0.01 and P<0.001) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-

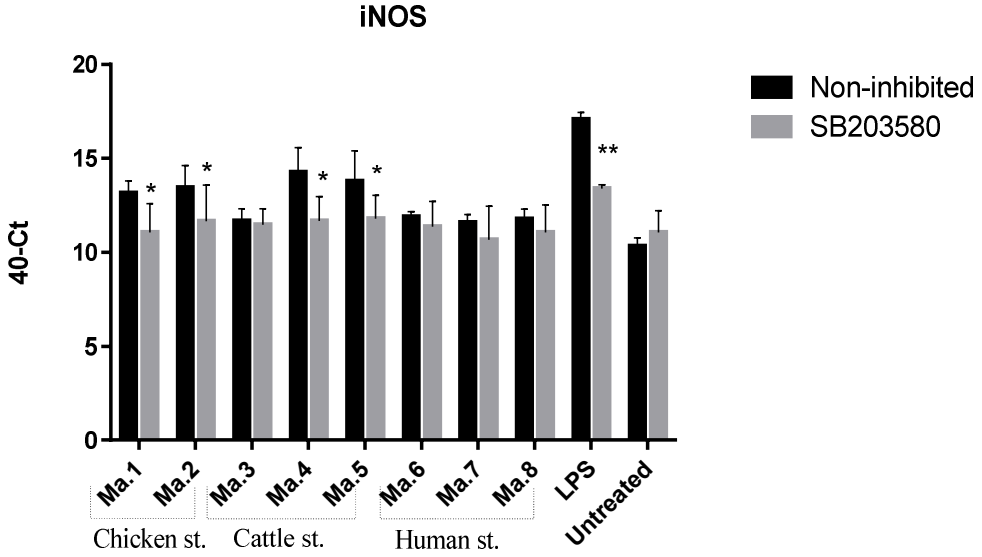
02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.7 The effect of p38 MAPK inhibition on cytokine and chemokine gene expression in HD11 cells

To determine the regulation of cytokine production by the p38 pathway in *M. avium*-infected chicken cells, the mRNA level of iNOS, IL-1β, CXCLi1, CXCLi2 and IL-6 genes were quantified following treatment of the cells with 50 μM SB203580, 30 min prior to the infection. We observed that when the p38 pathway was inhibited the expression level of these cytokines in HD11 cells in response to *M. avium* infection was reduced compared to non-inhibited cells.

4.2.7.1 iNOS

Treatment of HD11 cells with SB203580 prior to infection led to a significant reduction in iNOS gene expression in cells infected with Ma.1, Ma.2, Ma.4 and Ma.5 ($P<0.05$) in comparison to those not treated with the inhibitor (Figure 4.26).



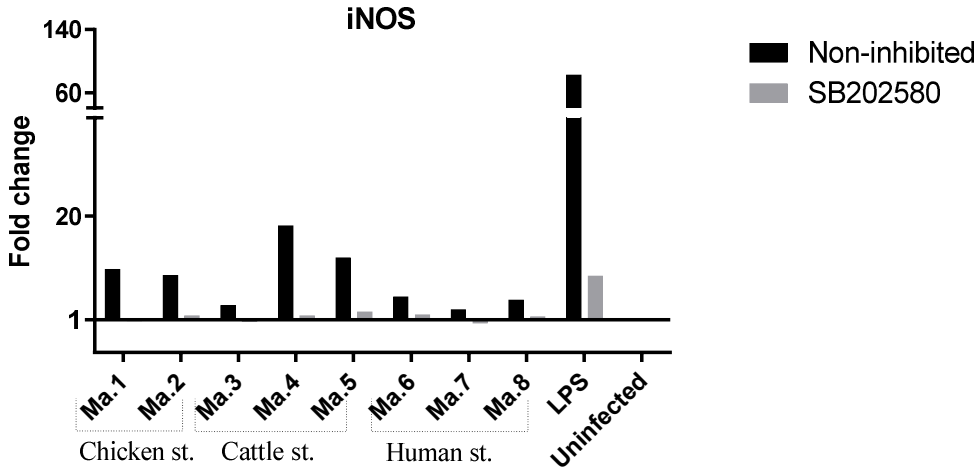


Figure 4.26: Effect of the p38 pathway inhibitor, SB203580, on iNOS induction by *M. avium* strains in HD11 cells.

The p38 pathway was inhibited in HD11 cells by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. Expression of iNOS mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (* and **) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of iNOS from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.7.2 IL-1β

Similarly, SB203580 reduced mRNA expression level of IL-1β in response to Ma.1, Ma.4 ($P<0.01$), Ma.2, Ma.3 and Ma.5 ($P<0.05$) (Figure 4.27).

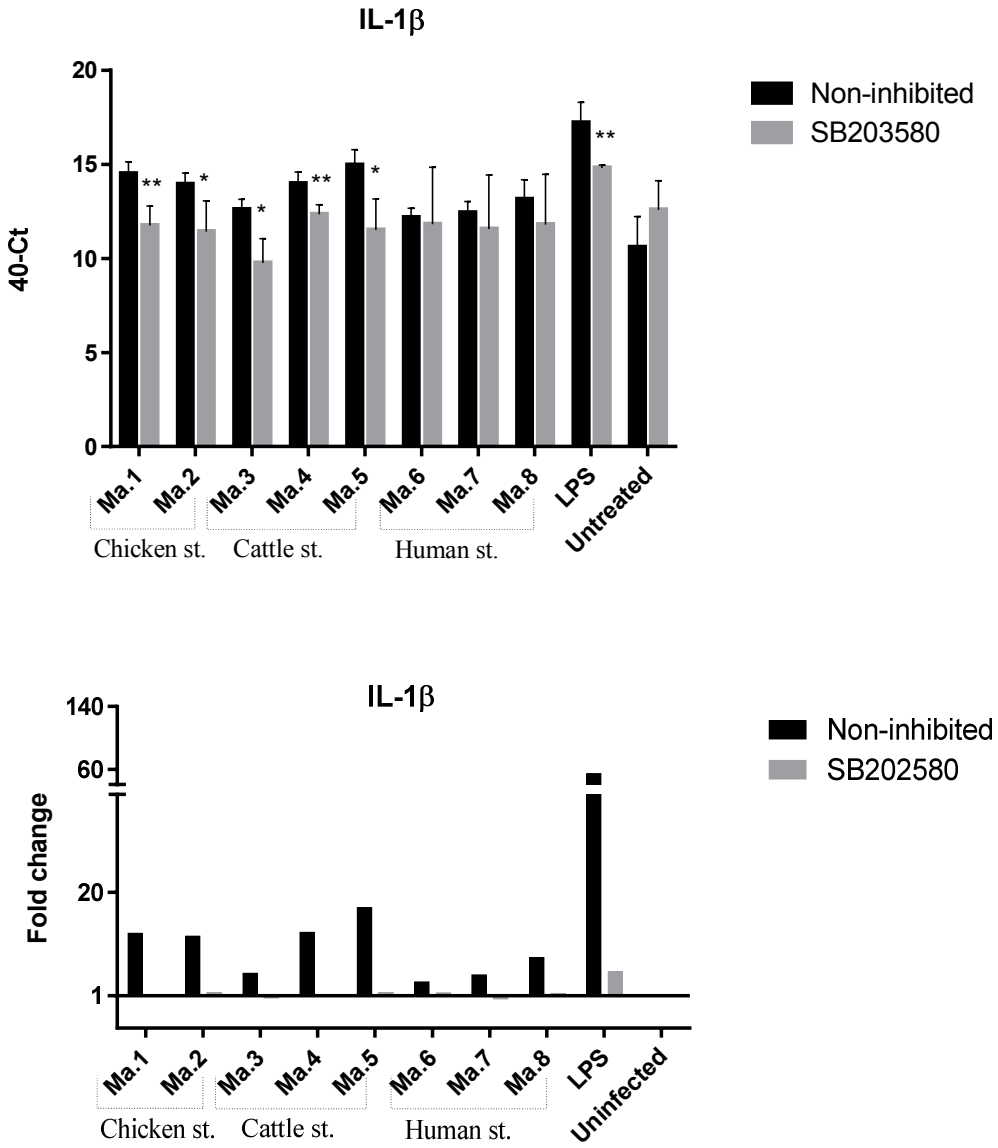


Figure 4.27: Effect of the p38 pathway inhibitor, SB203580, on IL-1β induction by *M. avium* strains in HD11 cells.

The p38 pathway was inhibited in HD11 cells by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. Expression of IL-1β mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*and***) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-1β from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7

(446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.7.3 CXCLi1

In addition, inhibition of the p38 pathway resulted in a significant reduction of CXCLi1 expression in response to infection with Ma.1, Ma.2, Ma.3, Ma.6, Ma.7, Ma.8 ($P<0.05$), Ma.4 and Ma.5 ($P<0.01$) (Figure 4.28).

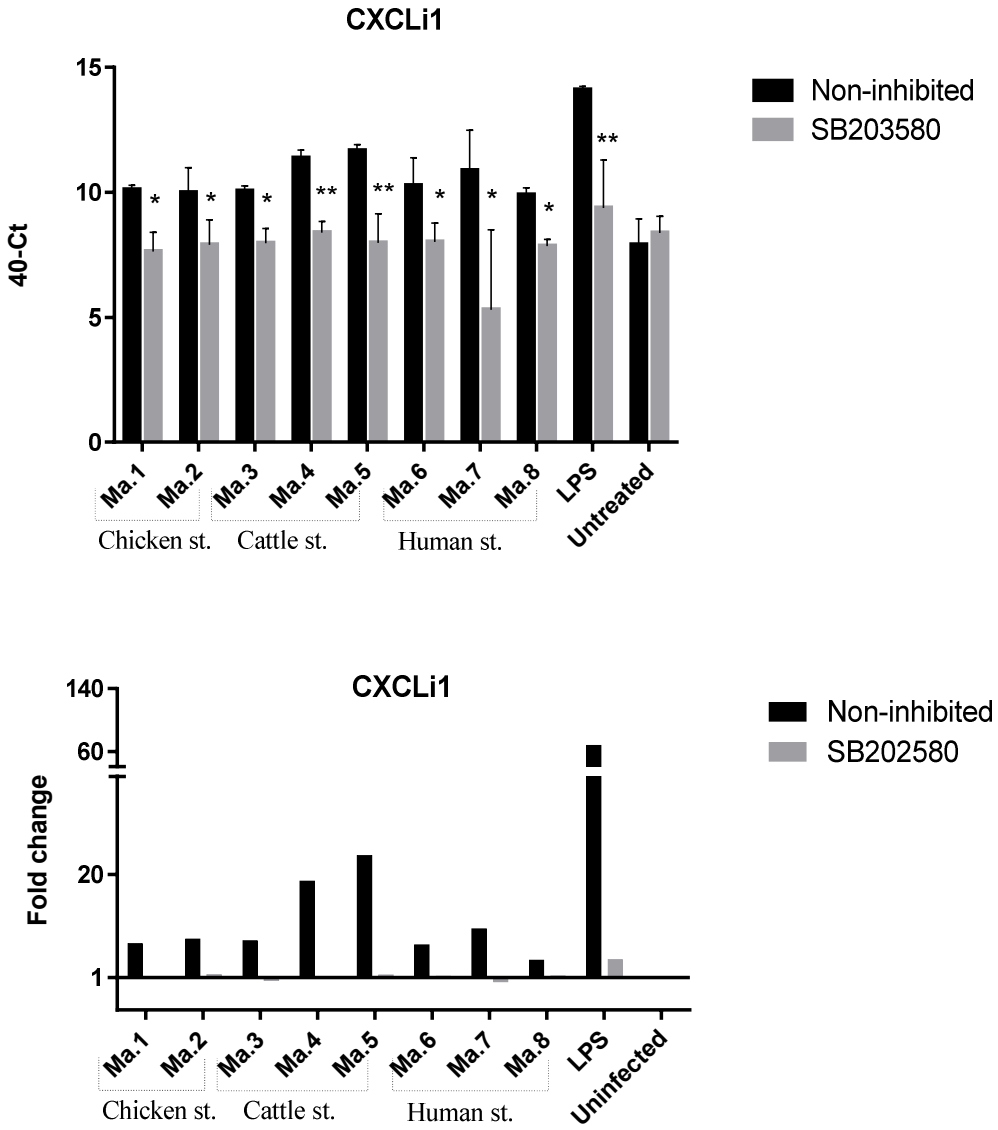


Figure 4.28: Effect of the p38 pathway inhibitor, SB203580, on CXCLi1 induction by *M. avium* strains in HD11 cells.

The p38 pathway was inhibited in HD11 cells by 50 μM SB203580, 30 min prior to infection with *M. avium* strains. Expression of CXCLi1 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*and**) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi1 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.7.4 CXCLi2

The significant reduction was also observed in the level of CXCLi2 expression in cells infected with all strains ($P<0.05$) following treatment of the cells with SB203580 (Figure 4.29).

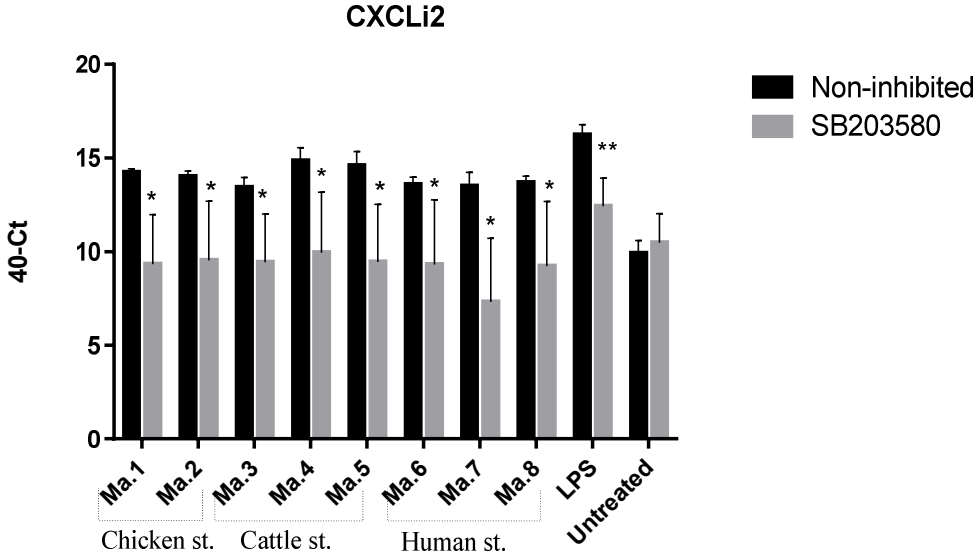




Figure 4.29: Effect of the p38 pathway inhibitor, SB203580, on CXCLi2 induction by *M. avium* strains in HD11 cells.

The p38 pathway was inhibited in HD11 cells by 50 µM SB203580, 30 min prior to infection with *M. avium* strains. Expression of CXCLi2 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values was deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$ respectively) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi2 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.7.5 IL-6

The p38 inhibitor, SB203580, also significantly reduced the expression of IL-6 in the cells infected with Ma.4 and Ma.5 ($P<0.05$) (Figure 4.30).

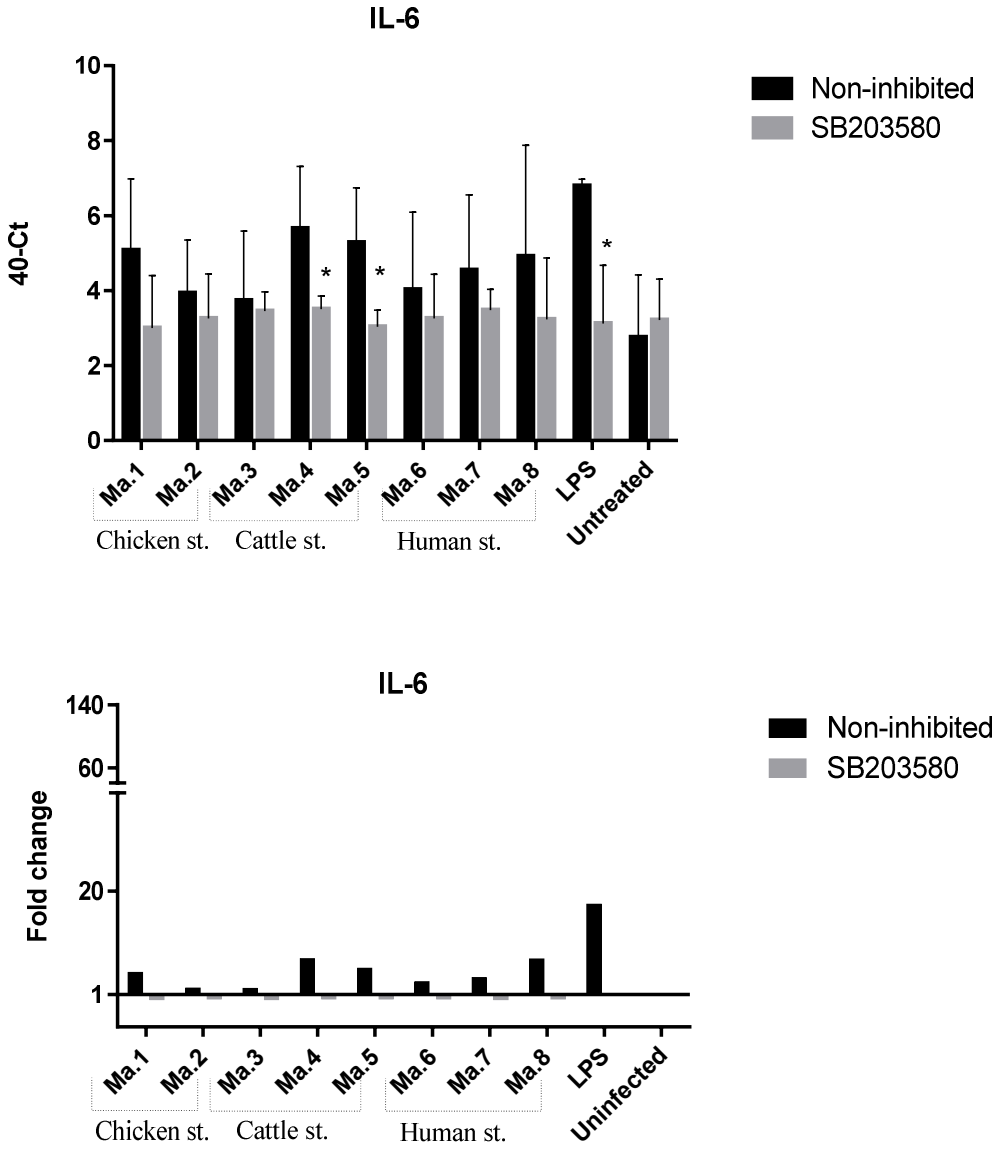


Figure 4.30: Effect of the p38 pathway inhibitor, SB203580, on IL-6 induction by *M. avium* strains in HD11 cells.

The p38 pathway was inhibited in HD11 cells by 50 µM SB203580, 30 min prior to infection with *M. avium* strains. Expression of IL-6 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*) refer to significant differences ($P<0.05$) between *M. avium*-infected cells treated with SB203580 (grey bars) and those not treated with SB203580 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-6 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-

way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.8 The effect of JNK inhibition on cytokine and chemokine production in THP-1 cells

To determine whether the JNK pathway is involved in regulation of cytokine production in cells challenged with *M. avium* strains, we used SP600125, a highly specific inhibitor of this pathway and the levels of TNF-α, IL-1β, IL-6 and CXCL8 cytokine concentration were measured in response to infection in THP-1 human cells.

4.2.8.1 TNF-α

The concentration of TNF-α production at 6 h post-infection was significantly reduced in THP-1 cells in response to challenge with Ma.1, Ma.2, Ma.3 ($P<0.05$), Ma.4 ($P<0.001$), Ma.5, Ma.6, Ma.7 and Ma.8 ($P<0.01$) following treatment with SP600125. This reduction was also observed in the level of this cytokine at 24 h post-infection in cells treated with Ma.1 ($P<0.05$), Ma.2, Ma.3 ($P<0.01$), Ma.4, Ma.5, Ma.6, Ma.7 and Ma.8 ($P<0.001$) (Figure 4.31).

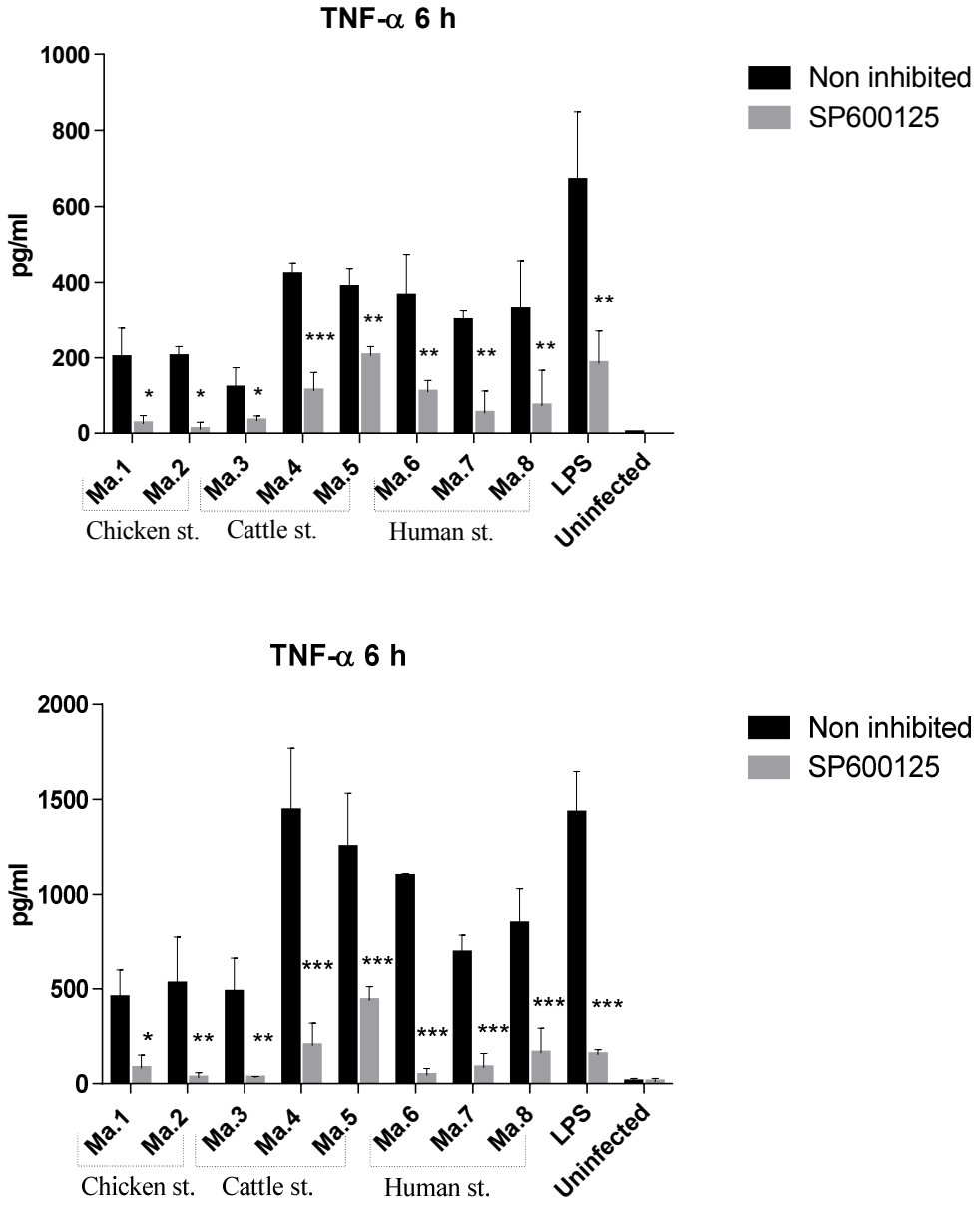


Figure 4.31: Effect of the JNK pathway inhibitor, SP600125, on TNF-α induction by *M. avium* strains in THP-1 cells.

The JNK pathway was inhibited in THP-1 macrophages by 50 µM SP600125, 30 min prior to infection with *M. avium* strains. TNF-α concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$) between *M. avium* strains-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.8.2 IL-1β

The level of IL-1β at 6 h post-infection significantly decreased in SP600125-treated cells only in response to Ma.4 ($P<0.01$), but not significantly in other strains, in comparison to cells without treatment. At 24 h post-infection, IL-1β production did not show any significant changes in cells pre-treated with SP600125 and infected with *M. avium* strains (Figure 4.32).

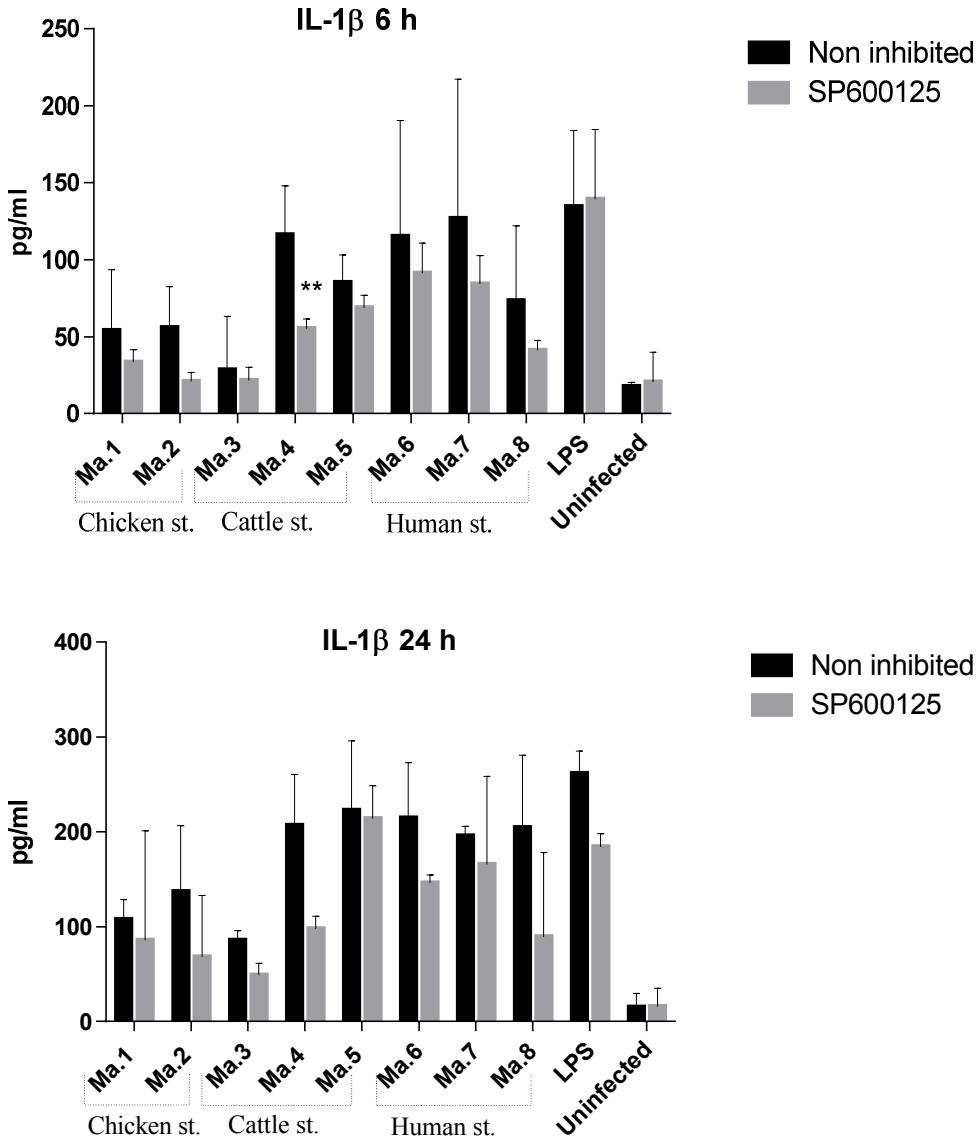
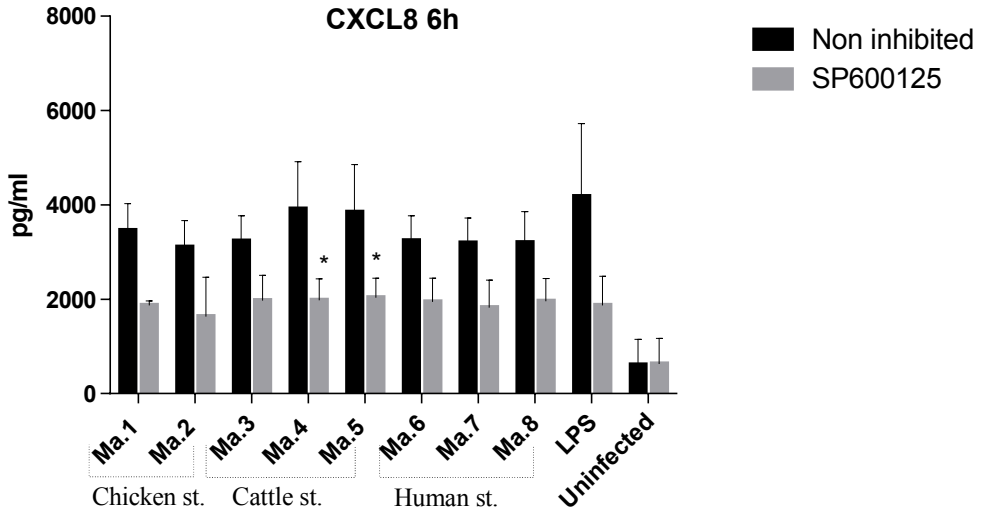


Figure 4.32: Effect of the JNK pathway inhibitor, SP600125, on IL-1β induction by *M. avium* strains in THP-1 cells.

