Gene expression changes in macrophages infected with pathogenic M. tuberculosis and non-pathogenic M. smegmatis and M. bovis BCG

By

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ..VC Mpongoshe... Date: April 2014

Abstract

The current anti-TB drugs have had success in decreasing the number of deaths caused by TB, however, this success is limited by the emergence of drug resistant TB strains. Therefore, a novel TB therapy that limits the development of resistance has become necessary in an attempt to effectively control TB. The anti-TB drugs directly target mycobacterial enzymes, and potentiate the development of this resistance, and have therefore provided the rationale for this study. The aim was therefore to identify host macrophage genes that affect *M. tb* intracellular survival. The proposed alternative anti-TB therapy potentially involves the application of RNA interference (RNAi) and RNA activation (RNAa) biological processes that will target host genes, thereby inducing an indirect bactericidal effect. We hypothesized that macrophage genes that are differentially expressed by pathogenic and non-pathogenic mycobacterial species may be important in the regulation of *M. tb* intracellular survival. The lipid-rich mycobacterial cell wall is implicated in the excessive clumping of the mycobacterial cells in liquid culture. In order to minimize this, Tween 80 detergent was supplemented (mycobacteria$^{\mathbf{T}}$). However, due to substantial evidence emphasising the detrimental effects of Tween 80 on the mycobacterial cell wall, mycobacteria were also cultured without Tween 80 (mycobacteria$^{\mathbf{NT}}$), in order to investigate if the perturbed mycobacterial cell wall induced by Tween 80 affects the transcriptional response of macrophages. We endeavoured to develop a new method to culture mycobacteria without Tween 80 that will still generate single cells. We further hypothesized that the macrophage gene expression profile induced by mycobateria$^{\mathbf{NT}}$ differs from the response induced by mycobacteria$^{\mathbf{T}}$.

Differentiated THP-1 (dTHP-1) cells were infected with pathogenic and non-pathogenic mycobacteria (for 3 h, 24 h and 48 h with *M. tb* and *M. bovis BCG*, and 3 h and 8 h with *M. smegmatis*) cultured in the presence or absence of Tween 80. The expression of 12 macrophage genes, selected based on their involvement in the phagocytic pathway and autophagy, as well as their general involvement in the immune response, was determined by qRT-PCR and further analysed on the REST programme. The expression of each target gene was normalised relative to the expression of the reference gene (Beta actin).

We observed that out of the 12 genes, TLR7 and VAMP7 were consistently downregulated in dTHP-1 cells infected with *M. tb*^{NT} and upregulated in dTHP-1 cells infected with *M. smegmatis*^{NT}. Their response to *M. bovis* BCG was inconsistent and not significantly different, and therefore could not be interpreted. Furthermore, CCL1 was upregulated by all the mycobacterial species. However, its expression was more pronounced in response to mycobacteria^{NT}, when compared to mycobacteria^{T}.

Differential gene expression of TLR7 and VAMP7 in response to pathogenic and non-pathogenic mycobacteria^{NT} suggests that these 2 genes may be potential targets for RNAa-based anti-TB therapy, even though we could not conclude whether their response was specific to macrophages. In addition, the observed difference in the expression of CCL1 induced by mycobacteria^{NT}, compared to mycobacteria^{T} suggests that the perturbation caused by Tween 80 on the mycobacterial cell wall most likely affected the response of macrophages to infection with mycobacteria. Furthermore, this study has demonstrated a feasible method by filtration to generate single cells from mycobacteria^{NT}, which should be considered for future mycobacterial infection studies.