The JNK pathway was inhibited in THP-1 macrophages by 50 μM SP600125, 30 min prior to infection with *M. avium* strains. IL-1β concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (**) refer to significant differences (P<0.01) between *M. avium* strains-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.8.3 CXCL8

Inhibition of JNK significantly reduced the induction of CXCL8 in THP-1 cells infected with Ma.4 and Ma.5 (P<0.05) at 6 h post-infection. In contrast, inhibition of this pathway had no significant effect on release of CXCL8 at 24 h post-infection in cells infected with *M. avium* strains (Figure 4.33).



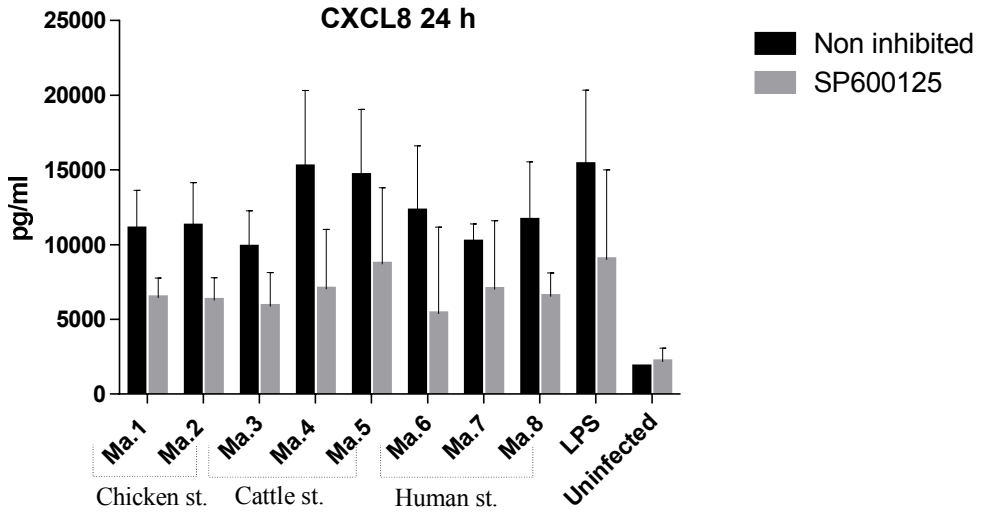


Figure 4.33: Effect of the JNK pathway inhibitor, SP600125, on CXCL8 induction by *M. avium* strains in THP-1 cells.

The JNK pathway was inhibited in THP-1 macrophages by 50 µM SP600125, 30 min prior to infection with *M. avium* strains. CXCL8 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*) refer to significant differences (P<0.05) between *M. avium* strains-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.8.4 IL-6

SP600125 also significantly reduced the level of IL-6 in these cells in response to infection with Ma.3, Ma.4 and Ma.5 (P<0.01) (Figure 4.34).

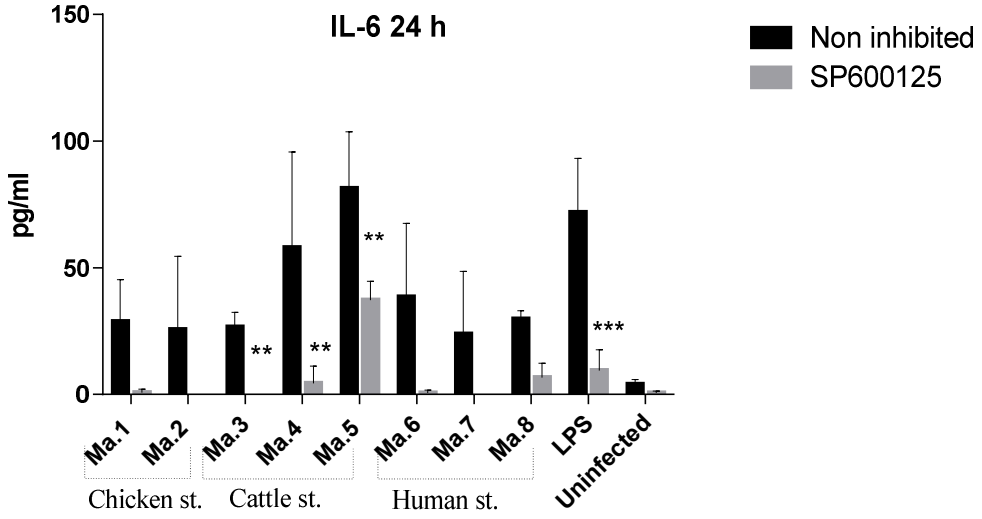


Figure 4.34: Effect of the JNK pathway inhibitor, SP600125, on IL-6 induction by *M. avium* strains in THP-1 cells.

The JNK pathway was inhibited in THP-1 macrophages by 50 µM SP600125, 30 min prior to infection with *M. avium* strains. IL-6 concentration in the cells supernatants were measured at 6 h and 24 h post-infection by ELISA. Asterisks (*, **and***) refer to significant differences ($P<0.05$, $P<0.01$ and $P<0.001$) between *M. avium* strains-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). The error bars indicate SEM of three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.9 The effect of inhibition of the JNK pathway on cytokine and chemokine expression in HD11 cells

We also evaluated the role of the JNK pathway in regulation of cytokine production in HD11 cells by treating the cells with the JNK inhibitor, SP600125, prior to infection with *M. avium* strains and gene expression of iNOS, IL-1β, CXCLi1, CXCLi2 and IL-6 quantified by qRT-PCR.

4.2.9.1 iNOS

Pre-treatment of HD11 cells with 50 µM SP600125 resulted in a significant reduction in iNOS gene expression in these cells in

response to infection with Ma.1, Ma.2 and Ma.5 (P<0.05) (Figure 4.35).

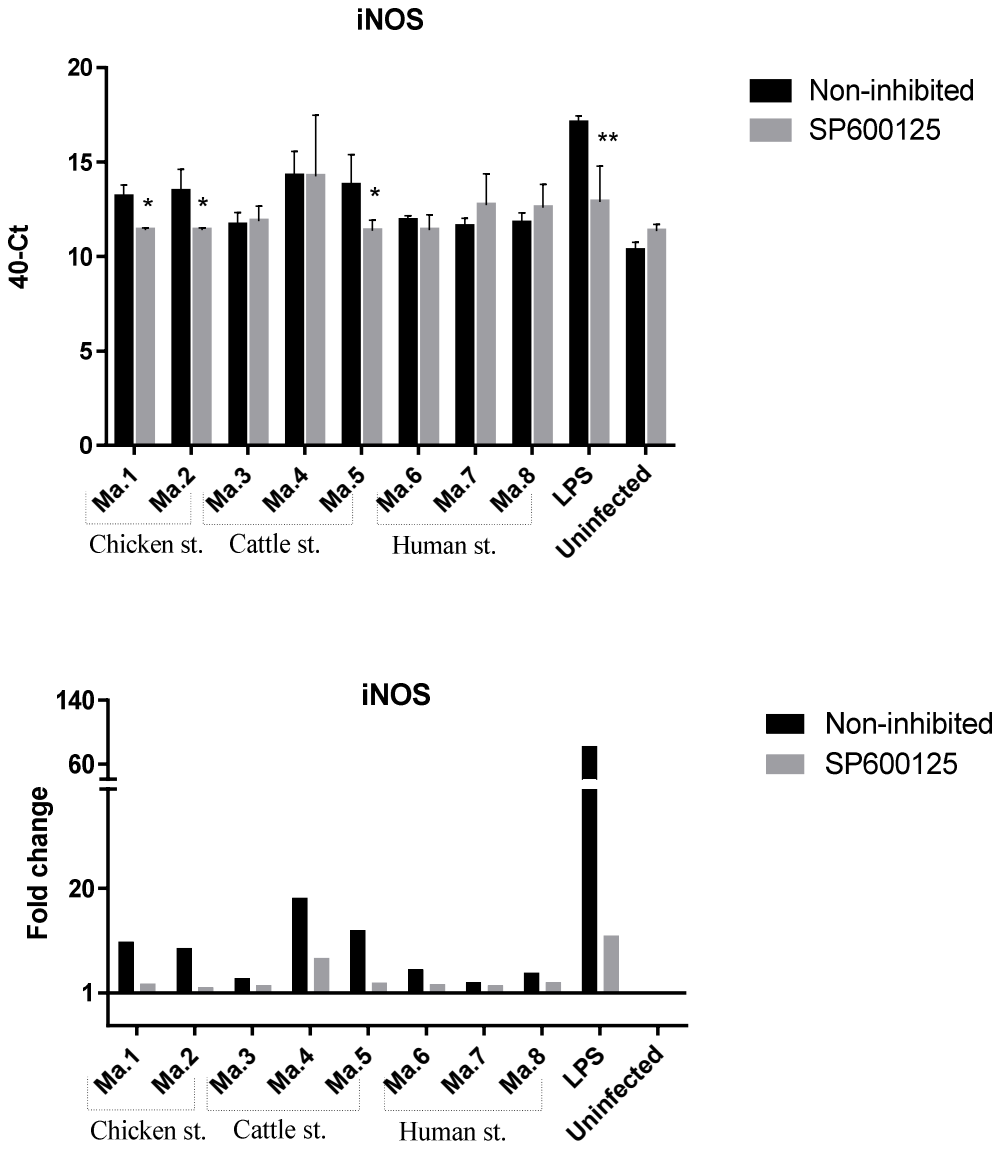


Figure 4.35: Effect of the JNK pathway inhibitor, SP600125, on iNOS induction by *M. avium* strains in HD11 cells.

The JNK pathway was inhibited in HD11 cells by 50 μM SP600125, 30 min prior to infection with *M. avium* strains. Expression of iNOS mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (* and**) refer to significant differences (P<0.05 and P<0.01 respectively) between *M. avium*-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA

levels of iNOS from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.9.2 IL-1β

We also observed a significant reduction of IL-1β in HD11 cells pre-treated with SP600125 and infected with Ma.2, Ma.4 and Ma.5 ($P<0.05$) (Figure 4.36).

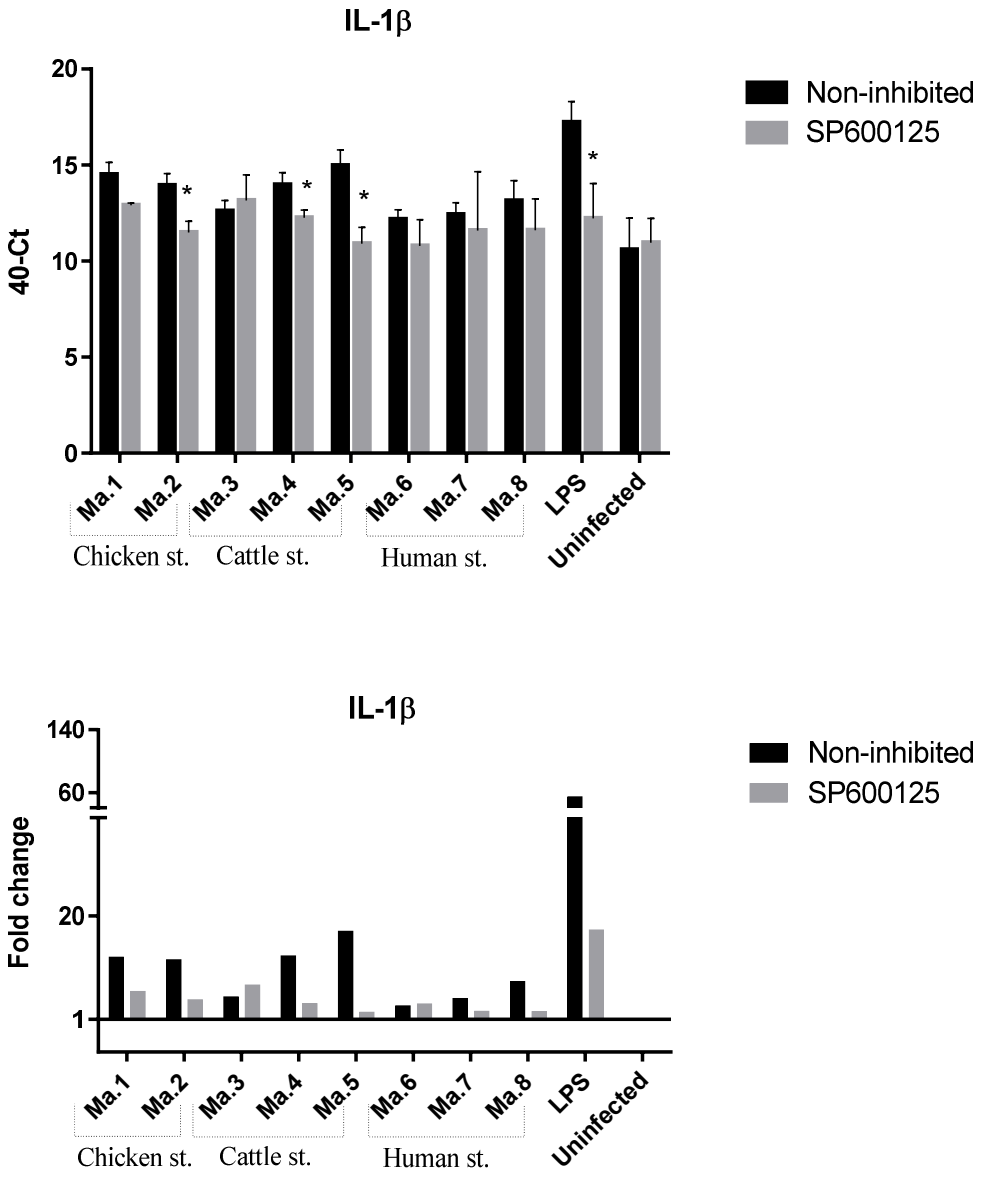
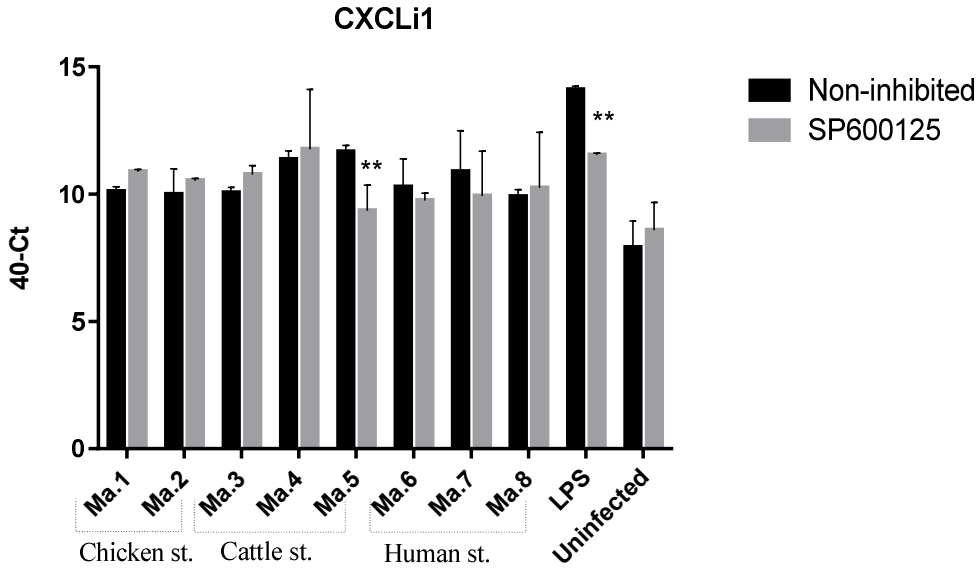


Figure 4.36: Effect of the JNK pathway inhibitor, SP600125, on IL-1β induction by *M. avium* strains in HD11 cells.

The JNK pathway was inhibited in HD11 cells by 50 μM SP600125, 30 min prior to infection with *M. avium* strains. Expression of IL-1β mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*) refer to significant differences (P<0.05) between *M. avium*-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-1β from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.9.3 CXCLi1

The mRNA level of cytokine CXCLi1 was significantly reduced in cells infected with Ma.5 (P<0.01) compared to those not treated with the inhibitor (Figure 4.37).



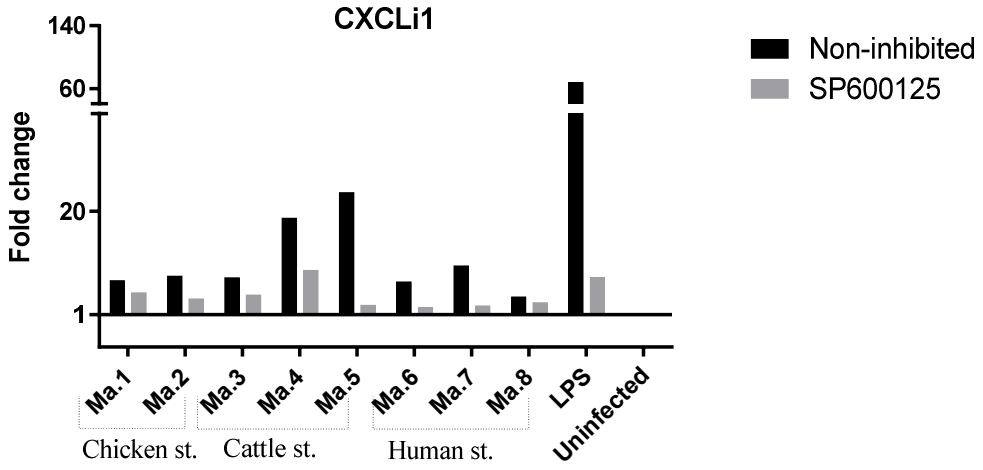


Figure 4.37: Effect of the JNK pathway inhibitor, SP600125, on CXCLi1 induction by *M. avium* strains in HD11 cells.

The JNK pathway was inhibited in HD11 cells by 50 µM SP600125, 30 min prior to infection with *M. avium* strains. Expression of CXCLi1 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*and**) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi1 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.9.4 CXCLi2

In the presence of SP600125, the mRNA level of CXCLi2 was significantly reduced in cells infected with Ma.2, Ma.4 and Ma.5 ($P<0.05$) compared with those in the absence of the inhibitor (Figure 4.38).

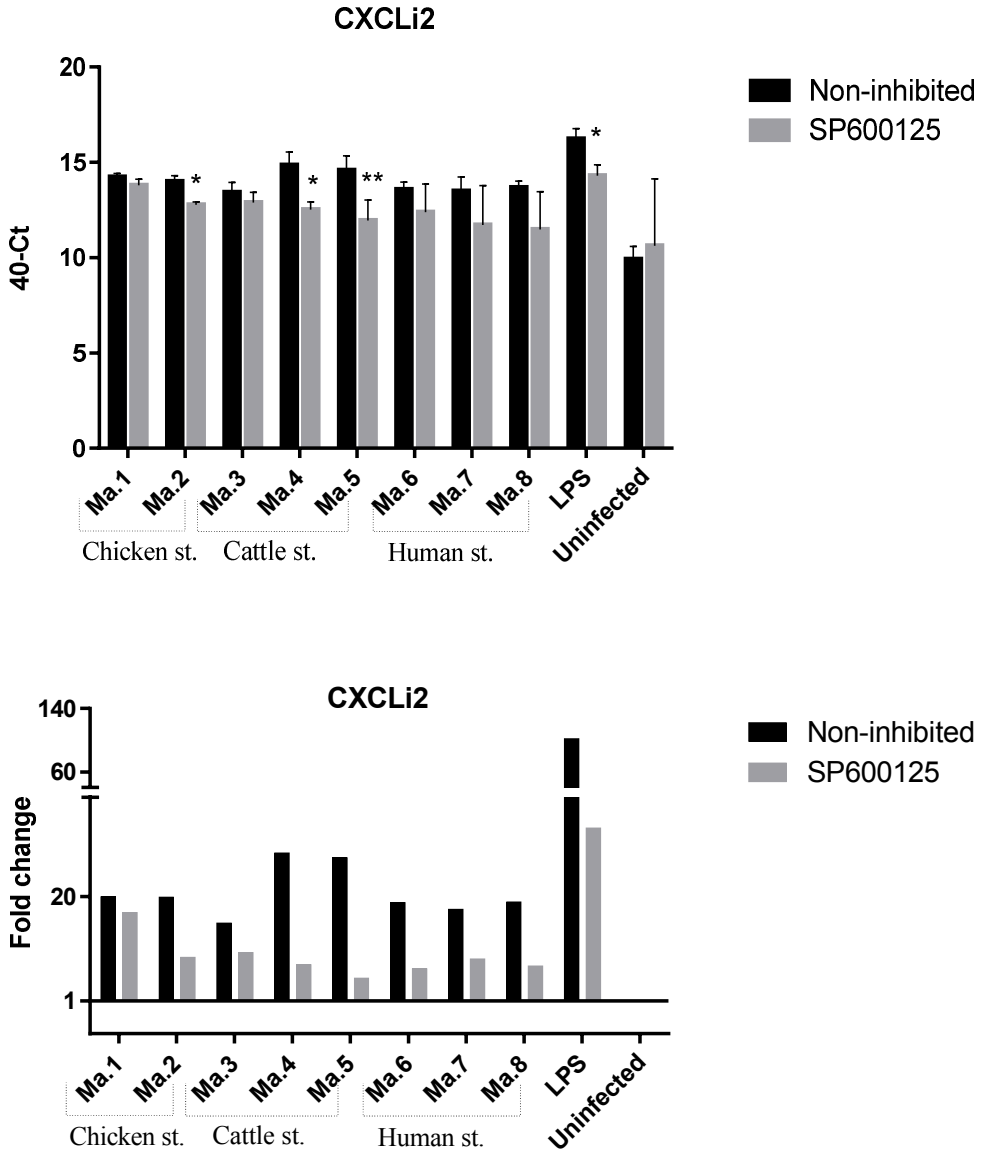


Figure 4.38: Effect of the JNK pathway inhibitor, SP600125, on CXCLi2 induction by *M. avium* strains in HD11 cells.

The JNK pathway was inhibited in HD11 cells by 50 µM SP600125, 30 min prior to infection with *M. avium* strains. Expression of CXCLi2 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Asterisks (*and**) refer to significant differences ($P<0.05$ and $P<0.01$ respectively) between *M. avium*-infected cells treated with SP600125 (grey bars) and those not treated with SP600125 (black bars). Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of CXCLi2 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6

(430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.2.9.5 IL-6

Following inhibition of JNK by SP600125 no significant differences were observed in IL-6 expression in HD11 cells in response to *M. avium* infection (Figure 4.39).

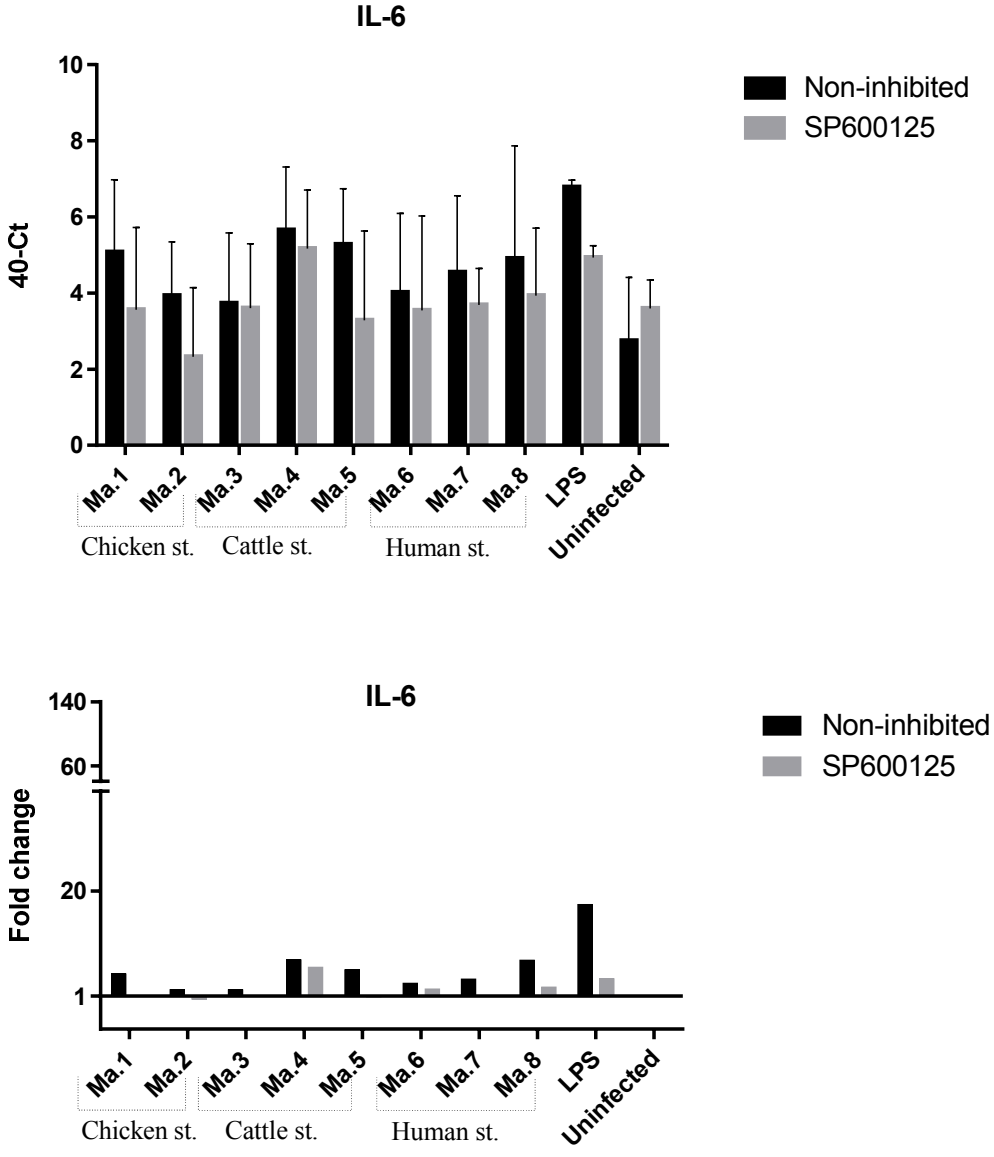


Figure 4.39: Effect of the JNK pathway inhibitor, SP600125, on IL-6 induction by *M. avium* strains in HD11 cells.

The JNK pathway was inhibited in HD11 cells by 50 μM SP600125, 30 min prior to infection with *M. avium* strains. Expression of IL-6 mRNA levels in HD11 cells were quantified in the absence and presence of the inhibitor at 6 h post-infection. The corrected cycle threshold (Ct) values were deducted from 40 (the negative end point) after standardization for input RNA. Data are also expressed as fold changes in mRNA levels of each cytokine when infected samples were compared to untreated HD11 control samples. The mRNA levels of IL-6 from untreated control samples are set as 1. Data were obtained from three independent experiments performed in triplicate. *M. avium* strains: Ma.1 (NC.08562-02), Ma.2 (NC.0855-07), Ma.3 (61.3623-05), Ma.4 (61.4627-08), Ma.5 (61.3728-08), Ma.6 (430/330), Ma.7 (446/301) and Ma.8 (460/132). Two-way ANOVA was used to determine differences between inhibited and non-inhibited cells.

4.3 Discussion

It is known that an effective innate immune response is required to assist macrophages in eliminating mycobacteria. Activation of the MAPK signalling pathway by components of mycobacteria (Chan et al., 2001, Jones et al., 2001b) and also by live *M. avium* (Reiling et al., 2001) has been studied. However, the role of each subfamily in regulation of cytokine production has been described poorly. Furthermore, knowledge of the signalling events regulating cytokine gene expression and the production of other immune effector molecules is limited.

In this study we examined the effect of widely used and highly specific inhibitors of MAPK signalling pathways on production of NO and pro-inflammatory cytokines and chemokines by human and chicken macrophages. PD98059 or U0126 were used to inhibit the ERK pathway, SB203580 was used as inhibitor for p38 pathway and finally SP600125 was used to inhibit the JNK pathway. The specificity of these inhibitors for individual cascades have been studied previously (Dudley et al., 1995, Chandel et al., 2000, Lee et al., 1994, Alessi et al., 1995, Favata et al., 1998, Bennett et al., 2001). We found that, the ERK and JNK pathways were important in regulation of pro-inflammatory cytokines whereas p38 did not appear to be involved in regulating TNF-α and IL-1β production in THP-1 cells. The results also indicated an interaction between the MAPK pathways studied in regulation of cytokine production in the HD11 cells stimulated with *M. avium* strains. In this chapter, firstly the role of the ERK pathway in regulation of cytokine production was

examined followed by the roles of p38 and finally JNK in signal transduction of cytokine production in THP-1 and HD11 cells.

The role of ERK in regulation of pro-inflammatory cytokines by human macrophages has been studied previously (Hasan et al., 2003). As previously mentioned in this study, *M. avium* strains were able to stimulate human macrophages and induce the production of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine CXCL8 in these cells. The level of these immune effector molecules increased with time over the whole 24 h period post-infection. The role of each pro-inflammatory cytokine and chemokine examined in this study in innate immune response to mycobacterial species has been described in chapter 1. ERK plays an important role in the regulation of cytokine production. Production of pro-inflammatory cytokines (Chan and Riches, 2001) and NO (Lahti et al., 2000) in response to LPS in murine macrophages has been reported to be regulated by the ERK pathway. In human macrophages it has been reported that ERK activation, but not p38, is essential for regulation of the TNF-α in response to *M. avium* (Reiling et al., 2001) and *M. bovis* (Mendez-Samperio et al., 2004). In addition, recently Chen et al. (2015) found that TNF-α is regulated by the ERK pathway in human mesothelial cells infected with *M. tuberculosis* by showing reduction of production of this cytokine both at protein and mRNA levels following inhibition of ERK by PD98059.

Pre-treatment of THP-1 cells with the ERK pathway inhibitor, PD98059, reduced the level of *M. avium*-induced pro-inflammatory cytokines and chemokines. This indicates that ERK is an important

regulator of pro-inflammatory cytokine production in THP-1 cells. Differential TNF-α regulation has been reported by the ERK pathway in mice. Although Kim et al., (2004) reported that inhibition of the ERK pathway by PD98059 had no effect on TNF-α production in the murine macrophage cell line, J774A.1 in response to LPS, an essential role of this pathway in regulation of TNF-α production has been reported by another group showing a reduction of TNF-α in murine bone marrow-derived macrophages in response to LPS (Valledor et al., 2000). Roach and Schorey (2002), demonstrated a decrease in production of TNF-α in response to *M. avium* and *M. smegmatis* in bone marrow-derived macrophages treated with PD98059 (Roach and Schorey, 2002). In line with their study, we observed that the concentration of TNF-α showed a reduction in response to infection with *M. avium* in PD98059- treated THP-1 cells. In addition, we found that the level of other examined cytokines also reduced following treatment of the THP-1 cells with PD98059 suggesting that ERK plays an important role in mediation of intracellular signalling to the infection with *M. avium* strains.

It has been reported that the ERK pathway might have a differential role in regulation of cytokine production. Although several cytokines showed reduction in cells pre-treated with PD98059, in previous studies, some cytokines have been shown not to be affected by this inhibitor (Souza et al., 2007). Pre-treatment of bovine monocytes with PD98059 resulted in a significant reduction of TNF-α and IL-12 mRNA in response to *M. paratuberculosis* (Souza et al., 2007). Also, PD98059 treatment of human monocytes showed reduction in

CXCL8 in response to LPS stimulation but did not alter by *M. tuberculosis* infection (Ameixa and Friedland, 2002). However, production of IL-10 in response to *M. avium* or LPS stimulation did not change following treatment of human macrophages with PD98059 (Reiling et al., 2001). Similarly, this inflammatory cytokine showed no change in monocytes challenged with *M. paratuberculosis* following inhibition of the ERK pathway by PD98059 (Souza et al., 2007). Therefore, it seems that activation of the ERK pathway is essential for some cytokines such as TNF-α as a crucial pro-inflammatory cytokine, but is not necessary for others like IL-10 which is an important anti-inflammatory cytokine.

The effect of inhibition of MAPK pathways on intracellular survival of mycobacteria has been reported previously. Shiratsuchi et al. (2008) found that *M. avium-intracellulare* (MAI) multiplication significantly increased following inhibition of ERK by PD98059 indicating that the ERK pathway plays an important role in limitation of intracellular growth of MAI. However, inhibition of p38 does not effect on the bacterial replication (Shiratsuchi et al., 2008). However, multiplication of pathogenic MAI increased following inhibition of p38 pathway in murine bone-marrow-derived macrophages (Tse et al., 2002). In addition, Tse et al. (2002) also found that ERK inhibition only has an effect on the growth of non-pathogenic mycobacteria but does not alter the growth of pathogenic mycobacteria. In human monocyte-derived macrophages it has been found that multiplication of MAI is not affected by inhibition of p38 while replication of pathogenic MAI increased

following ERK inhibition (Blumenthal et al., 2002). This controversial role of p38 in regulation of mycobacterial multiplication in mice and humans could indicate that the pathogenic mechanism of MAI might be different in these two species (Shiratsuchi et al., 2008). In addition, in human neutrophils, increased survival of bacteria and fungi has been reported following inhibition of the ERK pathway (Hii et al., 1999; Feng et al., 1999). Shiratsuchi et al. (2008) suggested that pathogenic MAI could limit the ability of human monocytes in controlling their replication through inhibition of ERK activation. However, the ability of pathogenic smooth-transparent morphotypes of MAI in reduction of p38 pathway activation does not affect the replication of these bacteria intracellularly (Shiratsuchi et al., 2008). In murine macrophages, it has been found that inhibition of the ERK pathway by PD98059 promoted intracellular replication of *M. avium* whereas their replication is reduced following inhibition of p38 by SB203580, suggesting that for control of the bacterial multiplication a proper balance of MAPK activation is needed (Klug et al., 2011). However, this regulation of intracellular replication was independent of endogenous TNF-α and IL-10 (Klug et al., 2011).

The type of stimulus might also be an important factor in regulation of cytokine production by ERK activation. Ameixa and Friedland, (2002) reported that neither inhibition of ERK by PD98059 nor inhibition of p38 by SB203580 had an effect on production of CXCL8 by human monocytes in response to infection with *M. tuberculosis*, while, in contrast, the level of this cytokine reduced following treatment of the cells with PD98059 in response to stimulation with

LPS (Ameixa and Friedland, 2002). It has been argued that protein tyrosine kinases could regulate the mechanism of CXCL8 expression both pre- and post-transcriptionally (Ameixa and Friedland, 2002). In the current study, we observed that, following inhibition of the ERK pathway, the expression of all the cytokines examined was blocked in these cells in response to *M. avium* infection. However, these cells still expressed higher levels of cytokines when stimulated with LPS compared to untreated control HD11 cells. Thus, involvement of MAPK pathways in regulation of cytokine production could be dependent on the type of stimulus. This hypothesis has also been supported by some other researchers. Chan and Riches, (2001) mentioned induction of iNOS by IFN-γ plus LPS in murine macrophages cell line modulated by all members of the MAPK pathway family, namely the ERK, JNK and p38 pathways (Chan and Riches, 2001). The same group found that ERK and JNK but not p38 play roles in mediation of iNOS production by a murine macrophage cell line following stimulation by IFN-γ and LAM of *M. tuberculosis* (Chan et al., 2001). In the present study, we found that all MAPK members play a role in regulation of iNOS expression in HD11 cells stimulated with *M. avium*. The type and nature of stimulus could thus be important in mediation of expression of iNOS. It would be interesting to investigate whether this stimulant-dependent regulation of iNOS applies to pro-inflammatory cytokines and chemokines in response to other mycobacterial species.

It has been reported that ligation with TLR2 of PIM isolated from *M. tuberculosis* and AraLAM isolated from avirulent mycobacteria could

lead to activation of ERK1/2 pathway and subsequently phosphorylation and activation of the NFκB and AP1 transcriptional factors in the RAW264.7 murine macrophage cell line (Jones et al., 2001a). In addition, in human monocytes, a 38KDa glycolipoprotein from *M. tuberculosis* H37Rv can activate the ERK and p38 pathways and induce TNF-α and IL-6 production through TLR2 and TLR4 (Jung et al., 2006). Further, Chan and his colleagues demonstrated that ManLAM from *M. tuberculosis* induces production of iNOS and NO through activation of the ERK and JNK pathways in Raw 264.7 cells (Chan et al., 2001). It has been suggested that most of the stimuli that activate p38 might also activate the JNK pathway (Roux and Blenis, 2004). However, the precise interaction between mycobacterial components and the type of signaling pathway is unclear and it is not understood which pathway is activated by single mycobacterial component. Information on *M. tuberculosis* infection indicates no clear pathway of signal transduction from stimuli to response for any particular stimulus.

To further validate the results from ERK inhibition by PD98059, we incubated the cells with a second well-known inhibitor of this pathway, U0126. Similar to data generated by use of PD98059, we found that all cytokines assessed, except IL-1β at 6 h post infection, showed significant reductions in THP-1 cells in response to *M. avium* strains compared to cells stimulated with *M. avium* alone. U0126 has previously been reported to inhibit production of TNF-α in response to *M. avium* (Bhattacharyya et al., 2002). In addition, human monocytes treated with the ERK inhibitor, U0126, also

showed inhibition of IL-1, CXCL8, TNF and prostaglandin E2 in response to LPS (Scherle et al., 1998). The difference observed in this study in IL-1β production in cells treated with PD98059 and U0126 could be due to the difference in their target site of inhibition. Although many similarities have been reported between the mechanisms of action of these inhibitors in suppression of ERK activation, there are some differences between them. For example, PD98059 is able to inhibit cyclo-oxygenases 1 and 2, enzymes (Borsch-Haubold et al., 1998) which function limits production of prostaglandin and leukotrienes, while U0126 does not inhibit these enzymes (Davies et al., 2000). Therefore, it would be interesting to know if there is any difference between the mechanisms by which they exert their effect on the process of production of a specific cytokine in the immune response to mycobacterial infection.

In the present study, treatment of chicken macrophage-like cells with ERK inhibitors showed significant reductions of all cytokines and chemokines tested following stimulation with *M. avium* strains. Kogut et al. (2008) assessed the role of the ERK pathway in cytokine expression in chicken heterophils in response to LPS and flagellin. They found that the levels of IL-6 and CXCLi2 expression were eliminated in chicken heterophils stimulated with LPS or flagellin following inhibition of ERK by either of its inhibitors PD98059 or U0126 (Kogut et al., 2008). It has also been reported that NO production in response to LPS diminished after inhibition of the ERK pathway with U0126 (He and Kogut, 2003). Our data showing blockage of cytokine and chemokine production following

ERK inhibition are consistent with previous studies in chicken cells suggesting a crucial role for this pathway in regulating signal transduction of cytokine gene expression in avian cells when infected with *M. avium* strains.

To evaluate the role of the p38 MAPK in regulation of cytokine production we observed that in SB203580-treated THP-1 cells, there was a variation among the cytokines produced in response to *M. avium* infection. Although IL-6 and CXCL8 were reduced significantly, significant increases were observed in the levels of TNF-α and IL-1β in response to some *M. avium* strains compared to the cells treated with *M. avium* alone. In accordance with our data, Kim et al. (2004) showed that TNF-α production by murine macrophages increased in response to LPS after the cells were treated with SB203580 (Kim et al., 2004). Similarly, an increase in the level of TNF-α has also been reported in *M. avium*-stimulated murine macrophages following treatment with SB203580 (Bhattacharyya et al., 2002). In line with these studies, and also the study by Blumenthal et al., (2002) which found an enhancement in the level of TNF-α in human macrophages infected with *M. avium* following treatment of the cells with SB203580 (Blumenthal et al., 2002), we observed an increase in the level of TNF-α and IL-1β in SB203580-treated THP-1 cells in response to LPS and *M. avium* while the levels of these cytokines were significantly reduced by inhibition of the ERK1/2 pathway. Our data, therefore suggest a negative role for p38 in regulation of TNF-α and IL-1β production in THP-1 cells. In contrast, some studies found that p38 has a role in

mediating TNF-α production in murine macrophages in response to LPS and IFN-γ (Ajizian et al., 1999) and human monocytes stimulated with LPS (Wang et al., 1999). This could be because Wang et al., (1999) used a different chemical, SB202190, and in a very low concentration, 3μM, to inhibit the p38 pathway. A significant increase of ERK phosphorylation has been reported in murine macrophages infected with *M. smegmatis* or *M. avium* pre-treated with the p38 pathway inhibitor, SB203580 (Bhattacharyya et al., 2002, Roach and Schorey, 2002). Therefore, cross-talk between ERK and p38 MAPK pathways might be suggested, which has previously been observed in bufalin-treated THP-1 cells (Kurosawa et al., 2000), and in endothelial cells of human umbilical vein stimulated with IL-1α (Houliston et al., 2001) and in murine bone marrow-derived macrophages in response to mycobacterial species (Roach and Schorey, 2002). The cross-talk and increased phosphorylation of ERK in SB203580-treated cells could be the reason that our experiment, using SB203580, resulted in an increase in the production of TNF-α and IL-1β at the level of protein in THP-1 cells.

Moreover, our findings that ERK and p38 pathways may play a differential role in regulation of a specific cytokine, corroborates data found by Feng et al., (1999). They found that induction of iNOS and IL-12 in macrophages by LPS might be differentially regulated by the ERK and p38 pathways (Feng et al., 1999). In their study, ERK suppressed IL-12 induction and played a minor role in iNOS expression, whereas p38 promoted IL-12 and was essential for iNOS

expression. So it would be worth investigating the relative role of each MAPK pathway in regulation of individual cytokines in response to other mycobacterial species.

We observed a difference between THP-1 and HD11 cells in IL-1β production following inhibition of the p38 pathway in response to *M. avium* infection. The differences in cytokine production have been reported previously with cells of the monocyte lineage (Tudhope et al., 2008). They reported that a p38 inhibitor was observed to be more effective in LPS-stimulated release of TNF-α in monocytes than in monocyte-derived macrophages and lung macrophages. In addition, inhibition of ERK by PD98059 resulted in greater reduction of TNF-α and IL-6 in monocyte-derived macrophages and alveolar macrophages than monocytes stimulated with LPS (Tudhope et al., 2008). Similarly, Means et al., (2000) found that following treatment of cells with PD98059, TNF-α gene expression was blocked in the murine alveolar macrophage cell line, C-8, in response to LPS stimulation but not in the non-pulmonary cell line, RAW264.7. In addition, this difference has also been observed in murine primary alveolar macrophages and peritoneal macrophages (Means et al., 2000). This indicates that regulation of cytokine production by a specific signalling pathway could be dependent upon the type of cell involved. More work is needed to be done to investigate if regulation of cytokine production follows different mechanisms in various cell types or different kinases are involved in mediation of this process among various types of cells.

JNK1, JNK2 and JNK3 are three well known genes of the JNK pathway (Gupta et al., 1996). The first two are expressed abundantly throughout the body while the other one is only expressed in certain organs such as heart, brain and testis (Utsugi et al., 2010). Impairment of T cell function has been detected in mice with deficiency in JNK1 and JNK2 (Yang et al., 1998, Dong et al., 1998). We found that inhibition of the JNK pathway by SP600125 resulted in reduction of some cytokines and chemokines examined in this study in both THP-1 and HD11 cells.

It has been reported that inhibition of the JNK pathway by specific inhibitors resulted in reduction of all the following cytokines, TNF-α, IL-1α, IL-1β, IL-6, granulocyte monocyte colony stimulating factor (GM-CSF) and NO production in murine alveolar macrophages in response to *M. immunogenum* 700506 infection (Chandra et al., 2013). In line with this study, we observed a reduction in production of all the cytokines examined, with the exception of IL-1β and CXCL8 at 24 h in THP-1 cells following their treatment with SP600125, suggesting an important role for the JNK pathway in regulation of cytokine production in these cells in response to challenge with *M. avium.*

Recently Chen et al., (2012) demonstrated that the level of TNF-α was significantly reduced in RAW264.7 cells infected with recombinant MPT83, derived from *M. tuberculosis*, following treatment with a JNK inhibitor but was partially reduced with ERK and p38 inhibitors. In addition, they observed that the JNK inhibitor showed pronounced inhibition on IL-6 and IL-12p40 while these

cytokines were not affected or only partially affected by use of PD98059 and SB203580, respectively (Chen et al., 2012). In the current study, we found that all cytokines under study were not affected similarly by inhibition of the p38 or JNK pathways. Therefore, it can be suggested that various cytokines could be differentially regulated by each of the MAPK signalling pathways. It has also been observed that p38 inhibition can lead to an increase in expression of IL-12 in murine macrophages while it reduced TNF-α production in these cells (Yang et al., 2010). Moreover, Utsugi et al. (2003) reported that the JNK pathway has a negative role in regulation of IL-12 induction by LPS in human macrophages. Overall, our findings are consistent with these data and support the concept that each cytokine may be differentially regulated by a different MAPK pathway. So, in the current study we found that U0126 inhibited TNF-α but had no impact on IL-1β production in THP-1 cells in response to *M. avium* challenge. In addition, pre-treatment of THP-1 cells with SB203580 resulted in increased levels of TNF-α and IL-1β in response to *M. avium* infection while IL-6 and CXCL8 decreased in these cells. Furthermore, production of IL-1β and CXCL8 at 24 h post-infection was not affected by JNK inhibition with SP600125. Taken together, our data indicate that production of each cytokine in response to *M. avium* infection may be differentially regulated in the context of the MAPK signalling pathway involved. It would be interesting to investigate if this differential regulation of cytokines in response to *M. avium* infection, could also been observable in infection with other mycobacterial species.

It seems that despite the complexity in the structure of the pathways, several signalling components are shared between the MAPK pathway subfamilies. In addition, it is suggested that regulation of TNF-α or IL-1β production does not follow a common pathway, but is dependent on type of strain, cell and stimulus.

In the current study, the level of IL-1β in THP-1 cells increased following inhibition of the p38 pathway by SB203580 while, in HD11 cells this pro-inflammatory cytokine was reduced by SB203580 in response to *M. avium* infection. So, it can be suggested that signalling events are significantly different in avian and human cells following *M. avium* infection in terms of signalling transduction regulated by p38 pathway. In addition, the results of the MAPK pathway experiments in this study suggest that ERK, p38 and JNK simultaneously regulate expression of cytokines in HD11 cells. In THP-1 cells, however, although activation of p38 is crucial in mediation of IL-6 and CXCL8, this pathway might have negative effect on TNF-α and IL-1β production in response to infection with *M. avium* strains. We also demonstrated that PD98059 is a stronger inhibitor of the ERK pathway in *M. avium*-induced cytokine production than U0126. So, it can also be suggested that the role of MAPK signalling pathways in regulation of cytokine production may be dependent on the cytokine examined and can vary when different steps of the pathway are inhibited and this could be observed even in same type of cells. Furthermore, a more detailed investigation of signalling pathways based on individual mycobacterial species is

needed in order to evaluate intracellular signal transduction specific to each species.

5 Cytokine gene expression profile and histopathological changes in chickens infected with *M. avium* strains

5.1 Introduction

To complement our work *in vitro*, we turned to the *in vivo* infection model to evaluate the ability of the *M. avium* strains to induce cytokine production in chickens.

The results of previous chapters using human and chickens cells (THP-1 and HD11) indicated a differential immune response between the different *M. avium* strains used in this study with Ma.3 as the lowest inducer of cytokine production and Ma.4 and Ma.5 as higher inducers of cytokine in both cell models. These three strains were used to inoculate chickens to evaluate the immune response of the chickens against these strains. It was therefore important to confirm whether the difference observed *in vitro* was also reflected in the *in vivo* response following infection of chickens.

5.2 Results

5.2.1 Colonisation of chicken liver and spleen following *M. avium* infection

The colonisation of the bacteria in blood, liver and spleen of infected chickens were determined by measurement of the number of CFU isolated from these organs at various time points following IV inoculation of the birds with the *M. avium* strains. On the day of bacterial inoculation, bacteria were found in the spleen and liver just 30 min post-inoculation

5.2.1.1 Liver

There was a significant difference between the number of bacteria isolated from the livers of chickens infected with Ma.3 at 30 min post-inoculation compared to Ma.4-infected birds ($P<0.001$) with very few bacteria of the latter strain enumerated at all. Also, chickens infected with Ma.5 showed a significantly higher number of CFU in their liver compared with Ma.4-infected chickens ($P<0.05$) at 30 min post-inoculation. The load of Ma.3 was reduced significantly ($P<0.01$) at 7 days post-inoculation in comparison to those at 30 min post-inoculation. By 21 days post-inoculation, the bacterial load of all strains reached its lowest level in liver samples from the infected birds (Figure 5.1).

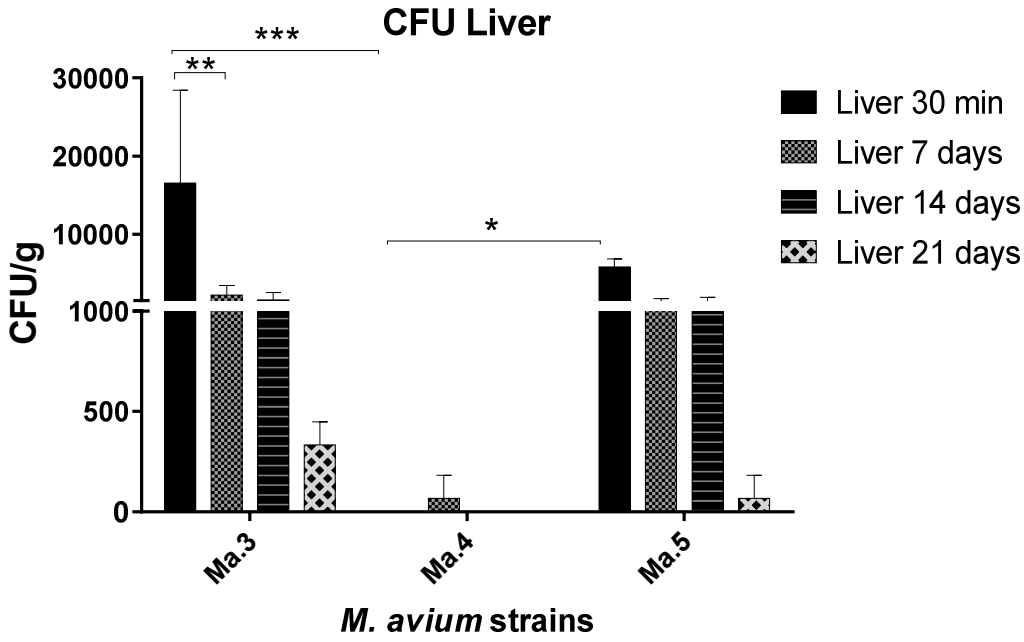


Figure 5.1: Number of CFU determined in liver of chickens following infection with *M. avium* strains.

Chickens were infected intravenously with $1x10^5$ per chicken of *M. avium* strains and three chickens from each group euthanized at different times post-infection. Liver samples were taken for bacteriological examination. Numbers of bacteria detected in liver at different time points (30 min, 7, 14 and 21 days post-infection). Each time point shows average ±SD of CFU/g from three chickens. Asterisks (*) and (***) refer to significant differences ($P<0.05$ and $P<0.001$ respectively) between CFU numbers at 30 min compared to Ma4-infected chickens and (**) denotes significant differences between CFU at 30 min compared to 7 days post-infection. Two-Way ANOVA was used to determine any significant differences between the groups.

5.2.1.2 Spleen

The number of CFU from the spleens of Ma.3-infected chickens at 30 min post-inoculation was significantly higher than that from Ma.4-infected chickens ($P<0.001$). Chickens infected with Ma.5 showed a significantly higher number of CFU in their spleen compared with Ma.4-infected chickens ($P<0.05$) at 30 min post-inoculation. The load of Ma.3 was reduced significantly ($P<0.01$) at 7 days post-inoculation in comparison to those at 30 min post-inoculation. By 21

days post-inoculation, the bacterial load of all strains reached its lowest level in spleen samples from the infected birds (Figure 5.2). No bacteria were recovered from blood samples at any time points post-infection.

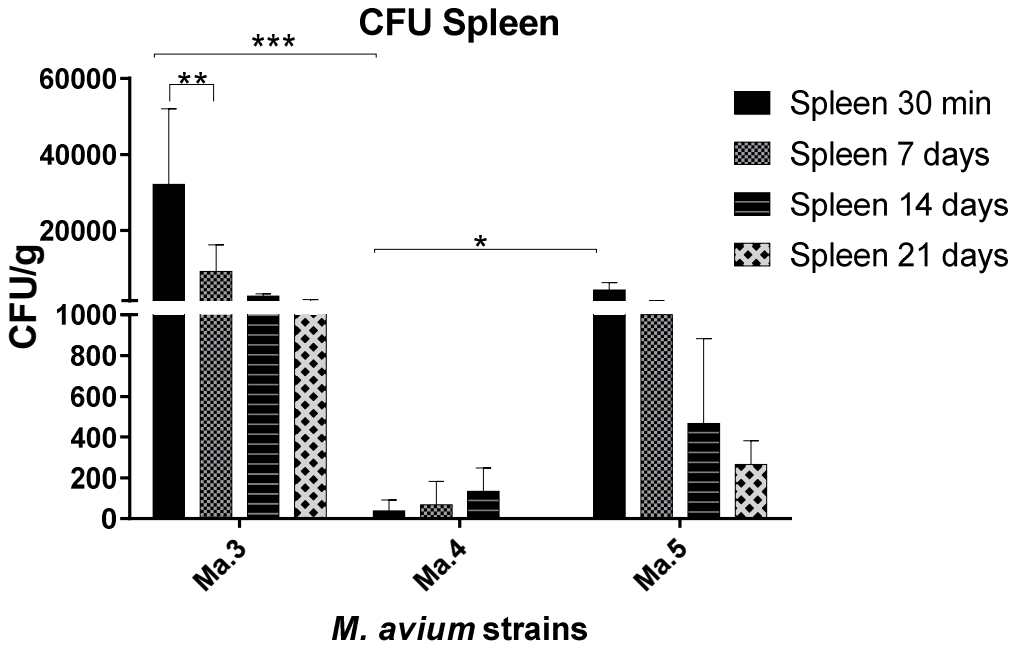


Figure 5.2: Number of CFU determined in spleen of chickens following infection with *M. avium* strains.