Uittreksel

Die huidige anti-tuberkulose middels se sukses lê daarin dat dit die aantal sterftes verminder maar hierdie sukses word weer beperk met die ontstaan van middel-weerstandige *M.tb* stamme. Daarom is nuwe middels nodig wat die ontwikkeling van middel-weerstandigheid beperk in 'n poging om effektiewe TB behandeling te bewerkstellig. Anti-tuberkulose middels teiken hoofsaaklik mycobakteriële ensiemsisteme en ontlok sodoende weerstandigheid in *M.tb* stamme en dit vorm die rasionale vir hierdie studie. Die doel was om gasheer makrofaag gene te identifiseer wat *M.tb* oorlewing intrasellulêr bewerkstellig. Die voorgestelde alternatiewe anti-TB behandeling sal dan behels die toepassing van RNA intervensie (RNAi) en RNA aktivering (RNAa) tegnologie wat gasheer selgene teiken (inaktiveer) en sodoende 'n bakterisidiese respons induseer. Die kanse is skraal dat mycobakterieë weerstandigheid sal kan ontwikkel onder hierdie omstandighede. Ons hipotetiseer dus dat makrofaag gene wat differensieel uitgedruk word deur patogeniese en nie-patologiese mycobakteriële spesies belangrik mag wees vir die oorlewing van *M.tb* intrasellulêr. Die lipiedryke selwand van mycobakterieë word geïmpliseer in die oormatige sameklomping van die bakterieë in vloeistofkulture. Om hierdie effek te minimaliseer word Tween 80 normaalweg tot die medium gevoeg (mycobakterieë^{T}). Maar weens genoegsame bewyse dat Tween-80 die selwand van bakterieë nadelig beïnvloed, is mycobakterieë ook in die afwesigheid van Tween 80 gekultureer (mycobakterieë^{NT}) om te bepaal of die nadelige effek van Tween 80 op die selwand die transkripsionele respons in makrofage beïnvloed post-infeksie. Dit was daarom ook ons doelstelling om 'n nuwe tegniek te ontwikkel om mycobakterieë te kultureer in die afwesigheid van Tween 80 wat ook enkelselle sal genereer vir beter gekontroleerde makrofaag infeksie. Ons hipotetiseer ook verder dat makrofaag geenuitdrukking-profiele verskil afhangende of infeksie gedoen is met mycobakterieë wat in die afwesigheid of teenwoordigheid van Tween 80 gekultureer is.

Gedifferensieerde THP-1 (dTHP-1) was geïnfekteer met patogeniese en nie-patogeniese mycobakterieë (vir 3 h, 24 h en 48 h met *M.tb* en *M.bovis BCG*, en 3 h en 8 h met *M.smegmatis*) gekultureer in die teenwoordigheid en afwesigheid van Tween 80. Die uitdrukking van 12 makrofaag gene, geselekteer op grond van hul betrokkenheid in die fagositose meganisme en in outofagie asook hul betrokkenheid in die immuunrespons, is

gekwantifiseer met qRT-PCR en daaropvolgens geanaliseer met die REST-program. Die uitdrukking van elke geen is genormaliseer relatief tot die uitdrukking van die verwysingsgeen (Beta actin). Daar is bevind dat van die 12 gene, TLR7 en VAMP7 deurlopend afgereguleer was in dTHP-1 selle geïnfekteer met *M.tb*^{NT} en opgereguleer was in dTHP selle geïnfekteer met *M.smegmatis*^{NT}. Selrespons met *M.bovis* BCG was onbeduidend en derhalwe kon geen gevolgtrekking hier gemaak word nie. Ook, CCL1 was opgereguleer met infeksie deur enige van die mycobakteriële spesies, maar CCL1 se uitdrukking was groter in respons tot mycobakterieë^{NT} wanneer vergelyk word met respons tot mycobakterieë^{T}.

Differensiële geenuitdrukking van TLR7 en VAMP7 in respons tot patogeniese en nie-patogeniese mycobakterieë^{NT} impliseer dat hierdie twee gene potensiële teikens kan wees vir RNAa-gebaseerde anti-TB behandeling, alhoewel ons nie kon beslis of hierdie respons spesifiek vir makrofage was nie. Ook, die verskille waargeneem in die uitdrukking van CCL1 geïnduseer deur mycobakterieë^{NT}, vergeleke met mycobakterieë^{T}, impliseer dat die steuring in die selwand veroorsaak deur Tween 80, heelwaarskynlik die respons van die makrofaag beïnvloed het. Hierdie studie beskryf ook ’n filtrasiemetode om enkele mycobakteriële selle te genereer wat oorweeg moet word by toekomstige mycobakteriële infeksiestudies.

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%:	Percentage
°C:	Degree Celsius
µl:	Microlitre
ACTB:	Beta actin
AFB:	Acid fast bacteria
ATCC:	American Type Culture Collection
BCG:	Bacillus Calmette Guérin
Bp:	Base pair
CBTBR:	Centre of Excellence in Biomedical TB Rsearch
CCL1:	Chemokine ligand 1
CCL2:	Chemokine ligand 2
CCR8:	Chemokine receptor 8
CD11b:	Cluster of differentiation molecule 11 B
CD18:	Integrin beta-2
cDNA:	Complementary DNA
CFUs:	Colony forming units
CO_2:	Carbon dioxide
CR3:	Complement receptor 3
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
dsRNA:	Double stranded RNA
dTHP-1:	Differentiated Acute monocyte leukemia cell line
ER:	Endoplasmic reticulum