Chickens were infected intravenously with $1x10^5$ per chicken of *M. avium* strains and three chickens from each group euthanized at different times post-infection. Spleen samples were taken for bacteriological examination. Numbers of bacteria detected in spleen at different time points (30 min, 7, 14 and 21 days post-infection). Each time point shows average ±SD of CFU/g from three chickens. Asterisks (*) and (***) refer to significant differences ($P<0.05$ and $P<0.001$ respectively) between CFU numbers at 30 min compared to Ma4-infected chickens and (**) denotes significant differences between CFU at 30 min compared to 7 days post-infection. Two-Way ANOVA was used to determine any significant differences between the groups.

5.2.2 Gene expression profile of chicken liver samples after experimental infection with *M. avium* strains

The profile of mRNA gene expression of selected cytokines and chemokines from the livers was determined using qRT-PCR. All Ct

values have been standardized and corrected for RNA input level variation using 28S rRNA as a reference gene. The corrected Ct values were deducted from 40 (the negative end point) such that greater Ct values indicate greater expressed cytokine mRNA levels.

5.2.2.1 30 min and 7 days post-infection

We found that there were no significant differences between the levels of all tested cytokine gene expression at both 30 min and 7 days post-infection in the livers of *M. avium*-infected chickens in comparison with non-infected birds as shown in (Figure 5.3, Figure 5.4, Figure 5.5 and Figure 5.6).

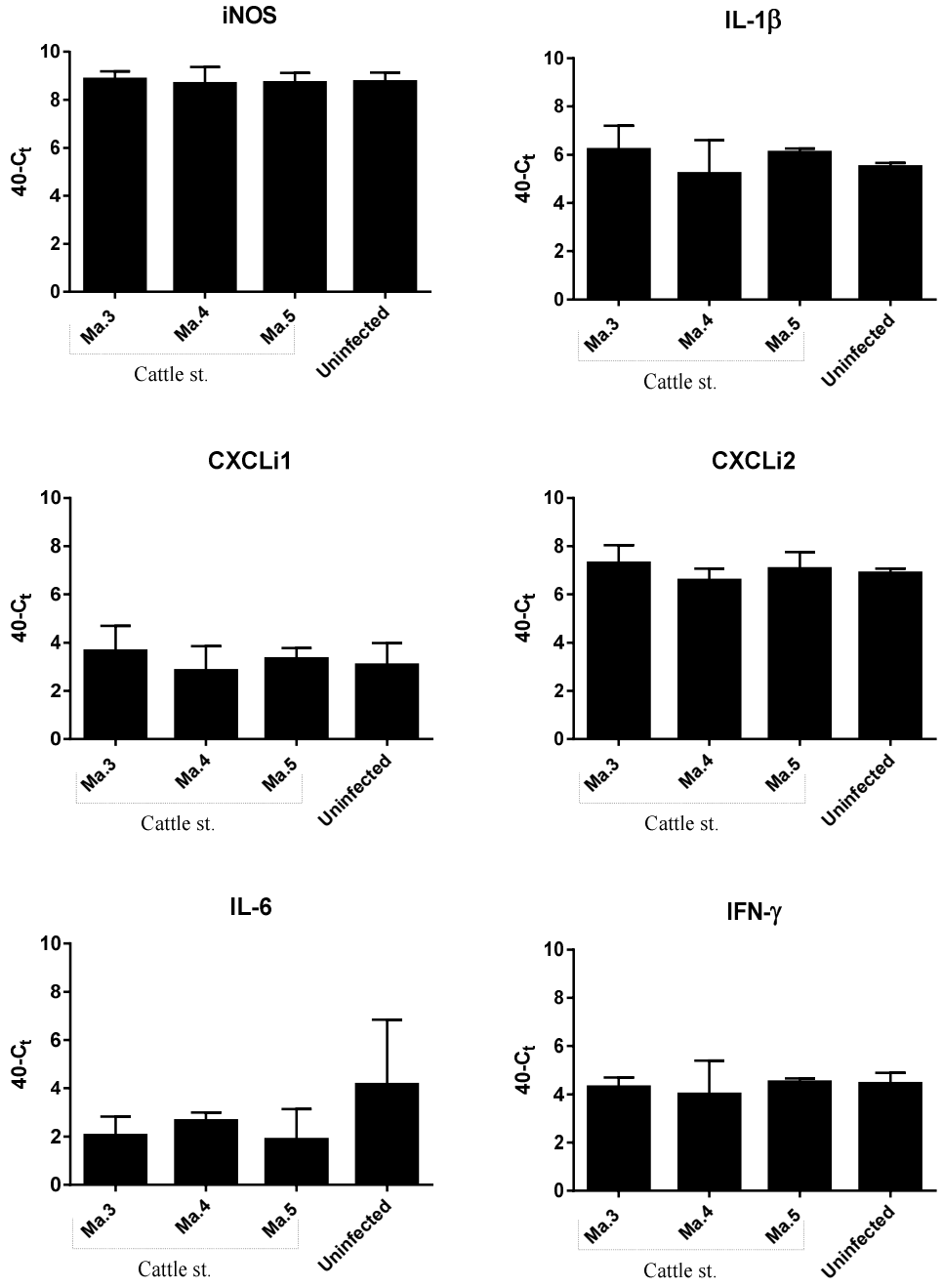


Figure 5.3: Cytokine and chemokine gene expression profile in the liver of chickens at the day of infection (30 min following inoculation) with *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 30 min after infection. The levels of cytokine expression in liver were quantified using qRT-PCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

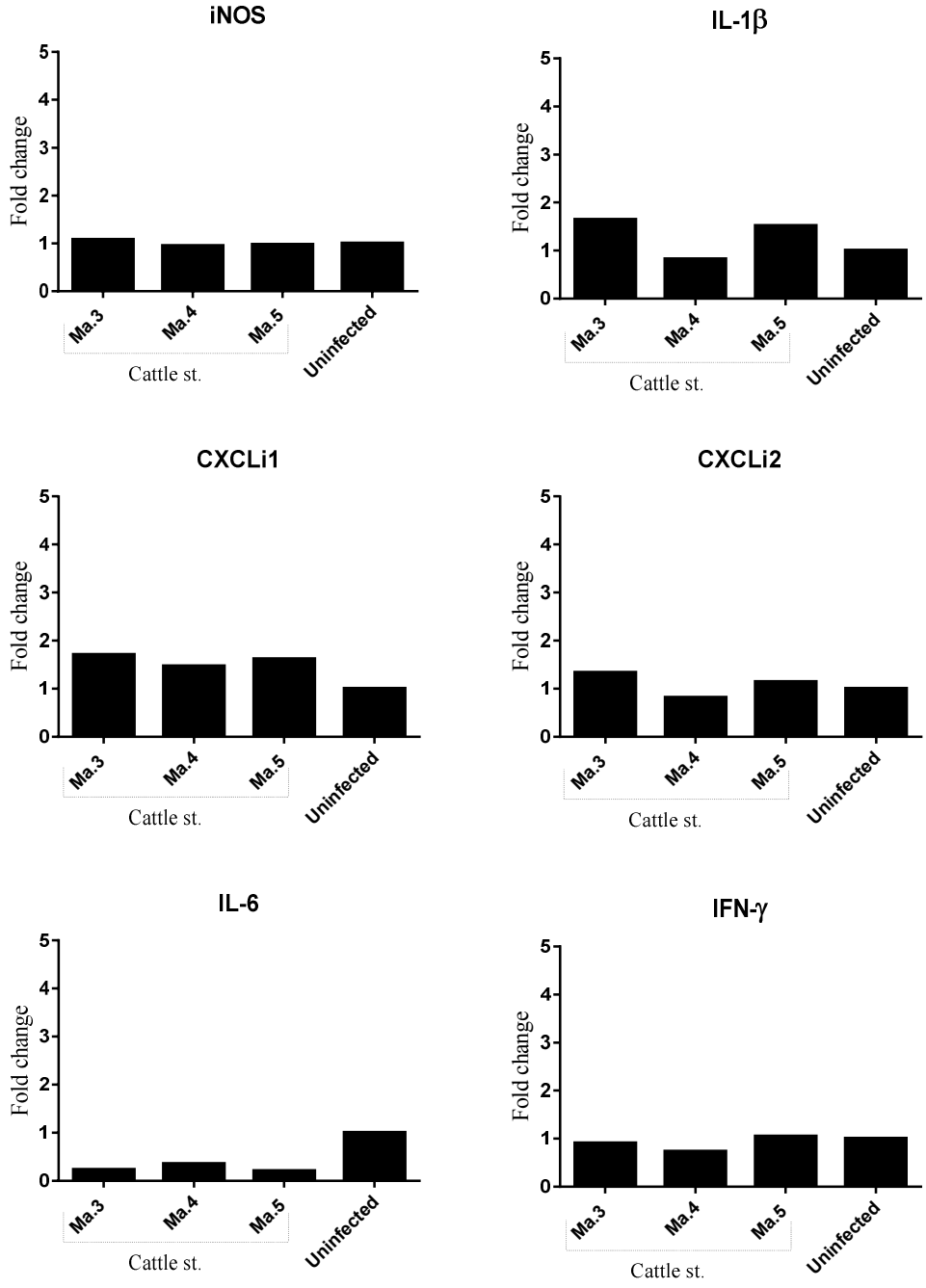


Figure 5.4: Cytokine and chemokine gene expression profile in the liver of chickens at the day of infection (30 min following inoculation) with *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 30 min after infection. The levels of cytokine expression in liver were quantified using qRT-PCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from the uninfected group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

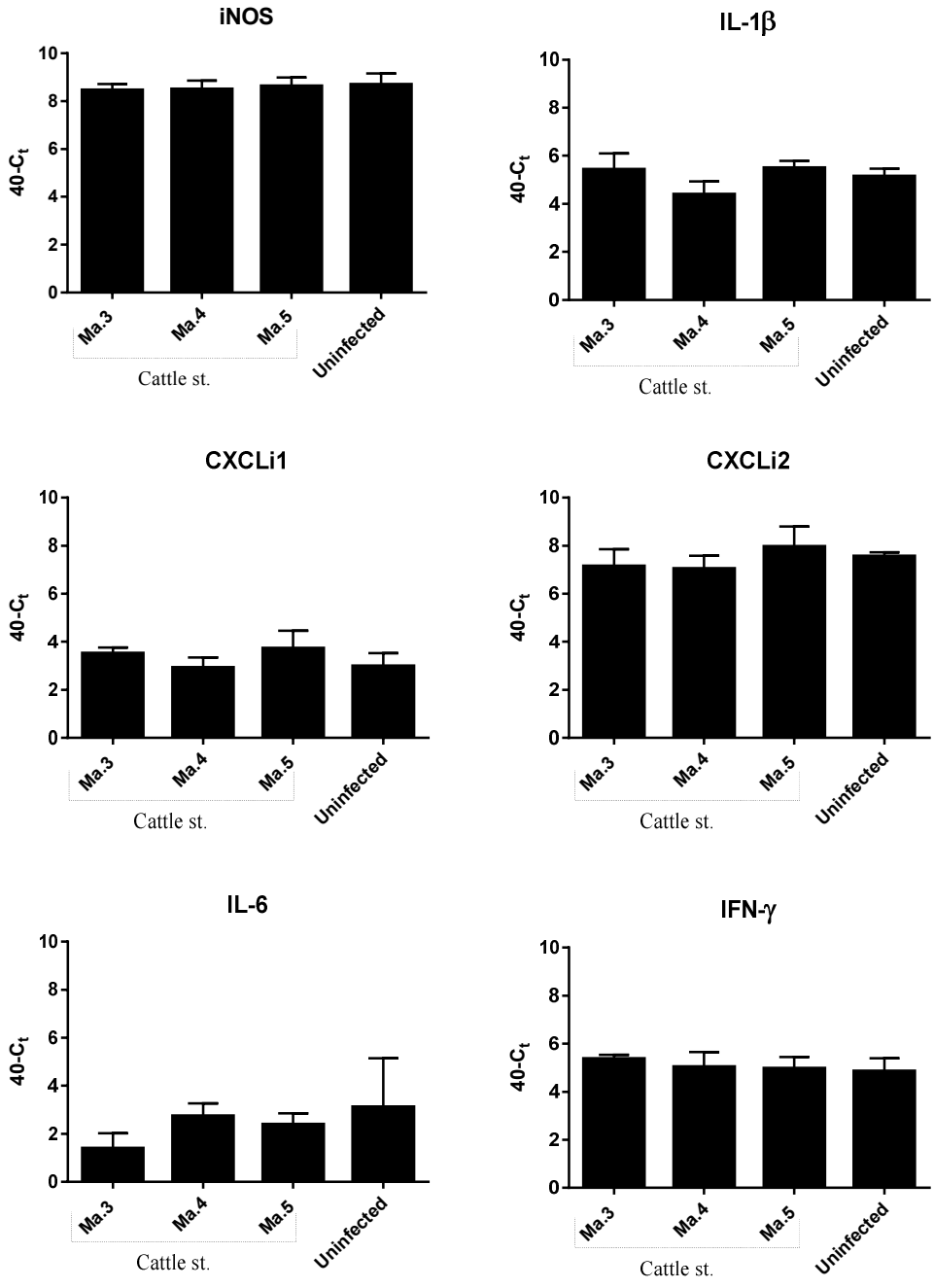


Figure 5.5: Cytokine and chemokine gene expression profile in the liver of chickens 7 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 7 days post infection. The levels of cytokine expression in liver quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

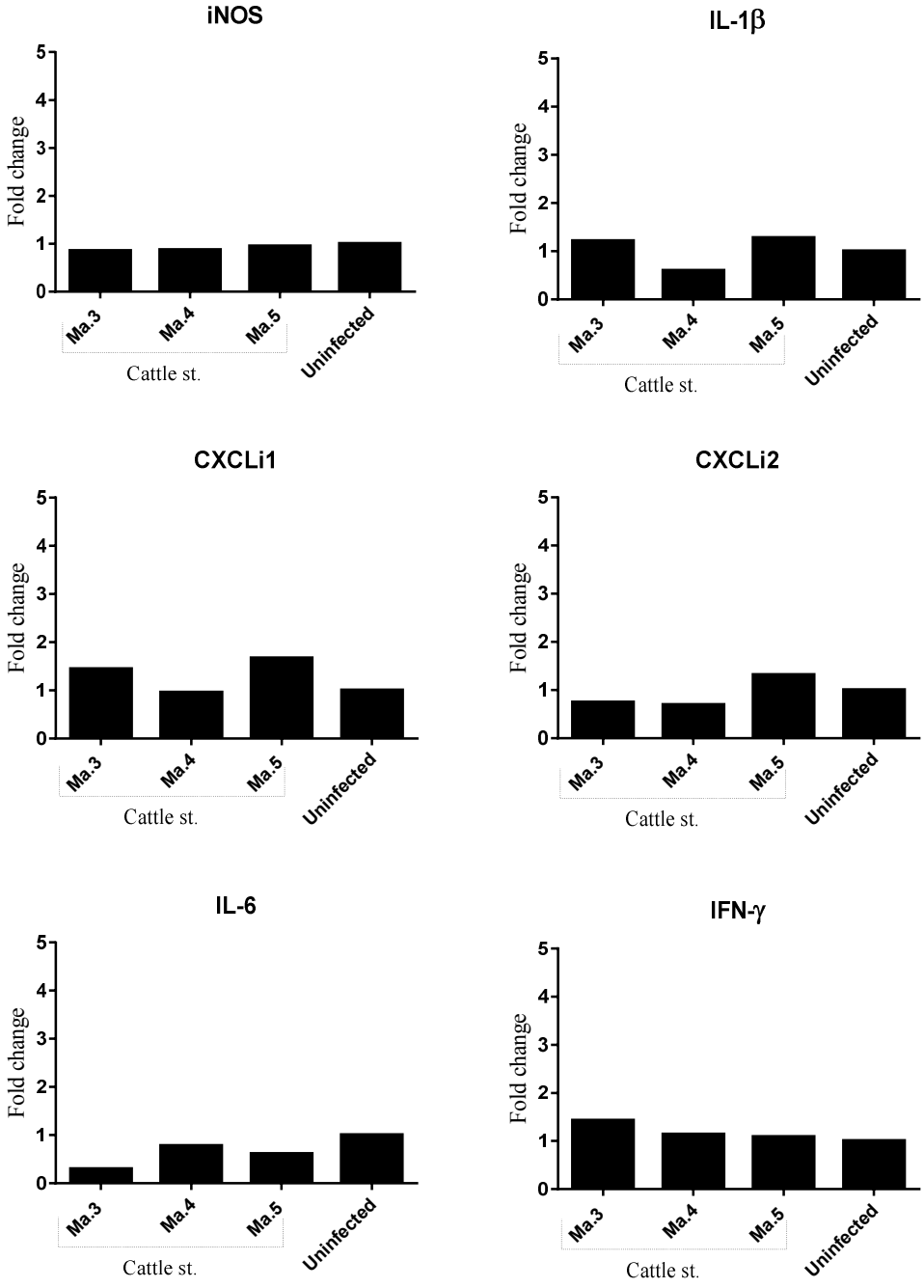


Figure 5.6: Cytokine and chemokine gene expression profile in the liver of chickens 7 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 7 days post infection. The levels of cytokine expression in liver quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected chicken group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.2.2 14 days post-infection

However, as shown in Figure 1.7-1.8 at 14 days post-infection there was a significant ($P<0.05$) increase in expression at the mRNA level of IFN-γ, in chickens infected with *M. avium* strains (<2.38, <1.94 and <2.15 fold changes for Ma.3, Ma.4 and Ma.5, respectively) in comparison with uninfected birds. This significant increase was not observed with the other cytokines at this time point (Figure 5.7 and Figure 5.8).

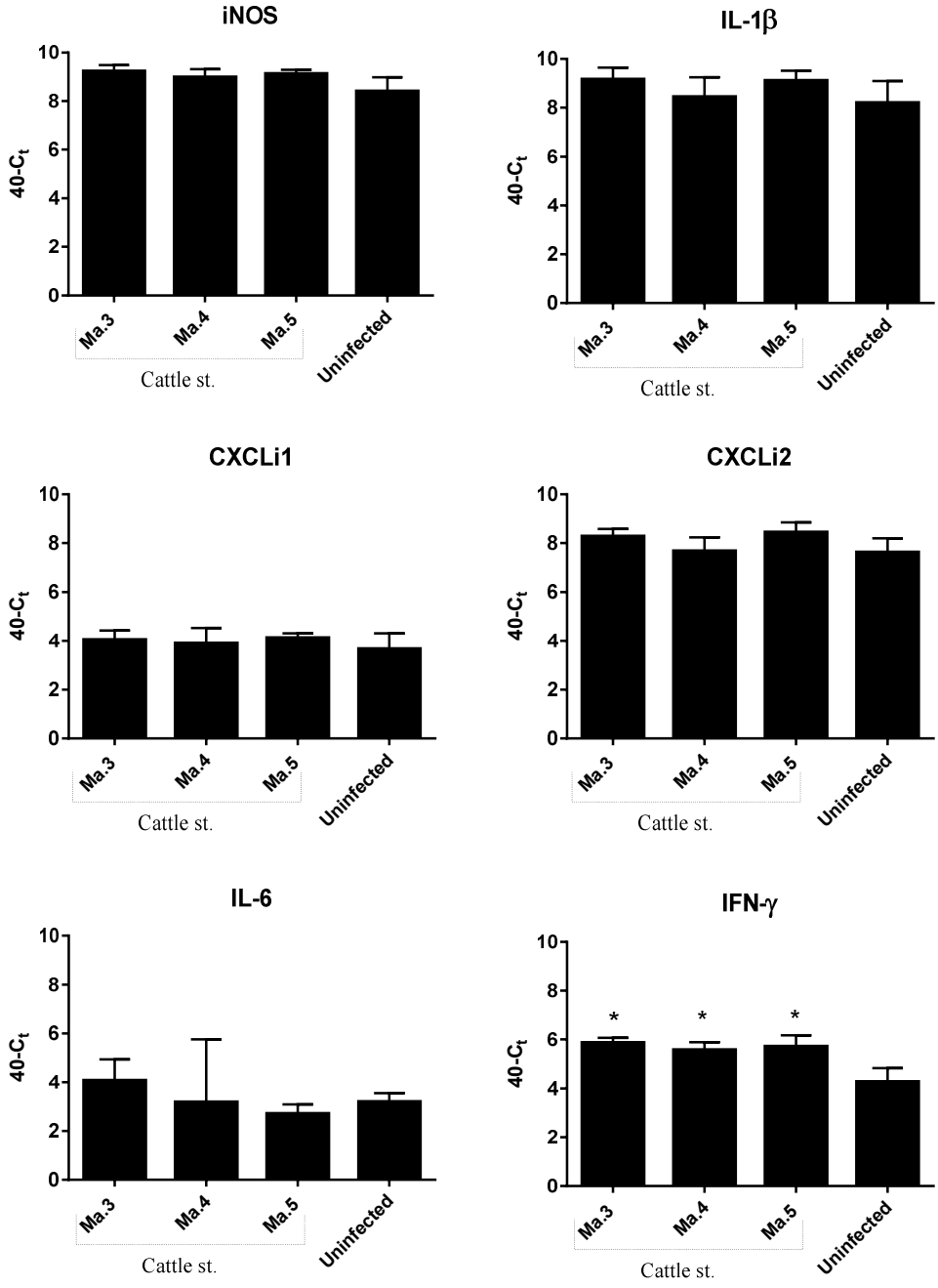


Figure 5.7: Cytokine and chemokine gene expression profile in the liver of chickens 14 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 14 days post infection. The levels of cytokine expression in liver quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. Asterisk (*) refers to significant differences ($P<0.05$) in *M. avium*-infected chickens compared to uninfected group. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

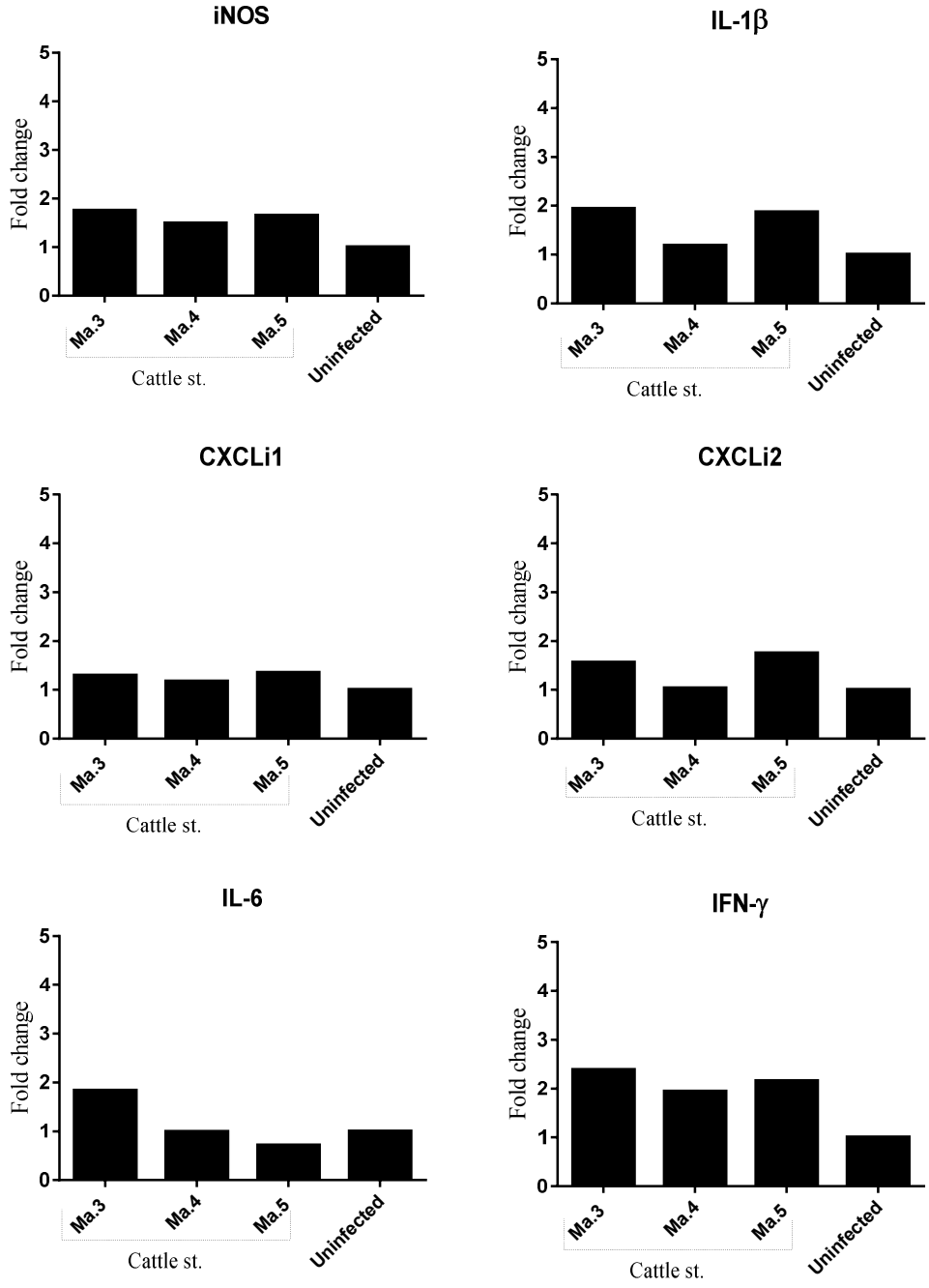


Figure 5.8: Cytokine and chemokine gene expression profile in the liver of chickens 14 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 14 days post infection. The levels of cytokine expression in liver quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected chicken group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.2.3 21 days post-infection

Furthermore, we also detected a significant up-regulation in mRNA level of all cytokines including iNOS (<2.72 fold change), IL-1β (< 7.04 fold change), CXCLi2 (<2.31 fold change), IL-6 (<4.23 fold change) and IFN-γ (<4.38 fold change) with the exception of CXCLi1 in liver samples from chickens infected with Ma.5, compared to non-infected group at 21 days post-infection ($P<0.05$) (Figure 5.9 and 5.10). Moreover, expression of IL-1β (<9.34 fold change) and CXCLi2 (<2.66 fold change) was significantly higher in liver of Ma.4-infected chickens and IL-1β (<6.45 fold change) in Ma.3-infected chickens compared to non-infected birds ($P<0.05$). However, no significant differences were observed between the different infected groups at any time point post-inoculation in terms of mRNA level of all the tested cytokine (Figure 5.9 and Figure 5.10).

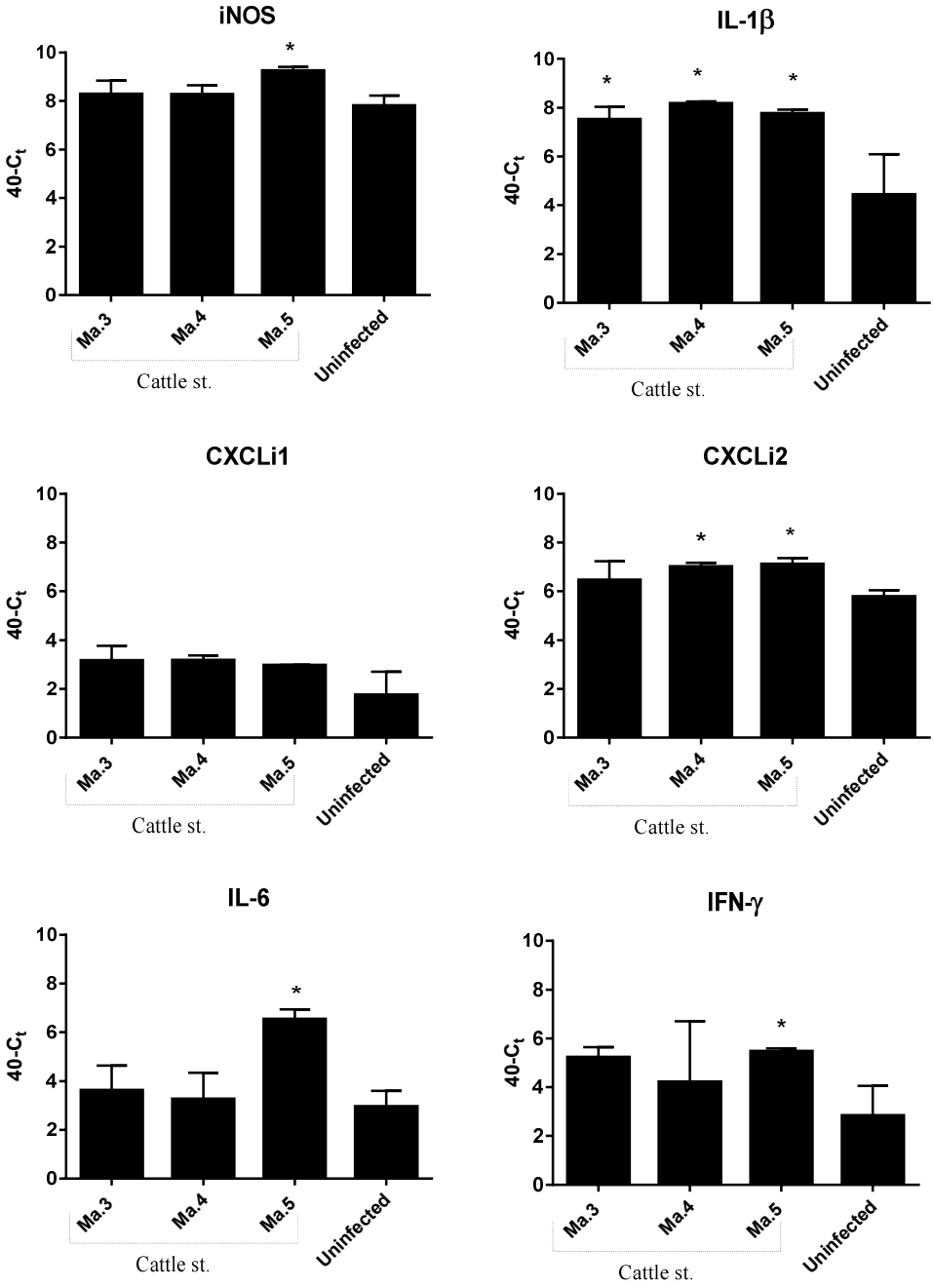


Figure 5.9: Cytokine and chemokine gene expression profile in the liver of chickens 21 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 21 days post-infection. The levels of cytokine expression in liver quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. Asterisk (*) refers to significant differences ($P<0.05$) in *M. avium* strains-infected chickens compared to uninfected group. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

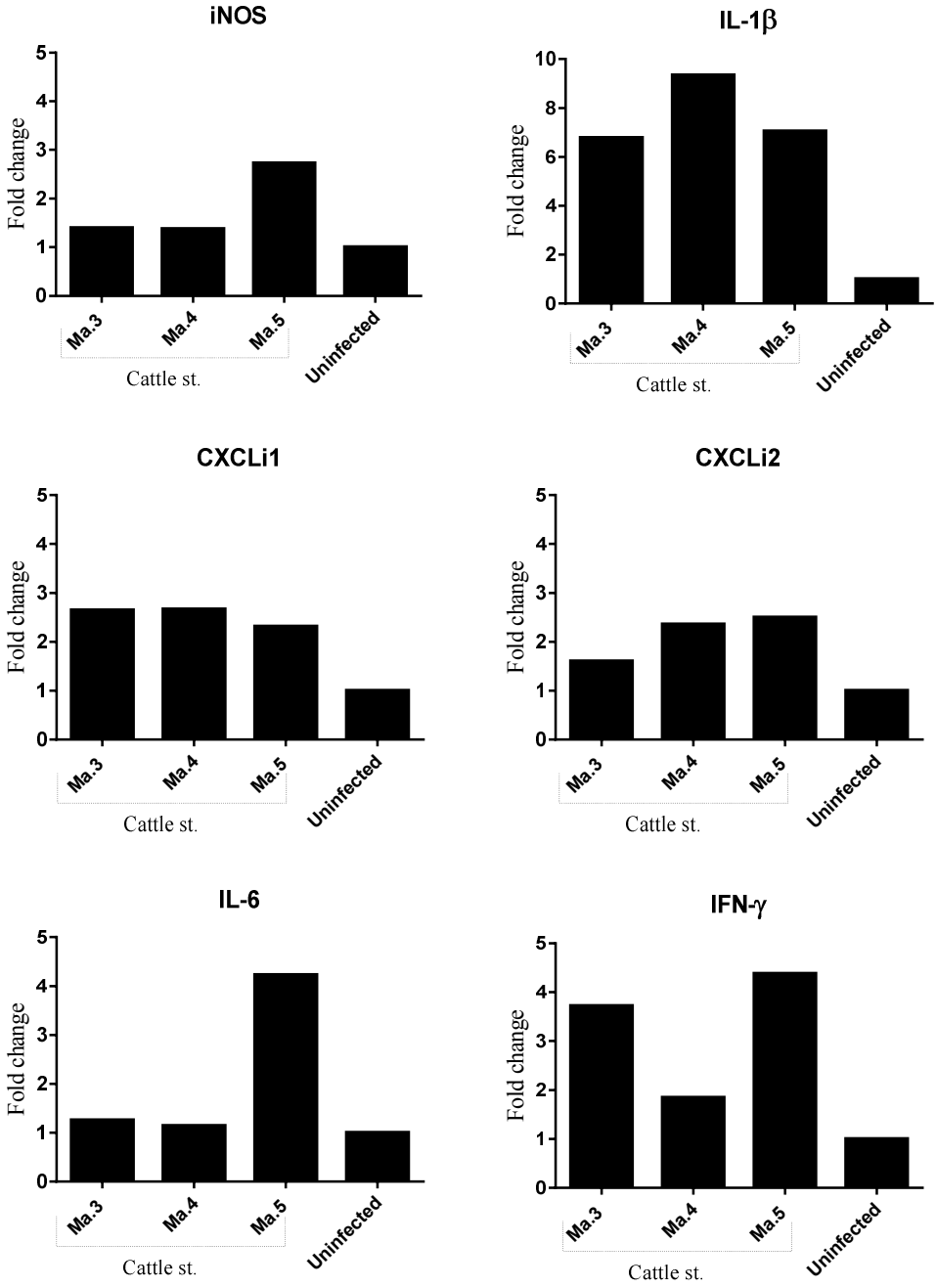


Figure 5.10: Cytokine and chemokine gene expression profile in the liver of chickens 21 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 21 days post-infection. The levels of cytokine expression in liver quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected chicken group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.3 Gene expression profile of chickens spleen samples after experimental infection with *M. avium* strains

The levels of cytokine and chemokine gene expression in the spleen of *M. avium*-infected birds were also quantified at different time post-infection using qRT-PCR.

5.2.3.1 30 min and 7 days post-infection

Similar to the findings from liver samples at both 0 and 7 days following infection, no significant changes were observed in gene expression in any of the *M. avium*-infected chicken groups in comparison with non-infected chickens (Figure 5.11, Figure 5.12, Figure 5.13 and Figure 5.14).

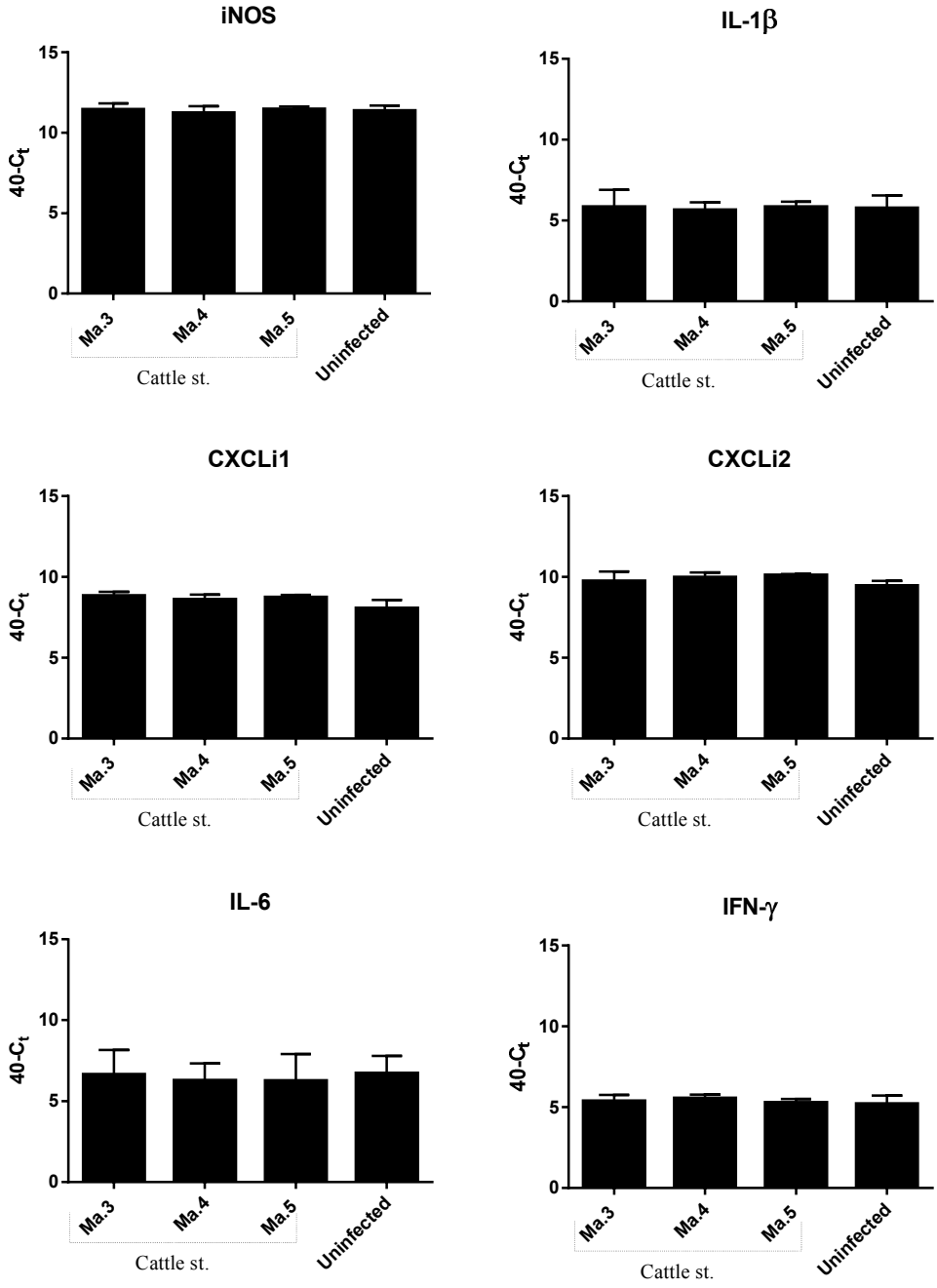


Figure 5.11: Cytokine and chemokine gene expression profile in the spleen of chickens at the day of infection (30 min following inoculation) with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed 30 min after infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

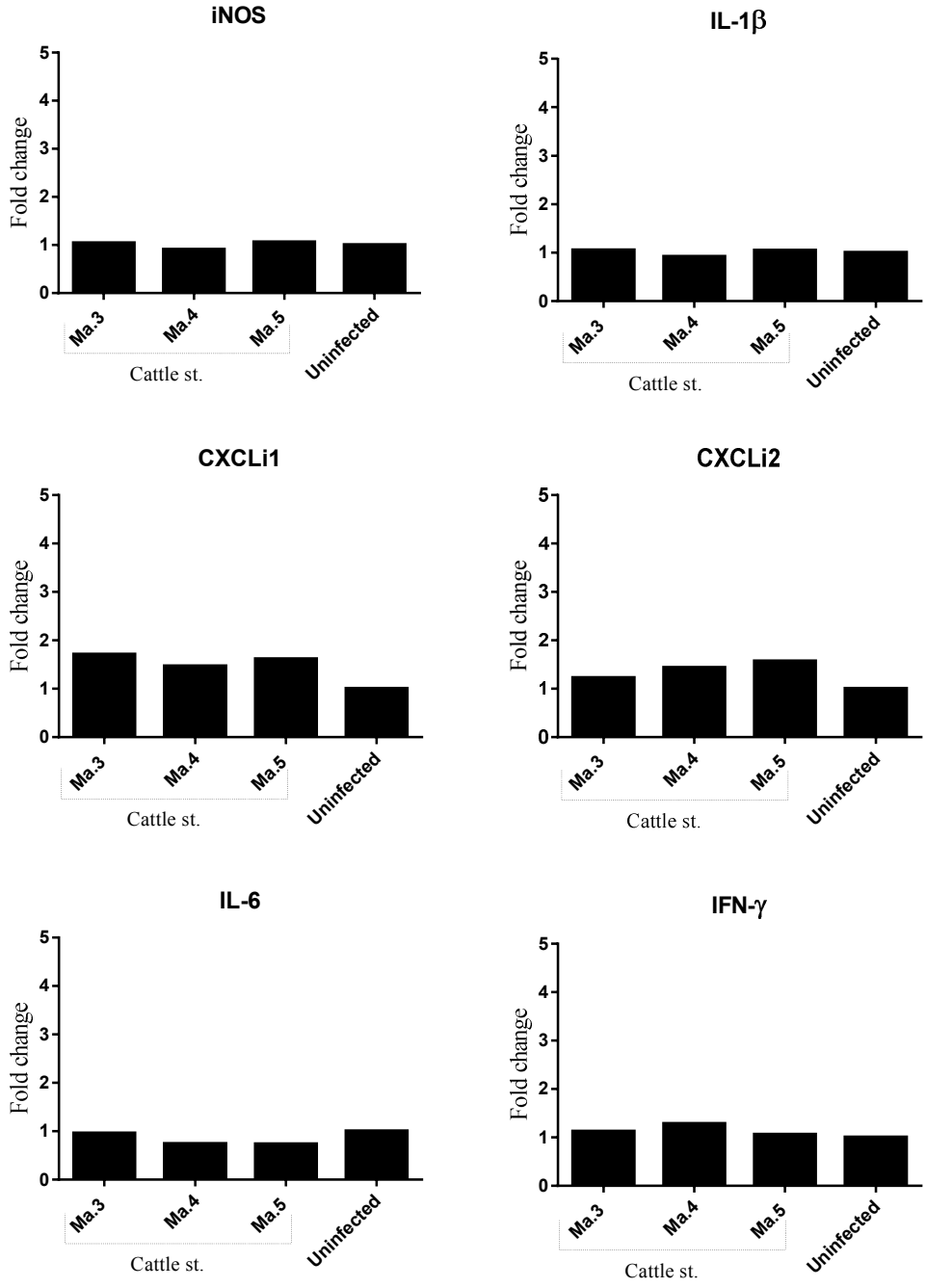


Figure 5.12: Cytokine and chemokine gene expression profile in the spleen of chickens at the day of infection (30 min following inoculation) with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed 30 min after infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA cytokine level in comparison to those from the uninfected group at the same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

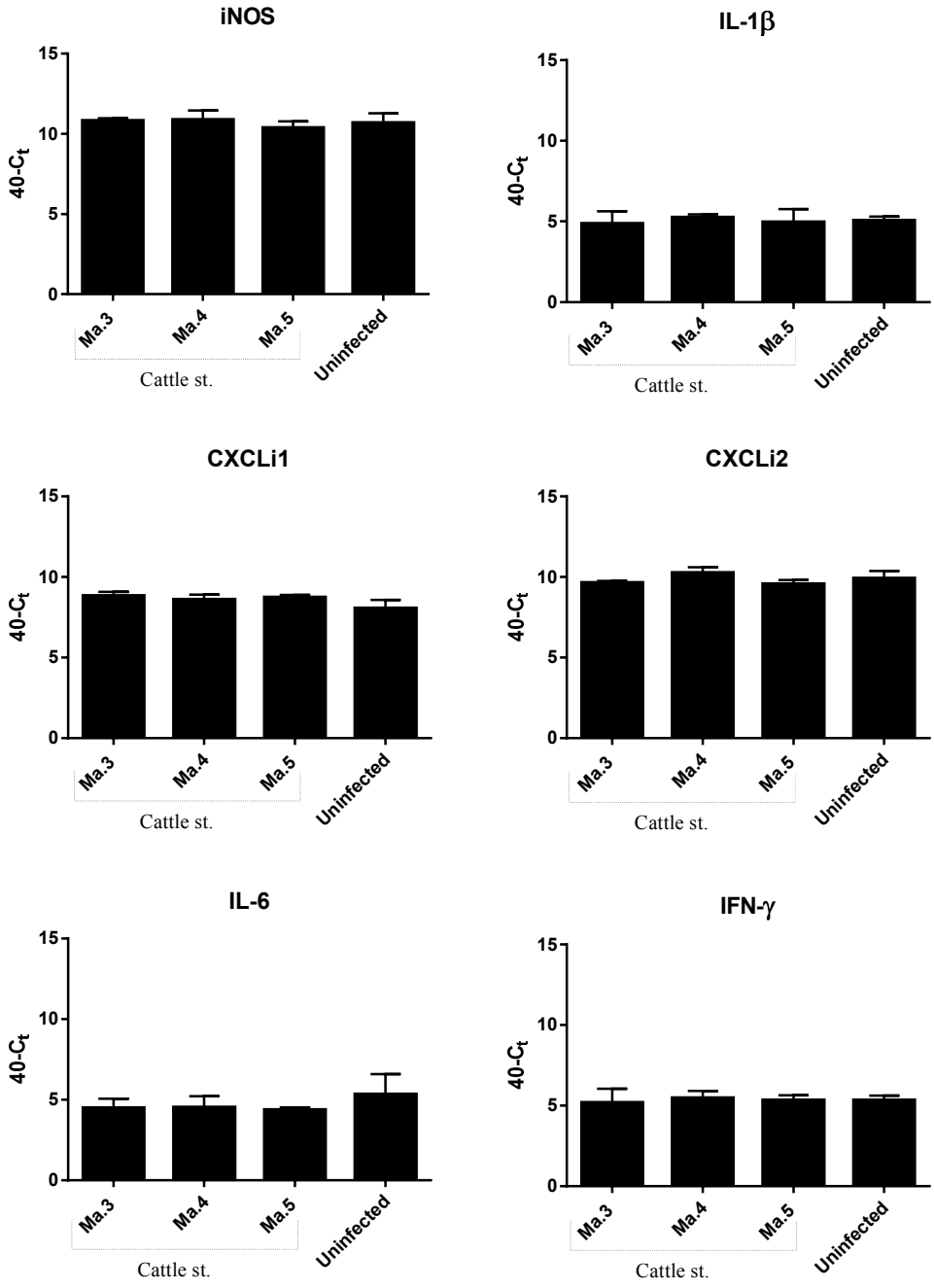


Figure 5.13: Cytokine and chemokine gene expression profile in the spleen of chickens 7 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 7 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

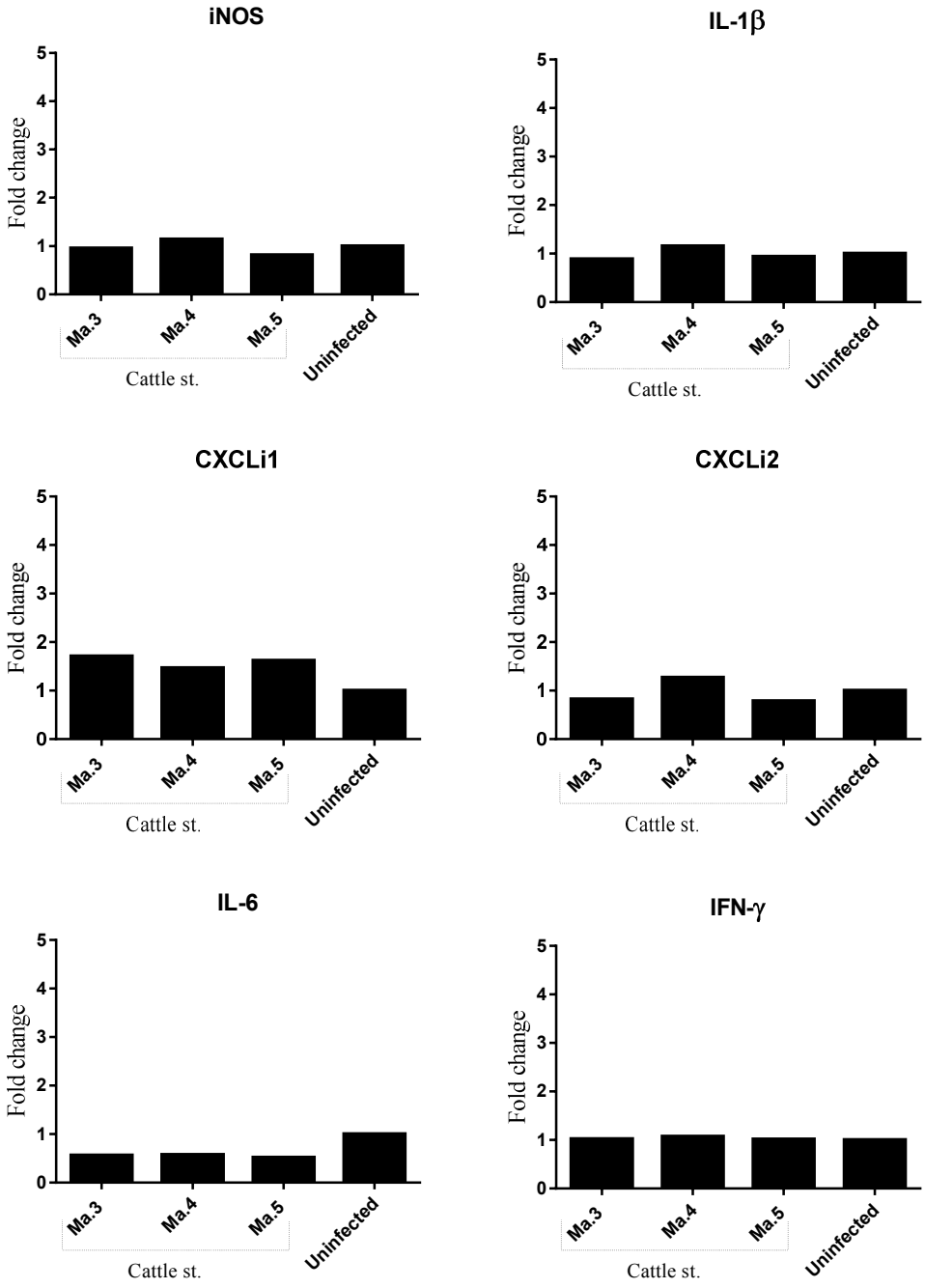


Figure 5.14: Cytokine and chemokine gene expression profile in the spleen of chickens 7 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 7 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected chicken group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.3.2 14 days post-infection

However, by day 14 the mRNA level of IL-6 (<3.34 fold change) and IFN-γ (<1.72 fold change) cytokines were significantly higher ($P<0.05$) in spleen samples from chickens infected with Ma.3 compared to their level in non-infected control group (Figure 5.15-5.16). In addition, compared to non-infected control chickens, Ma.4-infected chickens showed significantly higher ($P<0.05$) levels of IFN-γ (<1.96 fold change) in the spleen samples (Figure 5.15 and Figure 5.16).