ESAT:	6 kDa early secretory antigen target
ESX-1:	ESAT-6 secretion system-1
FCS:	Fetal calf serum
G:	Gauge
GAPDH:	Glyceraldehyde-3-phosphate
gDNA:	Genomic DNA
h:	Hour
H_2O:	Water
HIV:	Human immunodeficiency virus
IFN-γ:	Interferon- γ
IL-10:	Interleukin 10
IL-12:	Interleukin 12
IL-2:	Interleukin 2
IL-8:	Interleukin 8
kDa:	kilo Dalton
LC480:	LightCycler 480
M. avium:	*Mycobacterium avium*
M. bovis BCG:	*Mycobacterium bovis* BCG
M. marinum:	*Mycobacterium marinum*
M. paratuberculosis:	*Mycobacterium paratuberculosis*
M. smegmatis:	*Mycobacterium smegmatis*
M. tb:	*Mycobacterium tuberculosis*
MAC:	Mycobacterium avium complex
min:	Minutes
ml:	Millilitre
MOI:	Multiplicity of infection

MR:	Mannose receptor
MyD88:	Myeloid differentiation primary response
ng:	Nanogram
nm:	Nanometre
NOS:	Nitrogen oxidative species
^{NT}:	Cultured without Tween 80
OADC:	Oleic acid, albumin, dextrose and catalase
OD:	Optical density
OIS:	Oxidative intermediate species
PAMPS:	Pathogen-associated molecular pattern
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
pH:	Hydrogen ion concentration
PMA:	Phorbol 12-myristate 13-acetate
PPD:	Purified protein derivative
PRRs:	Pattern recognition receptors
qRT-PCR:	Quantitative Real-Time PCR
RD1:	Region of difference 1
REST:	Relative expression software tool
RIN:	RNA integrity number
RNAa:	RNA activation
RNAi:	RNA interference
rRNA:	Ribosomal RNA
S. flexneri:	*Shigela flexneri*
s:	Seconds
shRNA:	Small hairpin RNA

siRNA:	Small interfering RNA
SNAREs:	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SNPs:	Single-nucleotide polymorphisms
STX4:	Syntaxin 4
STX7:	Syntaxin 7
^{T}:	Cultured with Tween 80
TB:	Tuberculosis
TGF-β:	Transforming growth factor beta
TLR:	Toll-like receptor
TNF-α:	Tumor necrosis factor alpha
U/ ml:	Unit per millimetre
UV light:	Ultra violet light
VAMP7:	Vessicle-associated membrane protein 7
VHS:	Viral haemorrhagic septicaemis virus
WHO:	World Health Organization
x *g*:	Times gravity
X:	Times
ZN:	Ziehl Neelsen
α:	Alpha
β:	Beta
B-2-M:	Beta-2-Microglobulin
γ:	Gamma

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Literature review

1.1 Background

Tuberculosis (TB) is a disease that is caused by infection with *Mycobacterium tuberculosis* (*M. tb*). It is transmitted among individuals via inhalation of aerosols produced through coughing and sneezing by infected individuals (Shakri *et al*., 2012). This bacterium enters the body via the respiratory route and binds to specific pattern recognition receptors (PRRs) on the surface of lung macrophages, allowing it to gain entry into macrophages, where it resides and replicate (Aderem and Underhill, 1999; Chacón-Salinas *et al*., 2005; Ernst, 1998; Gordon and Read, 2002; Hu *et al*., 2000). PRRs are proteins involved in the recognition of molecular patterns that are associated with microbial pathogens, resulting in activation of the innate immune response for subsequent elimination of pathogens (Court *et al*., 2010; Schiller et al., 2006). *M. tb* pathogenicity is due to its ability to withstand the hostile environment of macrophages (Schlesinger, 1996; Simeone *et al*., 2012) and its intracellular survival depends on the host factors (Jayaswal et al., 2010; Simeone *et al*., 2012).