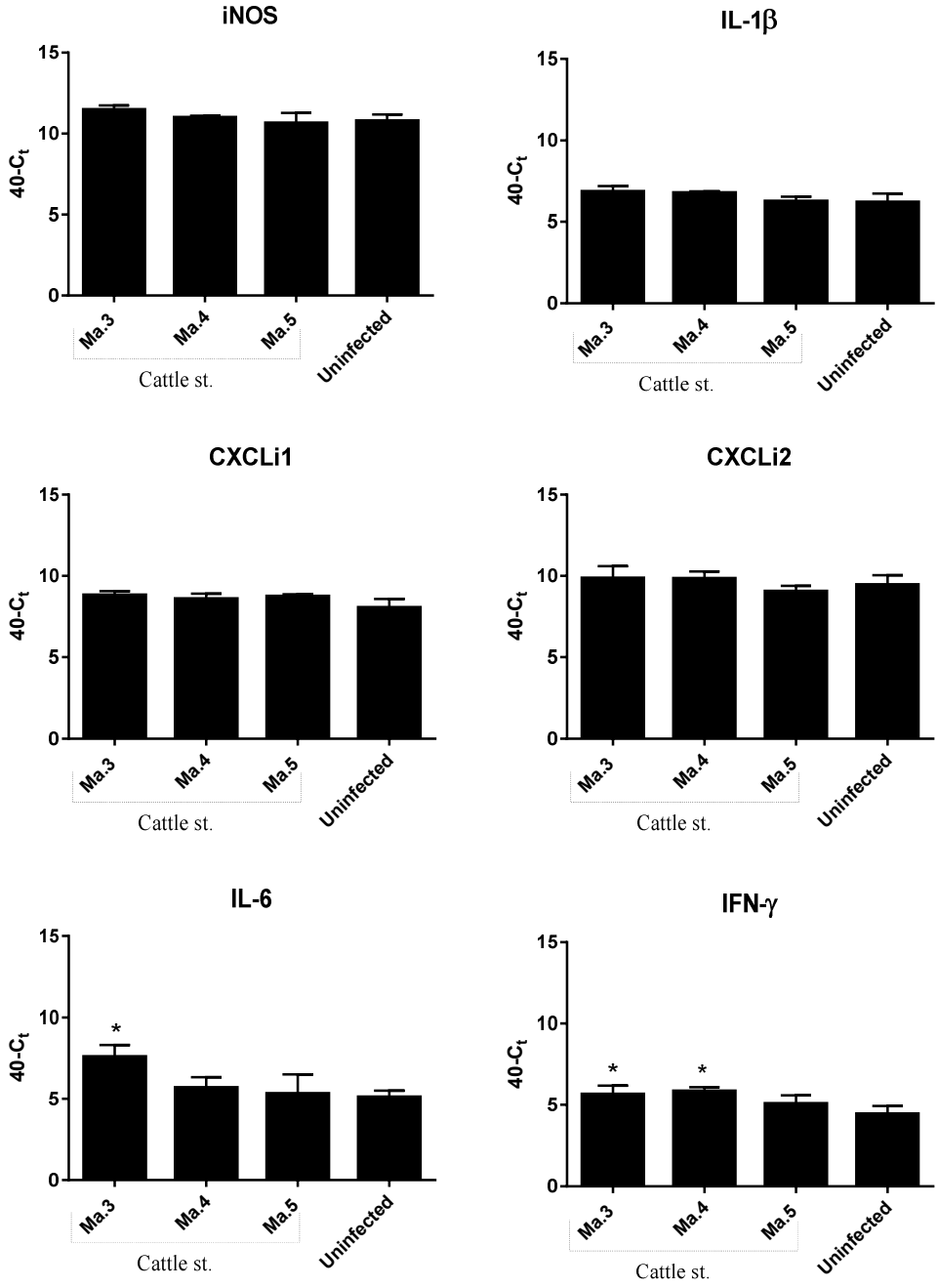


Figure 5.15: Cytokine and chemokine gene expression profile in the spleen of chickens 14 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 14 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. Asterisk (*) refers to significant differences ($P<0.05$) in *M. avium* strains-infected chickens compared to uninfected group. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

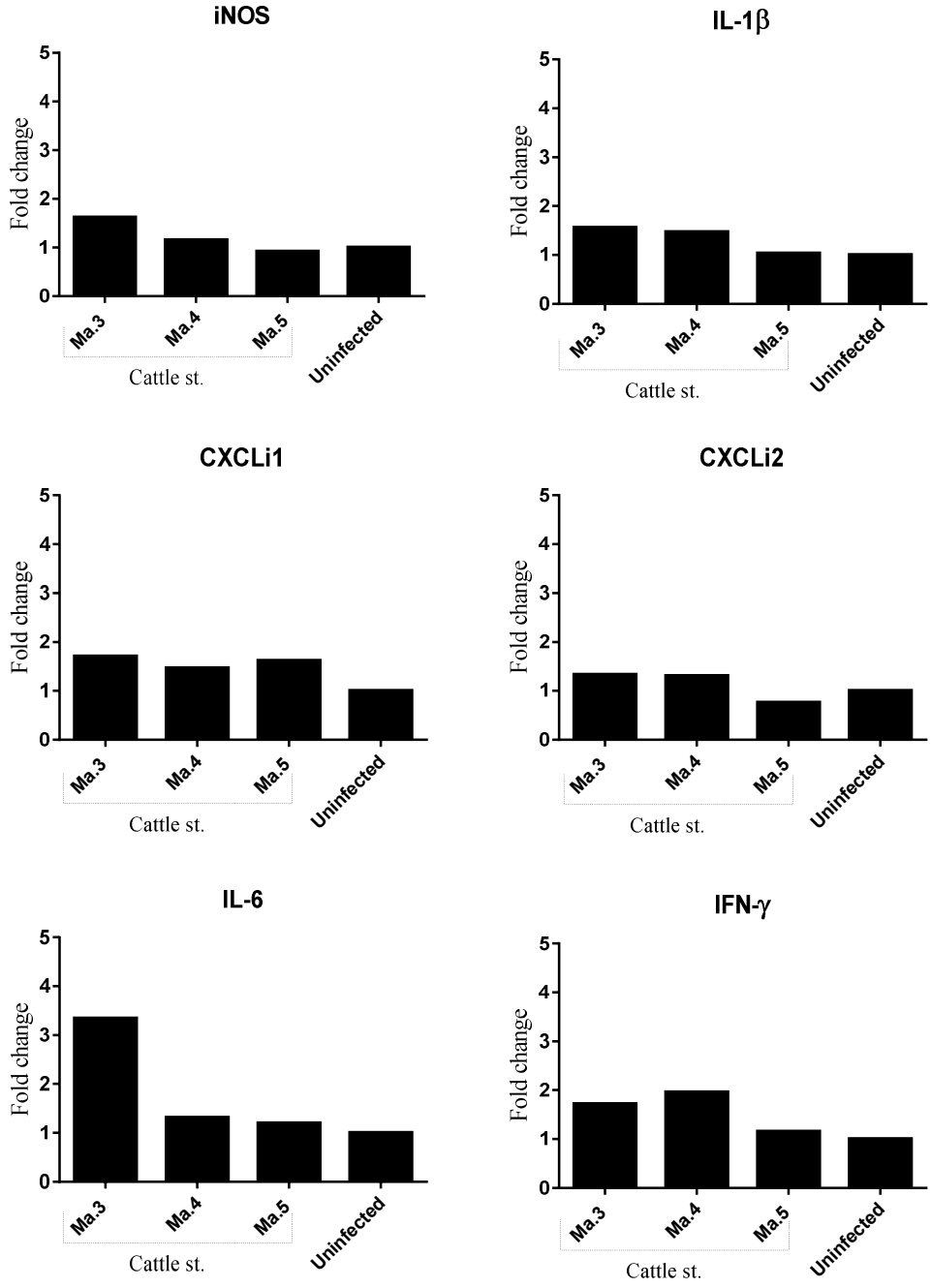


Figure 5.16: Cytokine and chemokine gene expression profile in the spleen of chickens 14 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 14 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected group at the same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.3.3 21 days post-infection

A significant up-regulation ($P<0.05$) in the levels of iNOS (<1.77 fold change) and IL-6 (<7.93 fold change) gene expression was detected in the Ma.5-infected group compared to non-infected chickens at 21 days following infection (Figure 5.17-5.18). There was no significant difference between the mRNA cytokine levels expressed in the spleen of any of groups infected with the different *M. avium* strains ($P>0.05$) (Figure 5.17 and Figure 5.18).

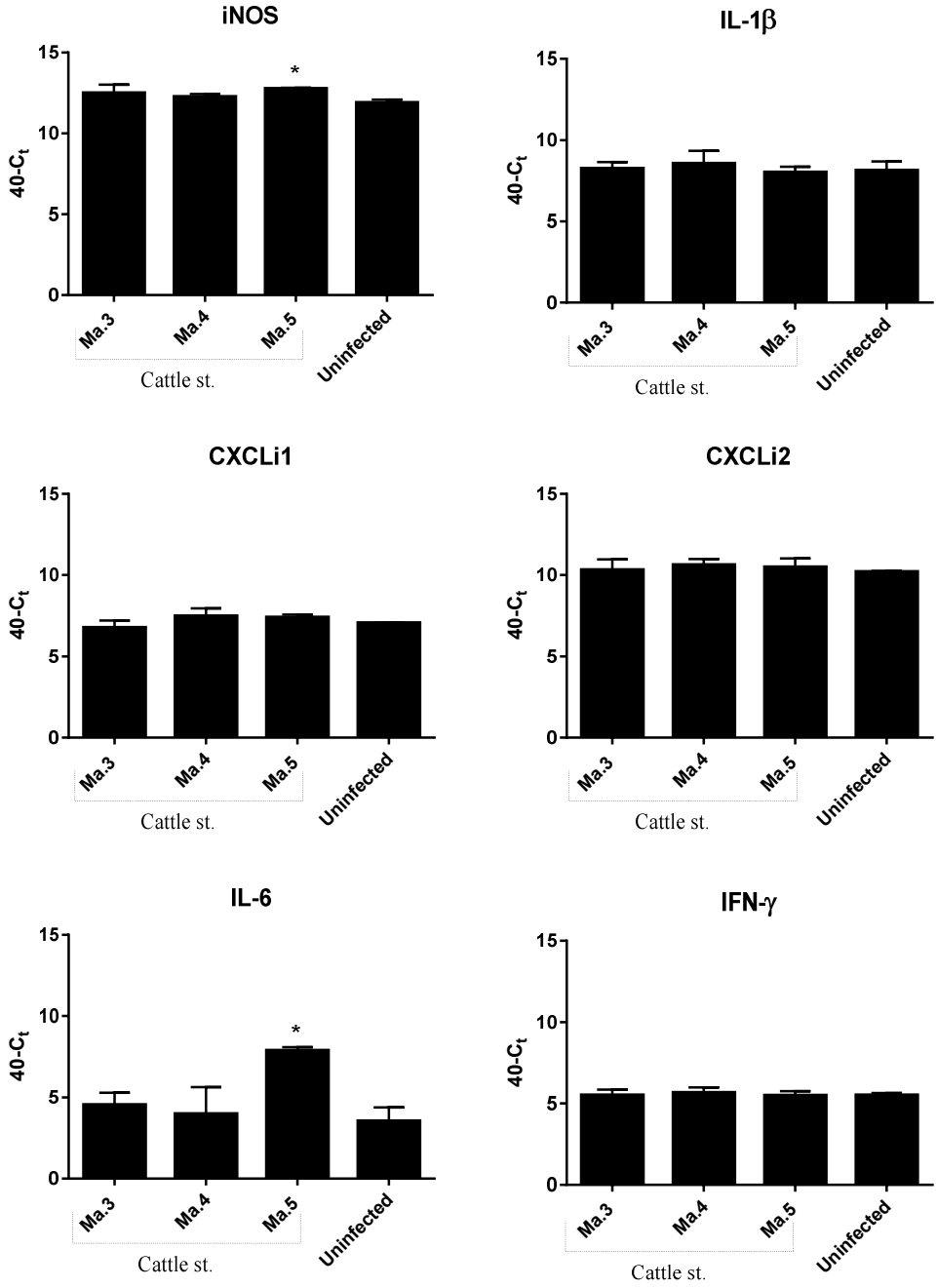


Figure 5.17: Cytokine and chemokine gene expression profile in the spleen of chickens 21 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 21 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected cycle threshold (Ct) values deducted from 40 (the negative end point) after standardization for input RNA. Asterisk (*) refers to significant differences ($P<0.05$) in *M. avium* strains-infected chickens compared to uninfected group. The error bars indicate SEM of triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08). One-way ANOVA was used to find differences between the groups.

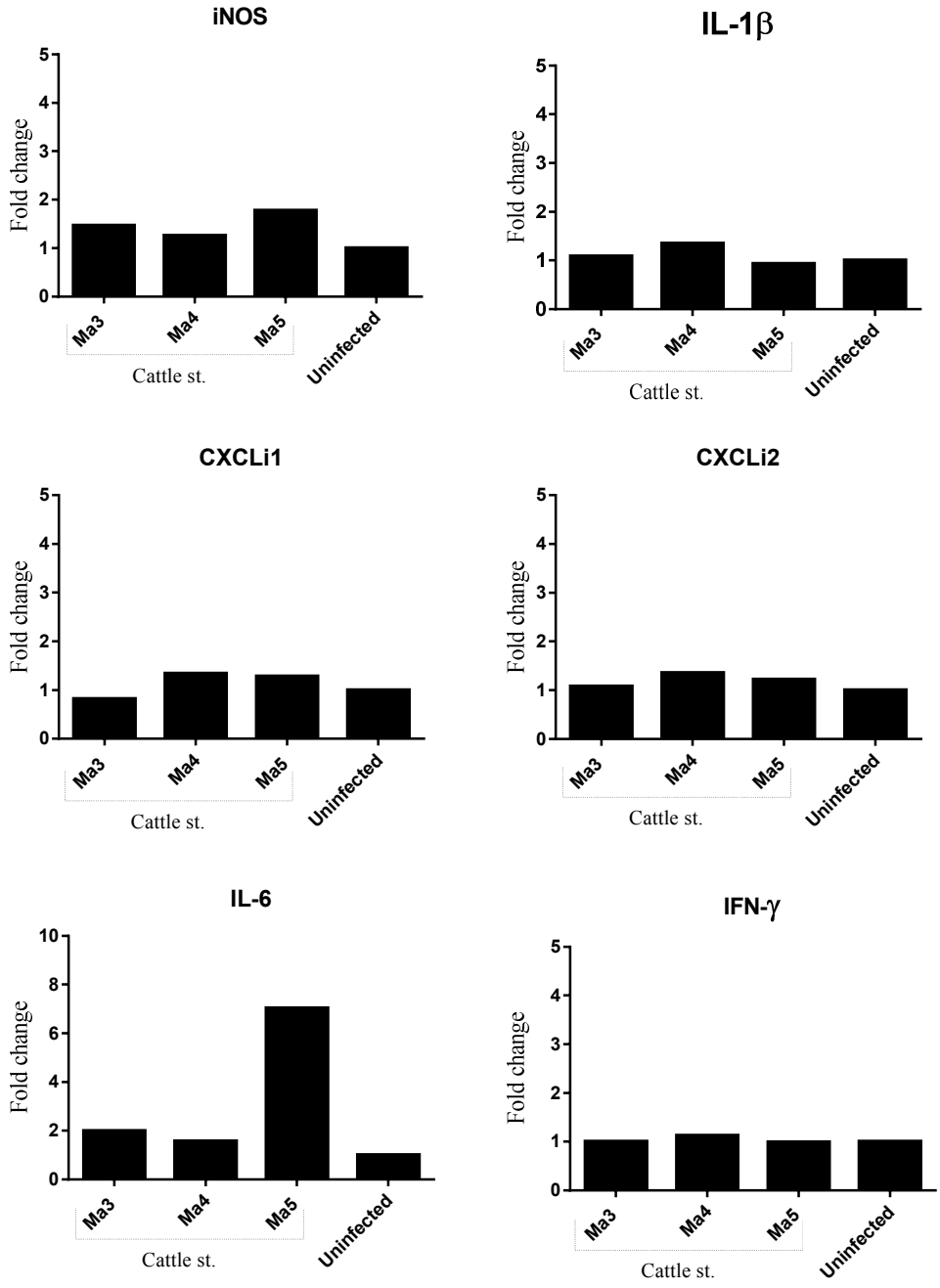


Figure 5.18: Cytokine and chemokine gene expression profile in the spleen of chickens 21 days post-infection with *M. avium* strains.

Chickens inoculated with 3 different strains of *M. avium* and killed at 21 days post-infection. The levels of cytokine expression in spleen quantified using qPCR. The corrected Ct values are shown as fold change in the mRNA level of cytokine in comparison to those from uninfected group at same time point. For this, the levels of cytokine mRNA in uninfected birds are set as 1. The data are obtained from triplicate samples from three chickens in each group. *M. avium* strains: Ma.3 (61.3623.05), Ma.4 (61.4627.08), and Ma.5 (61.3728.08).

5.2.4 Histopathological changes of liver and spleen of chickens infected with *M. avium*

In this experiment intravenous inoculation of the chickens with approximately $1x10^5$ CFU of *M. avium* strains did not result in any observable clinical signs and gross pathological lesions in the infected birds over the duration of experiment.

No microscopical changes were observed in liver of infected and non-infected chickens at the day of inoculation (Figure 5.19).

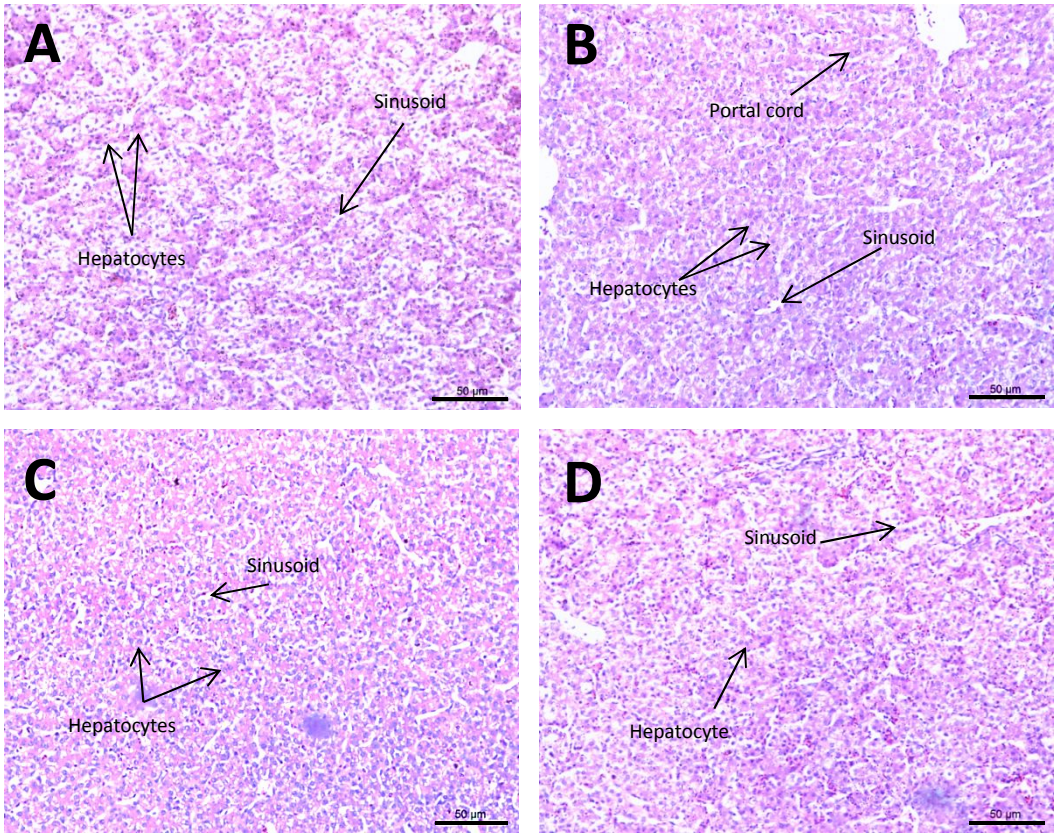


Figure 5.19: Representative histopathological micrographs from the livers of chickens at 30 min after infection with different *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 30 min after infection. Liver samples were collected, fixed, processed and slides were made from their paraffin blocks. The slides were stained with haematoxylin and eosin, (A) Ma.3, (B) Ma.4, (C) Ma.5 and (D) uninfected control chickens. Scale bar: 50µm.

However, microscopic examination of livers at 21 day post-inoculation revealed variable degrees of lymphocytic infiltration in these tissues from chickens infected with all three *M. avium* strains (Figure 5.20).

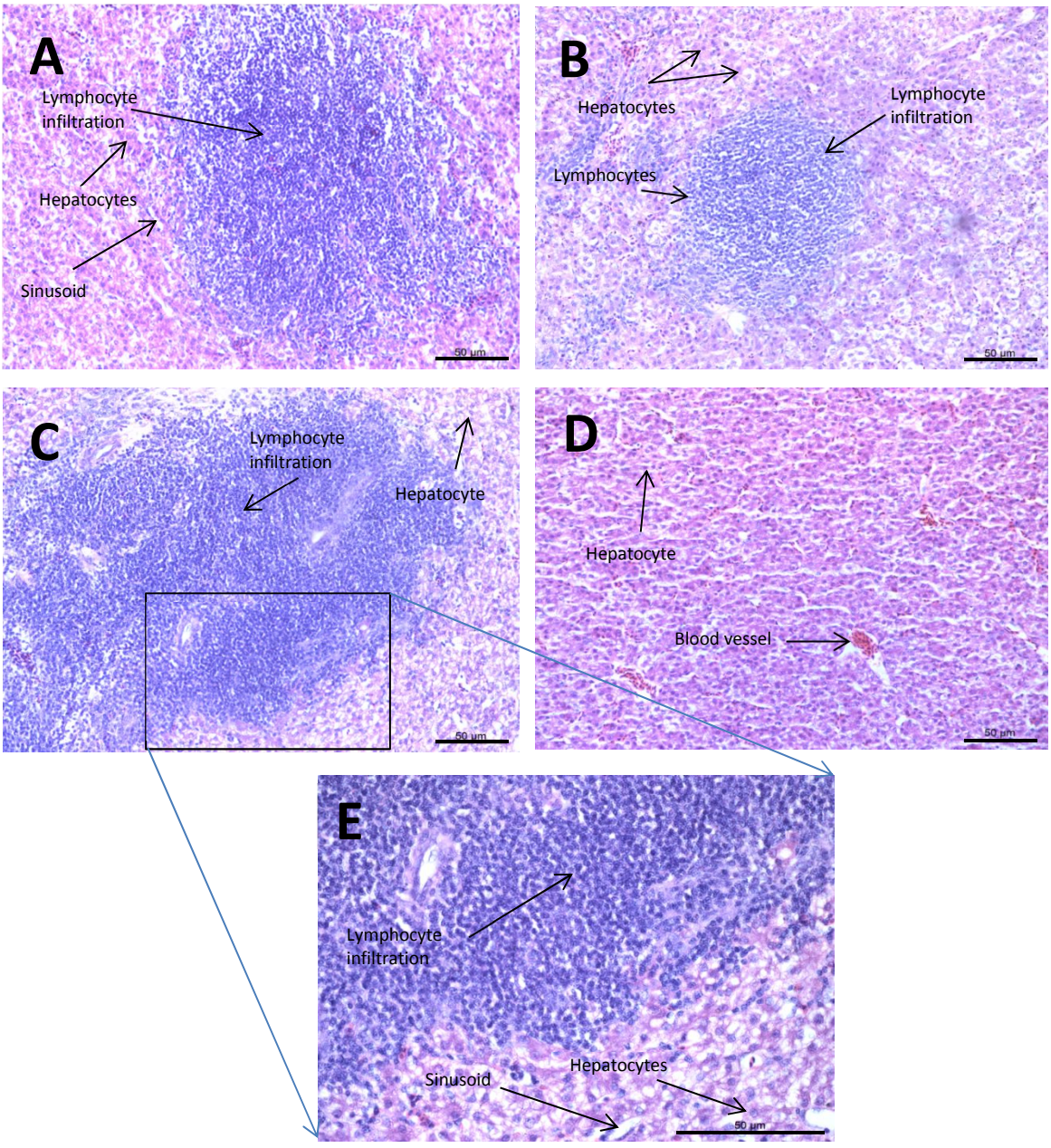


Figure 5.20: Representative histopathological micrographs from the livers of chickens 21 days after infection with different *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 21 days after infection. Liver samples were collected, fixed, processed and slides were made from their paraffin blocks. The slides were stained with haematoxylin and eosin, (A) Ma.3, (B) Ma.4, (C) Ma.5 and (D) uninfected control chickens, (E) higher magnification of a portion of C. Scale bar: 50µm.

Microscopically, no pathological changes or gross changes in the overall structures of the white and red pulp were observed in the spleen of any of the *M. avium*-infected chickens either on the day of inoculation (Figure 5.21) or at 21 days following infection (Figure 5.22).

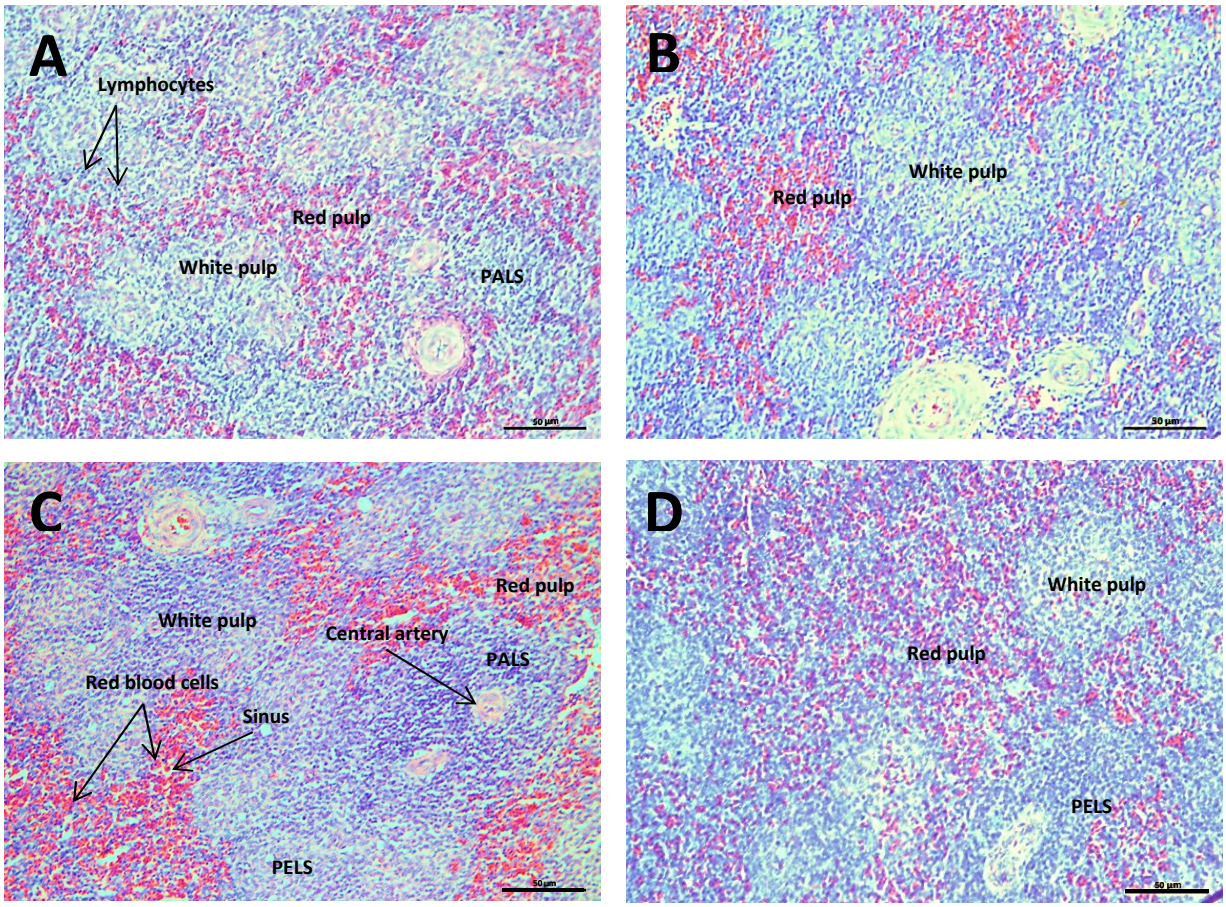


Figure 5.21: Representative histopathological micrographs from the spleens of chickens at the day of infection (30 min following inoculation) with different *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 30 min after infection. Spleen samples were collected, fixed, processed and slides were made from their paraffin blocks. The slides were stained with haematoxylin and eosin, (A) Ma.3, (B) Ma.4, (C) Ma.5 and (D) uninfected control chickens. Periellipsoidal lymphatic sheath (PELS), Periarterial lymphatic sheath (PALS), Scale bar: 50µm.

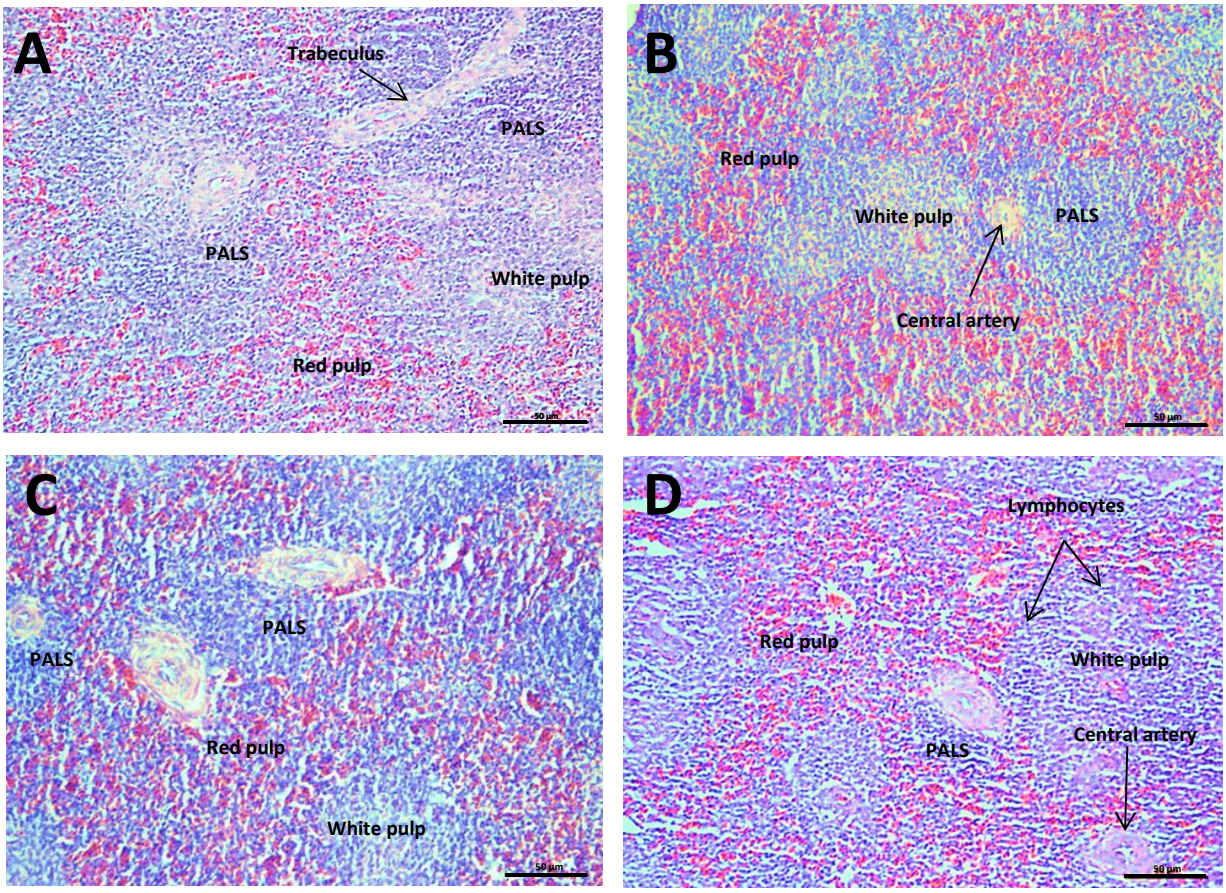


Figure 5.22: Representative histopathological micrographs from the spleens of chickens 21 days after infection with different *M. avium* strains.