Despite the discovery of TB over a century ago, the availability of the *Mycobacterium bovis* Bacillus Calmette Guérin (*M. bovis* BCG) vaccine and anti-TB drugs, this disease is still one of the major daunting challenges in public health (Mehra *et al*., 2013; Simeone *et al*., 2012). The major factors interfering with TB control are mainly attributed to the inability of *M. bovis* BCG vaccine to provide permanent protection (Ottenhoff and Kaufmann, 2012), HIV co-infection (Matthews *et al*., 2012), the lengthy period of TB treatment regime (Padmapriyadarsini et al., 2011) as well as the emergence of drug resistant *M. tb* strains (Fakruddin, 2013; WHO; 2010; Yokobori *et al*., 2013). The latter is the primary basis of the existing battle in drug development research (Nguyen and Pieters, 2009). A novel approach that will limit the extent of resistance has therefore become necessary as an attempt to effectively control TB.

1.2 Macrophages as primary cells of *M. tb* infection

Macrophages are phagocytes and primary cells of *M. tb* infection. Their ability to internalise, process and present antigens of invading pathogens for subsequent degradation makes them essential for host defence against infection (Harding and Boom, 2010; Martín-Orozco *et al*., 2001; Wynn *et al*., 2013). Macrophages are involved in activation and maintenance of the immune responses (Chaurasiya and Srivastava, 2008; Giacomini et al., 2001; Wang *et al*., 2003). Their primary role is to kill invading microbes; however, it has become clear that they are unable to permanently eliminate pathogenic mycobacteria such as *M. tb* (Giacomini *et al*., 2011; Jayaswal *et al*., 2010; Reljic *et al*., 2010; Spira *et al*., 2003; Volpe *et al*., 2006). Their killing mechanisms are not entirely understood, however, they involve the fusion of phagosomes with lysosomes (Fairbairn *et al*., 2001; Zimmerli *et al*., 1996), production of proinflammatory cytokines including IL-12, IFN-γ and TNF-α (Giacomini *et al*., 2011, 2001; Reljic *et al*., 2010) and induction of apoptosis as means of protecting the host from intracellular pathogens (Behar *et al*., 2011).

1.3 Macrophage - *M. tb* interaction

Humans are equipped with an immune system (divided into innate and adaptive) that protects the body against diseases by eliminating invading pathogenic and non-pathogenic microorganisms (Nguyen and Pieters, 2009). Upon infection, macrophages become activated and produce cytokines as part of the immune response, and the fate of infection depends on the level of cytokines produced (Giacomini *et al*., 2011, 2001; Reljic *et al*., 2010).

Cytokines are small protein molecules produced by the cells of the immune system, including macrophages. They are involved in cell signalling and have been classed as interleukins, interferons and chemokines based on the cell type they are secreted from; their presumed function and target (Berrington and Hawn, 2007; Giacomini *et al*., 2011, 2001). They bind to target cell receptors by autocrine, paracrine or endocrine action and stimulate signalling pathways to induce a biological response (Renner *et al*., 1996; Sherry and Cerami, 1988; Suga *et al*., 1993). They are implicated in the response of the immune system against *M. tb* infection and are divided into proinflammatory and immunosuppressive groups, based on their roles in the immune system. The role of proinflammatory cytokines is to orchestrate the immune response, in order to eliminate the invading pathogens. They are involved in the

regulation of innate and adaptive immunity (Giacomini *et al*., 2011, 2001). In contrast, immunosuppressive cytokines induced by *M. tb*, suppress the immune system by counteracting its antimicrobial responses, thereby leading to progression of infection to TB disease (Giacomini *et al*., 2011, 2001)

Increased levels of proinflammatory cytokines result in either *M. tb* clearance or dormancy in the granuloma, whereas, an abundance of immunosuppressive cytokines lead to the progression of *M. tb* infection to TB disease (Figure 1.1) (Giacomini *et al*., 2011, 2001; Reljic *et al*., 2010; Rodríguez-Herrera and Jordán-Salivia, 1999). Furthermore, macrophages may induce a process known as autophagy, a defence mechanism initiated by the innate system for the elimination of intracellular pathogens (Gutierrez *et al*., 2004). Autophagy involves fusion of autophagosomes (organelles containing cytoplasmic material including phagosomes) with lysosomes, followed by lysosomal enzymatic digestion and elimination of the autophagosome contents (Gutierrez *et al*., 2004; Nakagawa *et al*., 2004).

The initial interaction between macrophages and *M. tb* is important for the fate of the infection (Giacomini *et al*., 2011; Reljic *et al*., 2010). Depending on the bacterial load and the virulence of the infecting mycobacterial species, macrophages can either be activated to combat infection or induce apoptosis (Placido *et al*., 1997; Riendeau and Kornfeld, 2003; Rodríguez-Herrera and Jordán-Salivia, 1999). Apoptosis, which acts as a bridge between innate and adaptive immune responses, is the process of programmed cell death employed by host macrophages to destroy the invading intracellular pathogens, by eliminating the supportive environment for bacterial growth (Keane *et al*., 2000; Riendeau and Kornfeld, 2003; Schaible *et al*., 2003). Infected macrophages attract immune cells that play a role in the immune response, to the site of infection (Ragno *et al*., 2001), resulting in formation of the granuloma. Granuloma is a hallmark of *M. tb* infection and it is an immune mechanism induced in attempt to contain the infecting pathogens that the immune system is unable to eliminate, such as *M. tb* (Hoshino *et al*., 2007; Tal et al., 2007; Thuong *et al*., 2008).

In response, the parasitization of *M. tb* in macrophages lead to subversion of bactericidal processes of macrophages, such as phagosome maturation and phagosome-lysosome fusion (Clemens and Horwitz, 1995; Koul *et al*., 2004; Xu *et al*., 1994), secretion of

proinflammatory cytokines by enhancing the production of immunosuppressive cytokines such as IL-10 (Giacomini *et al*., 2011, 2001; Koul *et al*., 2004), presentation of antigens (Garcia-Romo *et al*., 2013; Harding and Boom, 2010; Koul *et al*., 2004), induction of the respiratory burst (rapid release of the reactive oxygen species) (Ehrt and Schnappinger, 2009) and induction of apoptosis (Divangahi *et al*., 2009; Koul *et al*., 2004; Riendeau and Kornfeld, 2003; Spira *et al*., 2003). Although the molecular mechanisms by which *M. tb* disrupts phagosolysosome fusion are not entirely understood, Mehra *et al* (2013) reports that *M. tb* EsxH (effector molecule that has been linked to *M. tb* pathogenesis and survival within host cells) directly targets host Hrs (a component of the endosomal sorting complex required for transport (ESCRT)) to impair phagosomal traffick to lysosomes (Ilghari et al., 2011; A. Mehra *et al*., 2013). Another study, by Malik *et al* (2001) links the blockade of phagolysosome fusion to calcium deficiency in human macrophages. They report that this was due to *M. tb* inhibiting the activation of calcium-dependent effector proteins, calmodulin and calmodulin-dependent protein kinase II (Malik *et al*., 2001). In addition, it is known that subsequent to phagosome-lysosome fusion, mycobacteria remain in phagolysosomes for subsequent degradation by lysosomal enzymes (Figure 1.1). However, there has been accumulating evidence that *M. tb* can rupture the phagolysosomes, induce necrosis of infected macrophages and escape to multiply in new, non-fused phagosomes as well as in the cytoplasm. This phagolysosomal escape is associated with *M. tb* defence mechanism to evade the innate immunity (McDonough *et al*., 1993; Simeone *et al*., 2012). Furthermore, after several years of survival in a dormant state in granulomas, *M. tb* can reactivate and escape to spread to other tissues, resulting in infection progression to TB (Bold and Ernst, 2009; Silva Miranda *et al*., 2012). The interaction of macrophages with *M. tb* therefore represents a balance between macrophage antimicrobial activities and *M. tb* evasion mechanisms (Crevel *et al*., 2002).

1.3.1 Primary PRRs for *M. tb* phagocytosis

PRRs control the phagosomes/endosomes trafficking to lysosomes (Kang *et al*., 2005). *M. tb* is internalised by phagocytes such as macrophages via various surface receptors, of which complement and mannose receptors are primary for phagocytosis (Bermudez *et al*., 1999; Kang *et al*., 2005; Killick *et al*., 2013; Schlesinger, 1993; Spira *et al*., 2003).