Chickens were inoculated with 3 different strains of *M. avium* and killed 21 days after infection. Spleen samples were collected, fixed, processed and slides were made from their paraffin blocks. The slides were stained with haematoxylin and eosin, (A) Ma.3, (B) Ma.4, (C) Ma.5 and (D) uninfected control chickens. Periellipsoidal lymphatic sheath (PELS), Periarterial lymphatic sheath (PALS), Scale bar: 50μm.

5.3 Discussion

The chicken is an important animal model in immunology which has led to a number of important findings (Vainio and Imhof, 1995, Brown et al., 2003, Davison, 2003, Jansen et al., 2010). However, in the case of mycobacterial infection this animal model has rarely been used. Although Larsen and Moon (1972) reported a successful experimental infection of *M. paratuberculosis* in chicken and suggested chickens might be a susceptible species (Larsen and Moon, 1972), this pathogen is known as the causative agent of Johne's disease in cattle and has never been associated with avian tuberculosis (reviewed by Dhama et al., 2011). However, very few studies have been carried out on *M. avium* in chickens which is the most significant causative agent of tuberculosis in domestic birds (Thorel et al., 1990, Fulton and Sanchez, 2013). Although experimental infection of chickens with *M. avium* strains to evaluate virulence can result in death after a long course of disease lasting up to 115 days, the immune response of the chickens to the bacteria has not been studied (Collins et al., 1983).

In this chapter, first the clinical condition will be discussed followed by the bacterial kinetics, cytokine profiles and the histopathological study.

Mycobacteriosis is a chronic infectious disease of birds in which clinical signs include respiratory distress, diarrhoea, loss of weight, depression and reduction of eggs production (Kahn, 2005). In this study no clinical signs were observed during the course of the

experiment largely as a result of the experiment being of a relatively short duration (reviewed by Dhama et al., 2011).

We found that the number of *M. avium* bacteria in the spleen and liver of all infected chickens declined during the course of the experiment. This was unexpected. Mycobacterial growth in animal models has generally been studied using the mouse model. Several studies have reported the ability of mycobacterial species, particularly *M. tuberculosis* to grow and multiply in susceptible mouse lines (Rhoades et al., 1995, Manca et al., 1999). In contrast, it has been shown that mice are able to control BCG infection in the liver, spleen and lung (Saito et al., 2006). Pedrosa et al., (1994) infected mice with four different strains of *M. avium* originally isolated from AIDS patients. They found that two strains cleared readily and the other two strains proliferated in these animals (Pedrosa et al., 1994) indicating a difference in virulence between the two strains. Similarly, *M. abscessus*-R (rough wild-type) was able to proliferate in mice whereas *M. abscessus*-S (smooth attenuated mutant) was rapidly cleared from this animal model (Byrd and Lyons, 1999). In addition, *M. bovis* BCG showed a reduction in CFU in the liver, lung and spleen as early as two weeks post-infection after inoculation of mice intravenously with 10^6 CFU of the bacteria (Nobrega et al., 2007). However, very little is known about the susceptibility of the chicken to mycobacterial infection. Given that the strains used in this study were field strains and not known to be attenuated, the results in the present study indicate a degree of resistance of this chicken line to the *M. avium* strains used

in this study shown by their ability to control the infection. However, this is clearly not simply the result of the innate species resistance of the chicken in the same way that mice are generally relatively resistant to *Mycobacterium*, since chickens can be highly susceptible to *M. avium* disease and differences in susceptibility between lines of chickens may be responsible in addition to differences in virulence between the strains used here.

In mammals mycobacteria are able to disseminate to several organs of the body associated with the immune system including spleen and lymph nodes. Lymph nodes are considered the main sites of antigen presentation in mammals as following uptake of mycobacteria by phagocytic cells including dendritic cells and macrophages, these cells carry the bacteria and microbial components to the lymph nodes to be presented to T cells. In birds, however, draining lymph nodes are absent to recover the bacteria in these tissues. In birds, in addition to the spleen, the bacteria can disseminate to other tissues including liver, air sacs, lungs, bone marrow and gonads (Lennox, 2007). In the chickens it has been found that following *Salmonella* infection, splenic macrophages are the main site for expression of resistance and susceptibility phenotype rather than the intestine (Bumstead and Barrow, 1988). Similarly, the authors found that in in-bred chicken lines, the resistance or susceptibility to *M. avium* strains is also expressed in the spleen (Barrow and Bumstead, unpublished).

Nobrega et al. (2007) also showed that mycobacteria colonized the thymus of mice consistently, so this organ may be an additional

target organ for mycobacterial species. This occurred at the time when the bacterial load in the spleen and liver started to decline (Nobrega et al., 2007). The reduction of the bacterial load in the spleen and liver observed in the chickens in our study indicates that investigating the bacterial load in this organ at this time may have been useful. Despite the fact that the thymus is considered as an privileged organ, the presence of mycobacteria in the thymus in both human (Simmers et al., 1997, Stephen et al., 2003) and mice (Nobrega et al., 2007) has been reported. The macrophages and dendritic cells in this organ provide signals for differentiation through self-antigen presentation to differentiating thymocytes and this role is different from their function in other organs such as the spleen. So, by thymic colonisation, mycobacteria could be hidden from effective immune responses for a long period. Therefore, this would be worth investigating since the presence of mycobacteria in thymus could also affect T cell maturation.

The absence of bacterial growth from blood samples indicated that the chicken line used in this study was able to clear the bacteria from the blood rapidly. The clearance ability is likely to result from opsonic factors in the blood. This is a common occurrence with other bacteria, such as *Salmonella,* which are cleared rapidly from the blood by the liver and spleen (Barrow et al., 1987). It has also been reported with *E. coli* inoculated intravenously into immunized turkeys which is followed by a reduction in 5 logs in the bacterial count in the blood within 10 min post-inoculation (Arp, 1982). It is

likely that this is a common mechanism involving trapping the bacteria by tissue macrophages in spleen and liver.

The cytokine response of the chicken to *M. avium* infection has not been investigated thus far. In the control and prevention of mycobacterial diseases, cellular immunity plays a much more important role than humoral one (Bosio et al., 2000, Fulton and Sanchez, 2013). Interference with the production of cytokines and their function is one of the main mechanisms that mycobacteria use to survive inside macrophages (Hines et al., 1995). In order to improve our understanding of the immune response of chickens to *M. avium*, it was necessary to investigate the cytokine expression profile by qRT-PCR since it is not easy to detect the protein level of cytokines in poultry due to the limited availability of specific antibodies (Sijben et al., 2003). To quantify *in vivo* expression, we evaluated the mRNA level of various cytokines and chemokines including iNOS, CXCLi1, CXCLi2, IL-1β, IL-6 and IFN-γ expression in both liver and spleen tissue samples. We found that the levels of expression of these cytokines did not change at early time points post-infection. However, at 14 days following infection a significant up-regulation was observed in the level of IFN-γ in response to all the *M. avium* strains in comparison with non-infected birds in liver. In addition, at 21 days post-infection, most of the cytokines examined in this study, including iNOS, IL-1β, CXCLi2, IL-6 and IFN-γ, showed up-regulation in Ma.5-infected chickens liver samples compared to non-infected ones. This up-regulation was also observed in the mRNA level of CXCLi2 and IL-1β in chicken livers

infected with Ma.4 and IL-1β in Ma.3-infected chickens. In Ma.4-infected chickens there were no bacteria recovered from the liver and spleen tissue samples. However, this group showed a significant increase in the mRNA level of IFN-γ at 14 days in liver and spleen and IL-1β and CXCLi2 at 21 days in the liver in comparison to non-infected chickens. This could be due to the existence of dead bacteria in the tissues and possibility of the stimulation of the immune response by bacterial antigens. Further experiments are needed to demonstrate which mycobacterial components have the capability to do this. Assessment of cytokine production, especially IFN-γ, might form the basis of valuable test to evaluate the strength of the protective immune response to *M. avium* in birds (reviewed by Dhama et al., 2011). IFN-γ plays a critical role in granuloma formation in mycobacterial infection and increases in the level of this cytokine indicate the possibility of a start in the formation of granulomata in the liver in this study. In immune responses to mycobacterial infection IFN-γ and IL-12 play crucial roles by activation of macrophages which leads to subsequent production of other cytokines and NO and killing the intracellular bacteria (Flesch and Kaufmann, 1987, Chan et al., 1992, Flynn and Chan, 2001). This macrophage activation is also important in acidification of phagosomes which also assists in destroying the bacteria (Via et al., 1998, Hostetter et al., 2002). The essential role of IFN-γ in the control of *M. tuberculosis* infection has been investigated in mice (Cooper et al., 1993, Flynn et al., 1993) and man (Barnes et al., 1993). Elevation of this cytokine has been detected in both pleural

fluid and blood of tuberculous pleuritic patients (Barnes et al., 1993) and in granulomata and pneumonitis areas of human pulmonary tuberculosis (Bai et al., 2004). Furthermore, significantly higher levels of IFN-γ have been detected *in vivo* in mice infected with BCG compared to non-infected animals (Fulton et al., 2000). Induction of IFN-γ production is largely dependent on IL-12 production. Infection of IL-12 p40 (-/-) mice, in which IFN-γ and other Th1 cytokines are absent, is lethal (Cooper et al., 1997). IL-6 also plays an important role in control of tuberculosis in mice. It has been shown that mice deficient in this cytokine succumbed to infection with *M. tuberculosis* (Ladel et al., 1997). Recently, Singh and Goyal (2013), have suggested that this cytokine is an important biomarker of infection with mycobacterial species. Increased levels of this cytokine have been reported in the plasma of patients with pulmonary tuberculosis (el-Ahmady et al., 1997). In contrast to these results, Nagabhushanam et al. (2003), argued that IL-6 induction by *M. tuberculosis* has an inhibitory effect on macrophage activation by IFN-γ. Susceptibility to *M. tuberculosis* infection has also been reported in mice deficient in IL-1β (Mayer-Barber et al., 2010). Overgrowth of *M. tuberculosis* and defective granuloma formation have been observed in mice deficient in IL-1R type 1 (Juffermans et al., 2000) and also in mice with double knock out of IL-1α and IL-1β (Yamada et al., 2000). The precise role played by IL-1β in resistance to mycobacterial infection is not clear. However recently it has been shown that this cytokine restricts replication of *M. tuberculosis* in murine and human macrophages through cell surface

expression of TNF-α receptor (TNFR) and activation of caspase-3 (Jayaraman et al., 2013). Taken together, our data showing elevation of the cytokines, specifically IFN-γ, IL-1β and IL-6, in response to infection with *M. avium* in chickens, are similar to those obtained from observing mycobacterial infection in humans and mice and are therefore likely to be key factors in controlling the infections.

In the current study we observed that at all time points the differences in cytokine quantification between the infected groups were not significant. As explained in chapter 3, this is in contrast to the differential immune response observed between different *M. avium* strains in induction of variable amounts of cytokine at the protein level in THP-1 cells and at the mRNA level in HD11 cells.

Our data suggest therefore that the chicken line used for the *in vivo* study could have been resistant to infection with *M. avium* rather than this resulting from using strains which were inherently attenuated. Another possibility is that the intravenous inoculation of the bacteria was not an optimal route of delivery for the *in vivo* infection although this has been used for *M. avium* previously to demonstrate differences in resistance between inbred chicken lines (Barrow & Bumstead, unpublished findings). Although the natural route of infection in mycobacteriosis is by the oral route, this route would not give results relevant to the main sites of infection in the early time period following inoculation. A number of chicken lines have been found which are resistant or susceptible to systemic salmonellosis, an infection caused by another well studied

intracellular bacterial pathogen (Bumstead and Barrow, 1988, Bumstead and Barrow, 1993, Wigley et al., 2002).

In microscopic examination of the liver, the cellular infiltration observed in this tissue from infected chickens, which was more noticeable at 21 days after infection, suggests the early stages of granuloma-like lesion formation. This phenomenon with lesion-like structures was not observed in non-infected chicken livers. Granulomata are typical lesions of mycobacterial diseases, including those seen in many species of birds including gallinaceous birds such as quail and turkeys, and also wild birds (Millan et al., 2010, Fulton and Sanchez, 2013). Granuloma formation, involving TNF-α, is important for cell mediated immunity (Lin et al., 2007), but studies on cell mediated immunity and the formation of granulomata in response to mycobacterial infection in various laboratory animals does show variability between the species. For example, although a good cellular immunity has been observed in mice, pigs and rabbits (Acland and Whitlock, 1986, Hibiya et al., 2008, Nakamura et al., 1984), no significant cell mediated immunity was detected in guinea pigs (Gupta and Katoch, 2005). The type of granuloma lesion also varies between the species. An extensive caseous necrosis has been observed in guinea pigs (Gupta and Katoch, 2005), whereas caseation was moderate in rabbits (Hibiya et al., 2011). However, in the murine thymus, no structural differences have been reported at eight weeks following infection with *M. tuberculosis* or *M. avium* (Nobrega et al., 2007). In pigs a systemic epitheloid granuloma can be observed, predominantly in the digestive organs and regional

lymph nodes and cell mediated immunity confers a degree of resistance against mycobacterial infection (Hibiya et al., 2011). In the current study, although no clinical signs or gross pathological lesions were observed in any chickens, the histopathological examination revealed an early immune response to the bacteria. Although the birds were inoculated with Log_{10} 5 bacteria and the number of bacteria reduced over time the formation of granulomata as a key feature of immune response in mycobacterial infection was observable in infected chickens particularly in the liver at 21 days post-inoculation. It would therefore be valuable to investigate whether the process of granuloma formation began in response to the live bacteria at early time points post-inoculation and continued even after the bacteria were killed and also whether dead bacteria are capable of stimulating the formation of granulomata.

It is known that for protection against tuberculosis infection, live attenuated bacilli are used and dead bacteria do not protect when administered as vaccines (Wilson and Milles, 1964). It has been shown that it is vital to use live bacteria for vaccination to obtain protective efficacy in a wide range of experimental animal models. Despite the fact that, following challenge with mycobacteria, formation of granuloma is a key feature of protection, the involvement of dead mycobacteria in stimulation of granuloma formation remains unclear. In addition, although *M. tuberculosis* have been found to be localized mainly in the necrotic core of the granuloma in guinea pigs (Hoff et al., 2011), the viability of these bacilli has not been determined. Furthermore, Chambers et al.,

(1997) found that in contrast to live BCG, dead BCG bacteria failed to induce localized swelling in mouse footpad model (Chambers et al., 1997). However, it has been reported that injection of dead bacilli into animals may lead to formation of lesions with variable degree of severity. While a local abscess developed after subcutaneous administration, intravenous injection of dead bacilli led to deposition of the bacilli in the viscera and subsequent formation of small follicular lesion similar to tubercles which sometimes lead to caseation and the disease called Necrotuberculosis (Miller, 1905). It is likely that following release of intracellular toxins as a result of bacterial death and lysis by the activated macrophages, these toxins may act on tissues with the formation of characteristic lesions of tuberculosis. So, to some extent, it can be argued that although only live bacteria can multiply *in vivo*, virulent bacilli are not necessary for this disease, as the lesions have been produced by dead avirulent bacteria through intravenous injection of dead BCG strain in rabbit (Wilson and Milles, 1964). Therefore, an investigation of the relative ability of live and dead mycobacteria to induce granuloma formation in chicken would clearly be of interest.

6 General Discussion

The aim of this study was to evaluate and compare the immune responses induced by host cells to infection with different strains of *M. avium* isolated from animal and human sources. Very little work has been done to examine the immune response to *M. avium* and, to our knowledge; this is the first study to do this with the infection in chicken cells *in vitro* and *in vivo*.

In the current study cytokine production in response to *M. avium* challenge was studied in human and chicken macrophage-like cells. In addition, the role of each MAPK subfamily was evaluated in signalling transduction in the early stage of the immune response. The cytokine profile and cellular changes were also investigated in the chickens in response to infection with *M. avium*.

Understanding the interaction between mycobacteria and host macrophages will give greater insight into the pathogenesis of mycobacterial diseases (Mcdonough et al., 1993). The major objective of this study was to do this for both *in vitro* and *in vivo* infection.

In the present study we provide evidence that different strains within *M. avium* may behave differently in terms of induction of immune response in host cells.

6.1 Immune response to *M. avium* strains

Since NO plays an important role in killing intracellular bacteria (Shiloh and Nathan, 2000), it is suggested that the induction of higher concentrations of NO will lead controlling the infection and bacterial numbers. This was observed in our study where we found that macrophages infected with Ma.4 and Ma.5 produced higher

levels of NO and reduction of CFU number of these two strains by 24h in HD11 cells. This is clearly indicative that NO has a role in killing the intracellular bacteria.

A successful immune response against mycobacterial infection largely depends on the production of cytokines. It has been suggested that activation of macrophages and production of chemokines (e.g. CCL2, CXCL8) and cytokines (e.g. TNF-α and IL-1β) in response to mycobacterial infection initiates cellular ability to control the intracellular growth of mycobacteria (Flynn and Chan, 2001).

In the current study, a differential ability in the induction of cytokines was observed between various *M. avium* strains in both THP-1 and HD11 cells. Such differential responses have been reported between pathogenic and non-pathogenic mycobacterial species in several studies, generally indicating that pathogenic mycobacteria induce less cytokines compared to non-pathogenic species in cell lines, macrophages and animal models (Hoal-Van Helden et al., 2001, Lopez et al., 2003a, Manca et al., 2004, Theus et al., 2005, Sinsimer et al., 2008, Tanveer et al., 2009). Although Chacon-Salinas et al. (2005) reported a higher level of cytokine release in macrophages in response to the *M. tuberculosis* Beijing strain in comparison with *M. tuberculosis* genotypes Canetti and H37Rv, there are several studies to indicate that this highly pathogenic strain of *M. tuberculosis* induces lower levels of cytokines compared to less pathogenic strains (Wang et al., 2010, Sarkar et al., 2012). In a recent study, Chakraborty et al. (2013) suggested

pro-inflammatory cytokine secretion could be a mycobacterial genotype-dependent process. They found that in THP-1 cells the highly virulent *M. tuberculosis* Beijing strain induced significantly lower level of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12, than other less virulent strains. In addition, this has also been observed between an avirulent species (*M. smegmatis*) and a more virulent species (*M. bovis* BCG) of mycobacteria infecting murine macrophages (Roach et al., 2002). A possible reason for this differential response is phosphatase activation. According to Roach et al., (2005) *M. avium* infection induced constant activation of the protein phosphatase 2A (PP2A) family of phosphatases in bone marrow derived macrophages in comparison to *M. smegmatis*. Moreover, inhibition of PP2A led to reversed suppression of macrophage activation infected with *M. avium* (Roach et al., 2005). Furthermore, Rajaram et al. (2011), suggested that the differential level of TNF induced by *M. tuberculosis* and *M. smegmatis* could be due to the differential action of lipomannan isolated from these two mycobacterial species. They found that stimulation of cells with lipomannan from *M. tuberculosis* resulted in destabilisation of the mRNA transcript of TNF and subsequently failure of TNF protein production (Rajaram et al., 2011). Despite these findings the exact mechanism of the differential response is still undefined. Therefore, further studies are required to determine how cytokine production is modulated by pathogenic mycobacteria. Taken together the differential immune responses observed in this study between *M. avium* strains support the concept that there is

variability in the level of pathogenicity among the strains and this was probably related to the immune response. Further experimentation is needed to investigate the possible differential ability of these strains that affects the level of host immune response.

It was also observed that this differential behaviour among the strains was not related to the source of the strains. Although Ma.3, Ma.4 and Ma.5 have been isolated from bovine, these three strains do not behave similarly in induction of cytokine production. The difference was obvious in both human THP-1 and chicken HD11 cells. So there is a possibility that these bacteria have been transmitted to the cattle from other animal species.

6.2 MAPK signalling transduction pathway

The role of the MAPK signalling pathway in regulation of signal transduction was examined in THP-1 and HD11 cells in response to *M. avium* infection. It was found that there was a general interaction between the MAPK pathways studied in regulation of cytokine production.

Several studies have reported MAPK as an essential signalling pathway in induction of pro-inflammatory cytokine production associated with mycobacteria infection (Blumenthal et al., 2002, Roach and Schorey, 2002, Schorey and Cooper, 2003). It is known that MAPK signalling pathways are activated by mycobacterial species via TLR engagement (Hasan et al., 2003, Blumenthal et al., 2005). Activation of MAPK by whole mycobacteria or mycobacterial components could lead to activation of transcriptional factors such

as NFκB and activator protein-1 (Wang et al., 2001, Pennini et al., 2006, Yang et al., 2006). Cytokines and chemokines are then released as a consequence of the activation of transcriptional factors.

The findings in this study demonstrate that pre-treatment of THP-1 cells with either of the ERK pathway inhibitors, PD98059 or U0126, reduced the level of *M. avium*-induced pro-inflammatory cytokines and chemokines. This indicated that ERK is an important regulator of pro-inflammatory cytokine production in THP-1 cells. These findings are in accordance with those of several research groups (Roach and Schorey, 2002, Bhattacharyya et al., 2002) who also reported reduction of cytokine production following inhibition of ERK pathway. In addition, in the current study, inhibition of ERK in avian cells resulted in reduction of production of NO and expression of cytokines and chemokines in response to stimulation with *M. avium,* suggesting an important role for ERK in regulation of signal transduction in these cells. In accordance with our data, Kogut et al. (2008) suggested an important role for ERK in regulation of cytokine production in avian cells following inhibition of ERK by either of its inhibitors PD98059 or U0126 (Kogut et al., 2008).

In contrast to inhibition of ERK, inhibition of p38 led to enhancement of TNF-α and IL-1β in SB203580-treated THP-1 cells in response to LPS and *M. avium* therefore suggesting a negative role for p38 in regulation of production of these cytokines in THP-1 cells. These findings are in accordance with Bhattacharyya et al. (2002) who reported enhancement of TNF-α in murine macrophages in response

to *M. avium* infection. Similarly, increase in the level of TNF-α was reported in response to *M. tuberculosis* infection in mice *in vitro* and *in vivo* following treatment with p38 pathway inhibitor, SB203580 (van den Blink et al., 2001). Since increases in level of TNF-α could reduce mycobacterial numbers, this pathway can be proposed as a candidate for designing more efficient therapeutic approaches, for example by cytokine therapy. However, it is worth noting that the effect of inhibition of p38 on cytokine production is cell-dependent which has been observed in this study and several other studies (Means et al., 2000, van den Blink et al., 2001, Tudhope et al., 2008). In addition, an *in vivo* study in mice suggested that inhibition of the p38 pathway could not be a suitable strategy to reduce bacterial survival and cytokine production in mycobacterial infection *in vivo* (van den Blink et al., 2001). In this study, the p38 inhibitor SB203580 was injected into mice infected with virulent *M. tuberculosis*. In addition to increased cytokine levels such as TNF-α that were observed in these mice, bacterial clearance was severely reduced in the SB203580-treated mice. This indicated that p38 pathway inhibition might play a role in bacterial survival *in vivo*. Very recently, (Qin et al., 2016) found that *in vivo* treatment of mice with the ERK inhibitor U0126 increased the number of *E. coli* and decreased survival of infected mice. However, the effect of MAPK inhibitors including ERK and JNK inhibitors on animal survival in mycobacterial infection is not fully understood.

The findings in the current study demonstrated that all cytokines under study could be differentially affected by inhibition of each of

ERK, p38 or JNK pathway. These data corroborate the findings from previous studies by Chen et al. (2012) who reported inhibition of TNF-α, IL-6 and IL-12 following inhibition of JNK while these cytokines were not affected or partially reduced by p38 or ERK inhibition. The ability of *M. tuberculosis* to inhibit IFN-γ, which is an essential cytokine in the immune response to mycobacteria and other pathogens via modulation of MAPK, is an important virulence mechanism of these pathogenic bacteria. Peng et al. (2011), found that "early secreted antigenic target of 6KDa", ESAT-6 a protein that is secreted by *M. tuberculosis,* can inhibit expression of IFN-γ by T cells through promoting functional activity of p38 MAPK. Therefore, it can be suggested that the p38 MAPK pathway plays an important role in the pathogenesis of mycobacterial infection and understanding the mechanism of modulation of immune responses by mycobacteria can provide further knowledge to develop more efficient therapeutic strategies.

Previous studies have described the involvement of MAPK subfamilies in the control of intracellular replication of *M. avium* in human macrophages (Blumenthal et al., 2002, Shiratsuchi et al., 2008). Treatment of human primary monocytes with the ERK inhibitor PD98059 increased mycobacterial multiplication in these cells (Shiratsuchi et al., 2008). In addition, in murine primary cells, inhibition of the p38 pathway resulted in enhanced mycobacterial survival following infection (Tse et al., 2002). Inhibition of the p38 pathway, however, does not affect mycobacterial multiplication in human cells (Blumenthal et al., 2002, Shiratsuchi et al., 2008). This

indicates a differential role of ERK and p38 pathways in regulation of mycobacterial replication in different species. Induction of cytokine production could be the main mechanism through which the degree of MAPK activity might influence the control of intracellular replication of mycobacteria (Blumenthal et al., 2002). Thus intracellular replication of *M. tuberculosis* and *S*. Typhimurium has been reported to be regulated by a network of kinases known as protein kinase B (Kuijl et al., 2007). Inhibition of this pathway could limit possible manipulation of the host signalling pathway by the bacteria and reduce intracellular growth of the bacteria.

An association between the virulence of *M. avium* morphotypes and the magnitude of activation of MAPK signalling pathways has also been studied previously (Blumenthal et al., 2002). It has been observed that there is an inverse correlation between virulence and the extent of signalling. A stronger signal of ERK1/2 and p38 phosphorylation has been detected in macrophages infected with a less virulent morphotype of *M. avium* compared to those infected with more virulent strains (Blumenthal et al., 2002). Therefore, this greater level of activation of signalling pathways could lead to a higher level of cytokine induction by less virulent strains of mycobacteria. Several studies have indicated that pathogenic mycobacteria are able to reduce cellular response and subsequently reduce the production of cytokines such as TNF-α and IL-1β (Falcone et al., 1994, Beltan et al., 2000). Roach and Schorey (2002), found that pathogenic and non-pathogenic mycobacteria infection in macrophages could differentially activate MAPK regulation. Non-

pathogenic *M. smegmatis* induced significantly higher MAPK activity and TNF-α production in murine bone marrow derived macrophage compared to pathogenic SmT *M. avium* 724 in these cells (Roach and Schorey, 2002). Moreover, macrophages challenged with avirulent SmO *M. avium* showed higher activity of MAPK and TNF-α production compared with the cells infected with virulent SmT *M. avium* (Tse et al., 2002). Although, it is accepted that MAPK signalling pathways are differentially activated by pathogenic and non-pathogenic mycobacteria, the effect of inhibition of MAPK on expression of cytokine is a controversial subject. Blumenthal et al. (2002), found that the effect of inhibition of MAPK on expression of cytokines was independent of virulence of *M. avium* morphotypes. However, it has been reported that cell signalling cascades are differentially involved in immune response to virulent and attenuated mycobacteria (Hasan et al., 2003). This could be either as a result of variation in cell surface interaction of mycobacteria or due to variation in trafficking with host cells (Armstrong and Hart, 1975). In the current study, cattle strains Ma.4 and Ma.5 induced a higher level of tested cytokine and chemokine in both THP-1 and HD11 cells and this could suggest that these two strains might be less virulent strains among all *M. avium* strains that been used in this study. In addition, the effect of MAPK inhibition in THP-1 cells in this study could be considered as strain dependent, while in HD11 cells this was not applicable. Therefore, it can be suggested that regulation of cytokine production could be cell dependent.

Subfamily members of MAPK pathways can be simultaneously or independently activated by mycobacterial components (Reiling et al., 2001, Jung et al., 2006). Accordingly, MAPK pathway modulation might lead to reduction of antimycobacterial cytokines and elevation of macrophages inhibitory cytokines as a result of the virulence of mycobacteria (reviewed by Jo et al., 2007). It has been reported that MAP kinases are involved in the activation of cells by virulent mycobacteria and these pathogenic mycobacteria are able to down regulate the signalling pathway (Hasan et al., 2003). As a consequence, it can be proposed that this ability of mycobacteria is an important virulence feature of these pathogens.

It would therefore be worthwhile to investigate the mechanisms that are used by more virulent strains to reduce activation of signalling pathways and subsequent reduction of cytokines production in host cells. It is known that pathogenic organisms have evolved several strategies for their survival and multiplication in host cells. *M. tuberculosis* and *M. bovis* BCG have been reported to inhibit expression of CD1 molecules in monocyte-derived dendritic cells via triggering phosphorylation of p38 MAPK pathway (Gagliardi et al., 2009). CD1 molecules play an important role in recognition of mycobacterial lipid antigens by T cells (Gagliardi et al., 2009). Interference with signalling pathways as a virulence factor of the pathogens has also been reported for many other organisms. For instance, *Yersinia* spp are able to interfere with host immune responses and stimulate cellular apoptosis. They perform this function via their YopJ protein (Palmer et al., 1998, Palmer et al.,

1999). This protein is able to inhibit activation of the NFκB and MAPK pathways in host cells (Orth et al., 1999). Some YopJ homologues have been found to perform a similar action in other pathogens. In *S.* Typhimurium the AvrA protein has been reported to inhibit NFκB during the early stage of infection (Collier-Hyams et al., 2002) and blocks the JNK signalling pathway at a later stage of the infection (Du and Galan, 2009). In addition, *Bacillus anthracis* has also the capability to inhibit the MAPK pathway via secretion of a toxin known as lethal factor (Duesbery et al., 1998). Taken together these findings support the concept that pathogenic organisms are able to modulate host immune responses by interference with signalling transduction in host cells using various mechanisms. It will be interesting to demonstrate what mechanism is used by pathogenic *M. avium* strains in particular to reduce host immune response.

Interference with signalling pathways is critically important for survival of the pathogenic mycobacteria in host cells in addition to suppression of immune response of the host cells (Koul et al., 2004). The precise mechanism of the ability of pathogenic mycobacteria to down regulate MAPK signaling pathways is not fully understood. It can be suggested that manipulation of MAPK activity could be a possible target of pathogenic mycobacteria. This ability can be considered as an important virulent mechanism of pathogenic mycobacteria which assists the mycobacteria to survive inside the macrophages. However, further experimentation is required to understand how pathogenic mycobacteria interfere with

MAPK activation and suppress production of immune mediated molecules and the non-pathogenic mycobacteria are not able to perform this function. Using microarrays might be useful to identify the possible differences in expression of genes between pathogenic and non-pathogenic mycobacterial species in order to provide a greater insight toward development of immunotherapeutic strategies in addition to designing a more efficient vaccine against mycobacterial diseases. Furthermore, different new technologies are now available which can reveal differences between pathogenic and non-pathogenic mycobacteria at the transcriptome level at different times. For instance, RNA sequencing has been used to produce a RNA snapshot from a genome at any given time. This technique can now provide much higher resolution than microarrays (Morozova and Marra, 2008, Grada and Weinbrecht, 2013).

Nair et al. (2011), found that the *M. tuberculosis* PPE18 protein can inhibit production of pro-inflammatory cytokines in host cells via inhibition of nuclear translocation of NFκB subunits p65 and p50. In addition, Man-LAM of *M. tuberculosis* has been suggested to block phosphorylation of MAPK by PMA in human THP-1 cells by upregulation of Src-homology2-domain containing tyrosine phosphatase 1 which thereby resulted in MAPK dephosphorylation (Knutson et al., 1998). Inhibition of the MAPK signaling pathway by *Salmonella* has also been reported. It was shown that the SptP protein of *S.* Typhi can perform this action through inhibition of activation of Raf as an activator of MKK and subsequently blocking the MAPK pathway (Lin et al., 2003).

In the current study inhibition of ERK in THP-1 cells by U0126 led to reduction of TNF-α but had no impact on IL-1β production in these cells in response to infection with *M. avium*. In addition, increased levels of TNF-α and IL-1β were observed following inhibition of p38 in THP-1 cells while the level of IL-6 and CXCL8 decreased in these cells. Furthermore, pre-treatment of JNK in THP-1 cells had no impact on the level of IL-1β and CXCL8 production at 24h post-infection. Taken together, the findings in this study suggest that production of different cytokines could be differentially regulated in response to challenge with *M. avium* in the context of the MAPK signalling pathway involved. In addition, signalling transduction of a cytokine could be different in various cell models. The p38 pathway negatively regulates production of IL-1β in THP-1 cells while this pathway plays important role in regulation of this cytokine in HD11 cells. It is likely that the regulation of cytokine signalling transduction is much more complex than was previously thought. It is therefore, worthwhile to investigate whether the cytokines are regulated differentially by MAPK pathways in response to other mycobacterial species infection. Moreover, investigation of the precise mechanism that is used by pathogenic species to modulate the cytokine signalling transduction could be of interest.

6.3 Chicken immune response to *M. avium* infection

Avian tuberculosis remains a major economic problem in poultry industries and is worldwide in distribution (Fulton and Sanchez, 2013). The chicken as a model of infection has rarely been used to evaluate immune response to *M. avium* infection which is the main

causative agent of avian tuberculosis in birds. In this study chickens were inoculated with three representative *M. avium* strains and the bacterial load, cytokine profile and histopathological examination were performed on tissue samples. The bacterial numbers reduced during the course of the experiment both in liver and spleen of infected birds. The chicken line used in the study was able to control infection with *M. avium*. The ability of this animal model to control experimental infection has previously been reported with *Salmonella* (Barrow et al., 1987) and *Campylobacter* in chickens (Jones et al., 2004), similarly to mycobacteria in mice (Nobrega et al., 2007). Since the examined strains are not known to be attenuated and were field strains, a degree of resistance to the infection for the chicken line can be suggested. Therefore, it would be worthwhile to investigate genetic resistance to *M. avium* infection which could provide a greater level of protection for flocks against disease which could be beneficial for both public health and economics. However, due to the complex nature of immunity breeding a more resistant chicken line whilst maintaining the appropriate level of productivity would be a difficult task. Thus, a different approach to improve resistance to pathogens is through the increased and improved use of adjuvants (reviewed by Lambrecht et al., 2009). Administration of phytochemicals could be a further way to improve host immunity. In addition to their role as antimicrobial agents (Hashemi and Davoodi, 2011), phytochemicals are able to improve host immunity against diseases such as coccidiosis by stimulating phagocytosis and production of reactive nitrogen species by macrophages and

granulocytes (Peek and Landman, 2011, reviewed by de Geus and Vervelde, 2013). Vaccination is a further obvious approach to protect chickens. Vaccination of chickens with *M. avium* fractions has been reported to reduce the number of lesions and the bacteria in liver but did not prevent infection (Falkinham et al., 2004). However, currently, commercial vaccines for protection against avian mycobacteriosis are not available. Viral- or DNA-vectored vaccines could be the next generation vaccines for chickens for administration also, for example, *in ovo* (Wu and Kaiser, 2011).

It is known that mycobacteria are able to modulate cytokine production and function as an important mechanism for intracellular survival of these bacteria in macrophages (Hines et al., 1995). IL-6 plays a major role in IFN-γ production and subsequent formation of granuloma in mycobacterial infection. An increase in bacterial load and delay in expression of IFN-γ have been observed in IL-6 knock out mice (Saunders et al., 2000). Since IFN-γ is crucial in induction of granuloma formation in mycobacterial infection, the elevation of this cytokine could be indicative of granulomata formation that was observed in the chicken livers in this study. In fact, this model represented very well the natural situation in other animal species where granulomata develop slowly in many individuals and, in fact, there is an argument that studying pathogenesis in a fully sensitive host does not give the complete picture. Similarly, experimental infection of resistant mice with *S.* Typhimurium showed that persistent infection might occur in these animals although they remain disease-free (Monack et al., 2004).

6.4 Future work

There are undoubtedly many ideas of interest based on the findings from this study that could be studied further to elucidate the full nature of the host immune response to *M. avium* in the chicken and explore new approaches to control. Future work might involve:

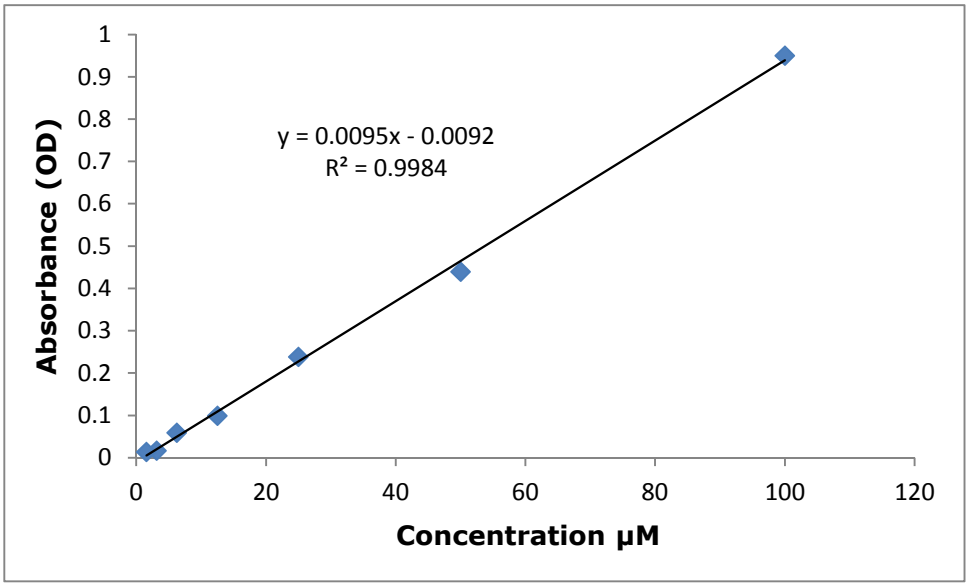
1- Using primary cells from chicken and humans such as monocyte-derived macrophages infected with different strains of *M. avium* rather than the cell lines used in this study.

2- Analysis of transcriptional factors particularly NFκB to investigate the possible differential activation following infection with *M. avium* infection in various cell types, for example, by the electrophoretic mobility shift assay.

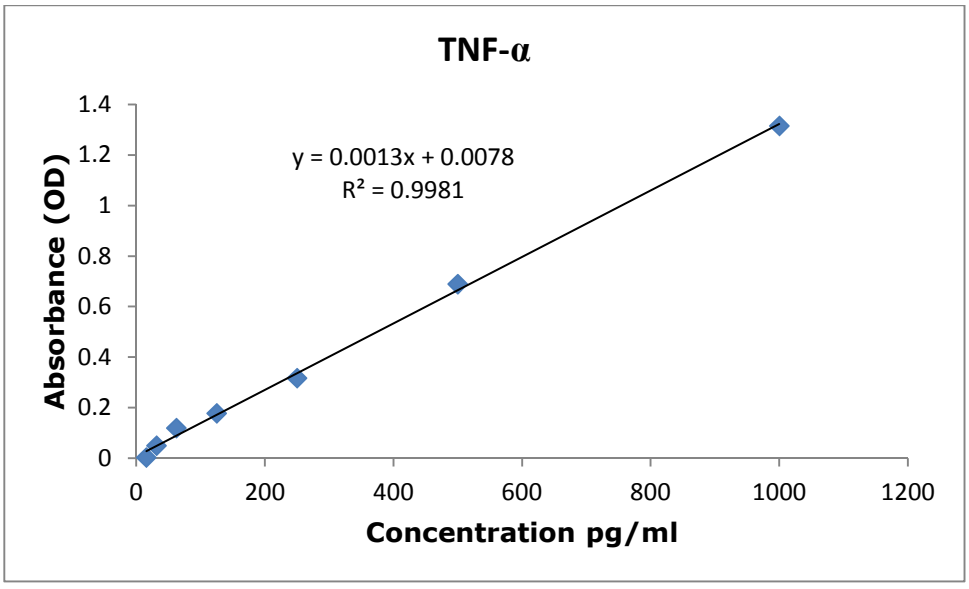
3- *In vivo* infection of a susceptible chicken line to further elucidate the immune response to the infection including extending the time course of the experiment.

4- The development of the granuloma in a resistant animal and the effects of dead bacteria on this process.

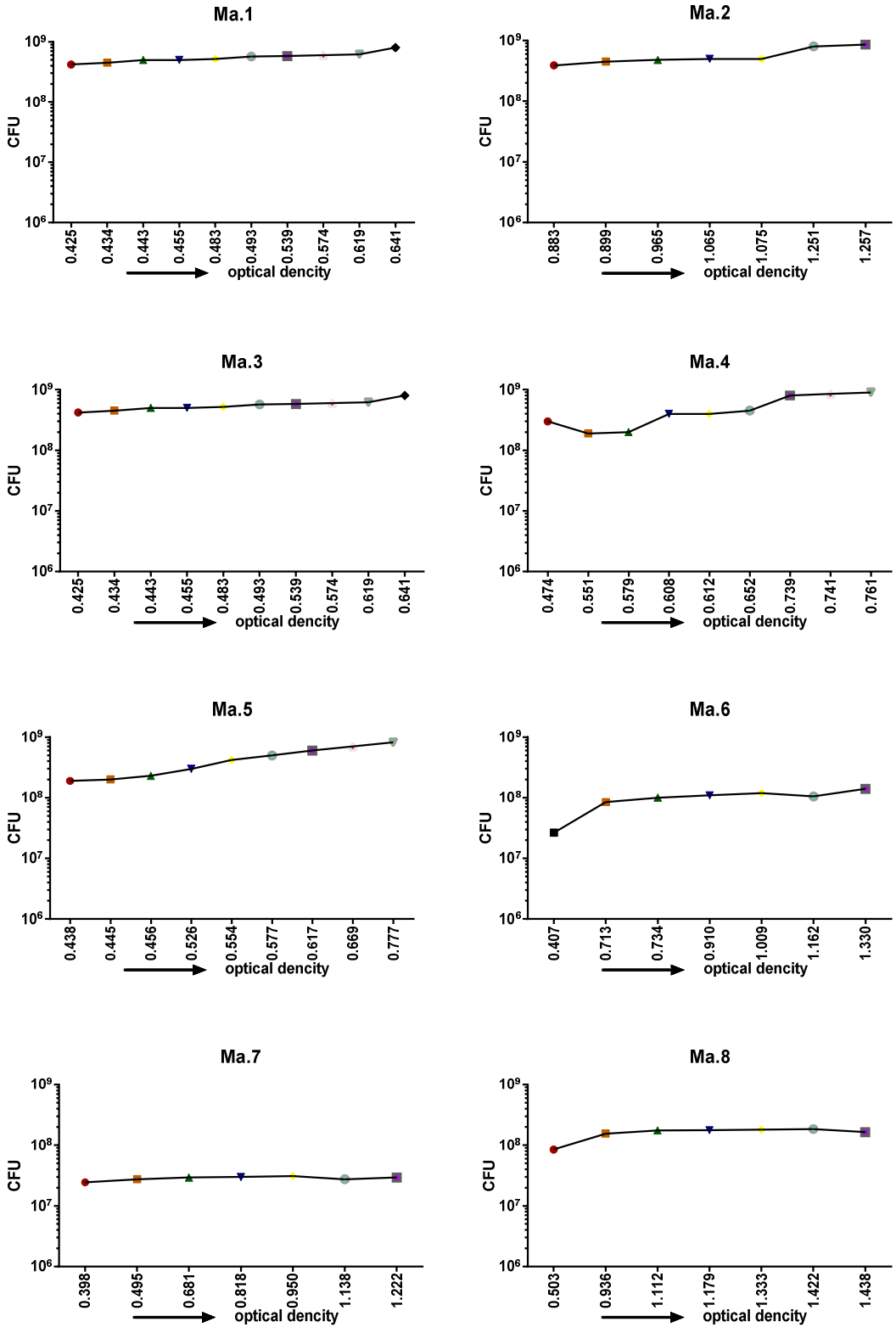
Appendices



Appendix 1: An example of a nitric oxide standard curve for assessment of concentration of nitrite in HD11 cells supernatants.



Appendix 2: An example of a TNF-α standard curve for assessment of concentration of this cytokine in THP-1 cells supernatants.



Appendix 3: Calibration graphs between the bacterial counts and the optical density of various *M. avium* strains.

Appendix 4: Solutions used in ELISA

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered.

Wash Buffer: 0.05% Tween 20 in PBS, pH 7.2-7.4.

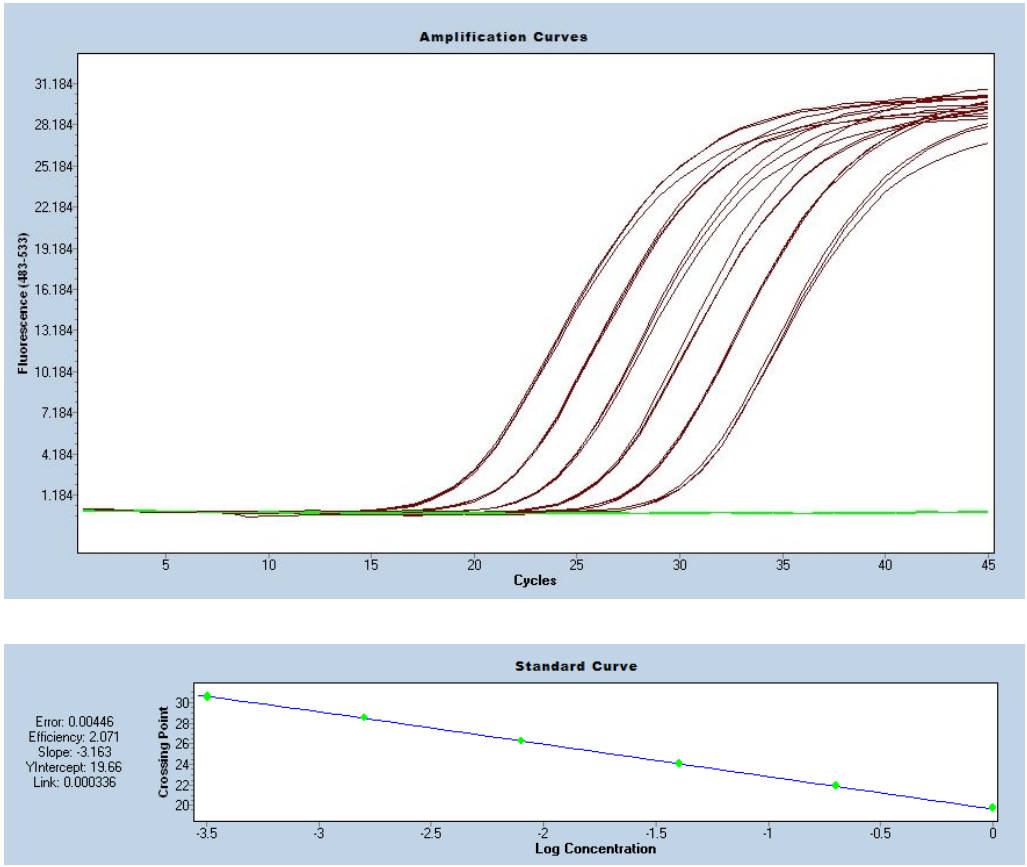
Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered.

Block Buffer: 1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered (for CXCL-8 measurement).

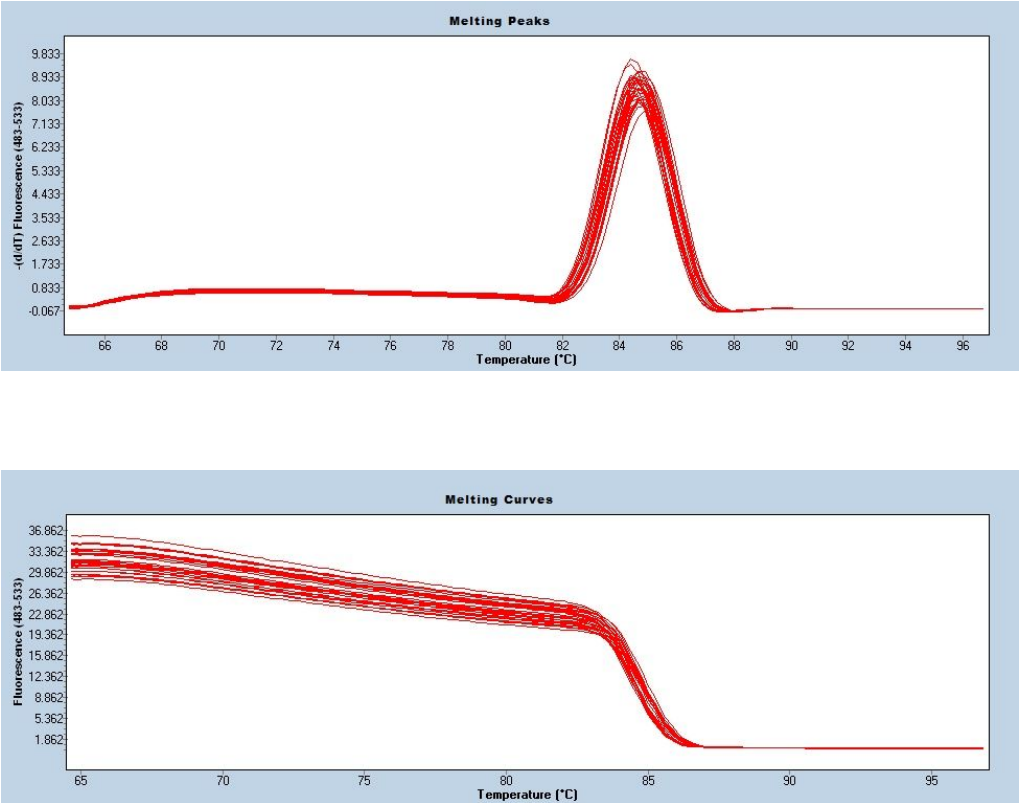
Reagent Diluent (for CXCL8 measurement): 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4, 0.2 µm filtered.

Substrate Solution: 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine).

Stop Solution: 2 N H_2SO_4.



Appendix 5: A snapshot of qRT-PCR standard curve for IL-1β in HD11 cells.

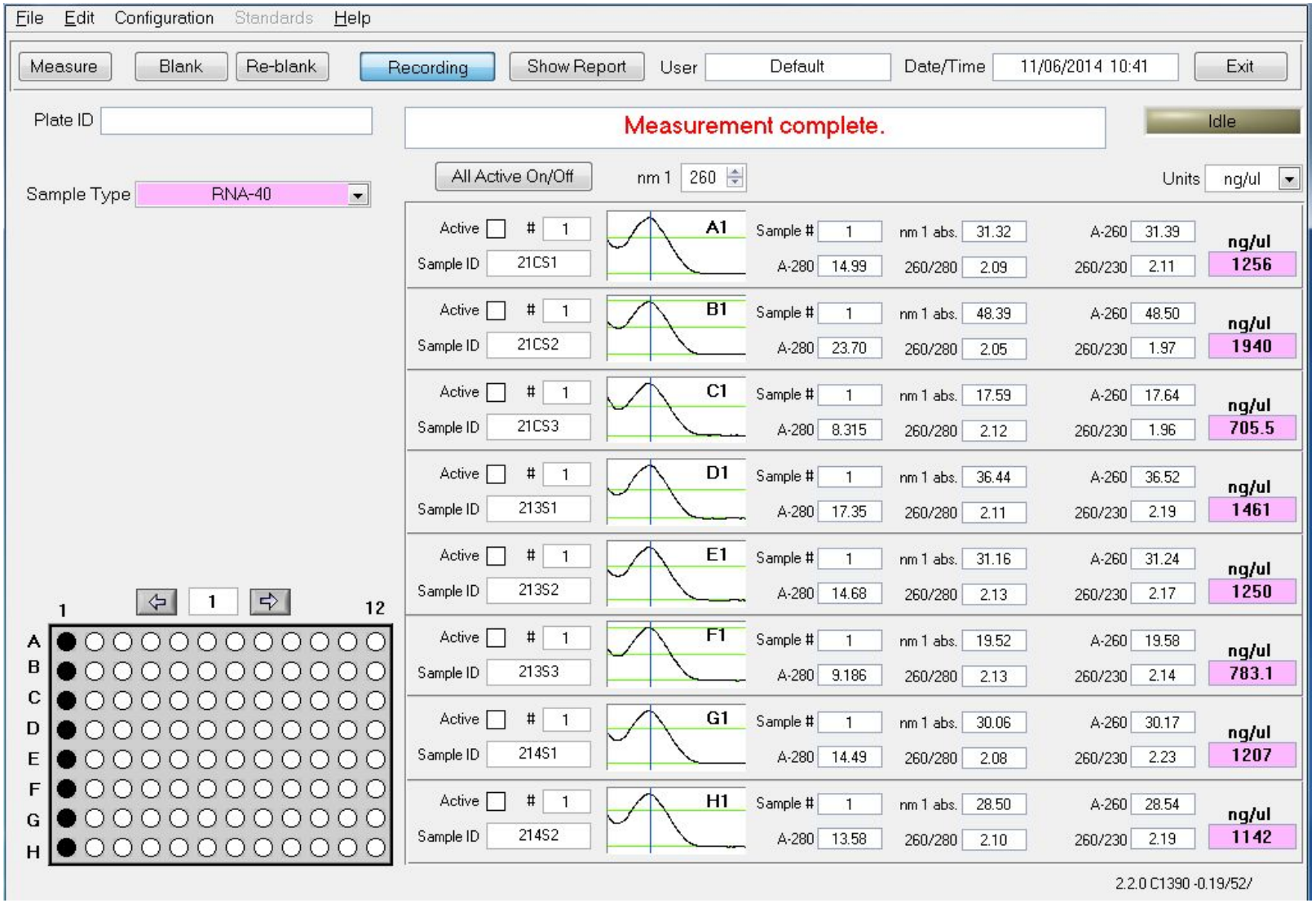


Appendix 6: A snapshot of dissociation (melting) curves of TNF-α expression in THP-1 cells quantified by qRT-PCR.

Appendix 7: Semi-quantitative scoring of the lesions in chicken livers

Liver 21 days post-inoculation	Chicken no.1	Chicken no.2	Chicken no.3
Non-infected chickens	-	-	-
Ma.3-infected chickens	++	++	++
Ma.4-infected chickens	++	++	+
Ma.5-infected chickens	+++	++	+++

- No lymphocyte infiltration
+ Few lymphocyte infiltration
++ Moderate lymphocyte infiltration
+++ Abundant lymphocyte infiltration



Appendix 8: Measurement of quantity and quality of RNA by NanoDrop spectrophotometer of spleen samples from chickens at 21 days post-inoculation.

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