CR3 is a heterodimer cell surface receptor consisting of CD11b and CD18 protein subunits (Ehlers, 2000; Melo *et al*., 2000; Velasco-Velázquez *et al*., 2003). CR3 plays a role in *M. tb* phagocytosis but not in the subsequent intracellular survival (Hu *et al*., 2000; Melo *et al*., 2000). *M. tb* is able to bind CR3 opsonically as well as non-opsonically (Melo *et al*., 2000; Velasco-Velázquez *et al*., 2003). CR3 is essential in clearing mycobacterial infection; however it does not induce killing of *M. tb* when it is bound non-opsonically (Le Cabec *et al*., 2000; Rooyakkers and Stokes, 2005). Moreover, several studies have reported that the entry of *M. tb* into macrophages through opsonic binding to CR3, results in the inhibition of IL-12 production due to extracellular calcium influxes that result from receptor ligation as well as from the release of the reactive oxygen species (ROS) (Marth and Kelsall, 1997; Sutterwala *et al*., 1997). *Ex vivo* studies are in contrast with the *in vivo*, regarding the importance of CR3 in *M. tb* phagocytosis. In an *ex vivo* study conducted in macrophages, it was observed that the ability of macrophages to bind and phagocytose *M. tb* was impaired when antibodies were produced against CR3, suggesting that CR3 is the predominant macrophage receptor for *M. tb* phagocytosis (Melo *et al*., 2000; Schlesinger *et al*., 1990; Spira *et al*., 2003; Velasco-Velázquez *et al*., 2003). Conversely, in *in vivo* studies it was observed that *M. tb* was able to enter macrophages in the absence of CR3, suggesting that it uses alternative receptors. Furthermore, the course of *M. tb* infection was not altered in mice lacking CR3 (Hu *et al*., 2000; Schlesinger *et al*., 1990).

The MR is found on the surface of macrophages and it is implicated in the inhibition of fusion of phagosomes with lysosomes. When MR is blocked or absent, phagosome-lysosome fusion is enhanced, indicating that the entry via MR limits fusion of phagosomes with lysosomes (Kang *et al*., 2005).

1.3.2 Cytokines involved in *M. tb* infection

1.3.2.1 Common proinflammatory cytokines in regulation of *M. tb* infection

Interferon gamma (IFN-γ) is the chief cytokine in the activation of macrophages, with the aim to combat the infection (Reljic *et al*., 2010) by contributing to antibacterial activities induced by macrophages (Denis, 1991; Giacomini *et al*., 2011). IFN-γ plays a major role in controlling *M. tb* infection in host cells (Herbst *et al*., 2011; O'Leary *et al*., 2011; Rooyakkers

and Stokes, 2005). Administration of IFN-γ to TB patients was observed to improve the clearance of infection. In addition, mutation in the IFN-γ receptor and antibodies to IFN-γ lead to the spread of *M. tb* infection (Seneviratne *et al*., 2007); Dorman *et al*., 2004). *In vitro,* IFN-γ was observed to inhibit the growth and replication of the pathogen, whereas *in vivo* it was essential in the formation of granuloma and the containment of *M. tb* infection (Cooper *et al*., 1993; Flynn *et al*., 1993; Sechler *et al*., 1988)

Interleukin 12 (IL-12) is involved in the generation of immune response against *M. tb* infection. It acts as an autocrine and paracrine to activate macrophages in order to induce antimicrobial response, including the production of IFN-γ (Giacomini *et al*., 2001; Robinson *et al*., 2010). Mice and humans deficient in IL-12 are highly susceptible to *M. tb* infection and are unable to control its growth, due to the absence of IFN-γ (Cooper *et al*., 1997; Jouanguy *et al*., 1999). Treatment with IL-12 is associated with the reduction of mycobacterial growth (Robinson and Nau, 2008).

TNF-α is a potent proinflammatory cytokine which together with interferon-gamma (IFN-γ) plays an important role in activating antimicrobial activities in macrophages, including inhibition of mycobacterial growth (Giacomini *et al*., 2011; Sharma *et al*., 2004). TNF-α is essential in the control of TB in humans and it is involved in the formation of granulomas Infection of mice deficient in TNF-α with *M. tb* was reported to impair granulomas, leading to resuscitation of *M. tb* infection to TB disease (Bean *et al*., 1999; Silva Miranda *et al*., 2012). Moreover, TNF-α is involved in induction of IFN-γ production, and apoptosis, during the early stages of *M. tb* infection (Beltan *et al*., 2000; Flynn *et al*., 1995; Giacomini *et al*., 2001; Wu *et al*., 2012).

1.3.2.2 Common immunosuppressive cytokines in regulation of *M. tb* infection

IL-10 is a potent immune suppressant, therefore, its abundance is associated with decreased ability of the host to control *M. tb* infection (O'Leary *et al*., 2011; Oswald *et al*., 1992; Redpath *et al*., 2001). IL-10 is known for its role in inhibiting the functions of macrophages (Redford *et al*., 2011), such as the production of proinflammatory cytokines, thereby allowing growth of *M. tb* inside macrophages. IL-10 strongly suppresses IL-12 expression, resulting in

reduced production of IFN-γ and failure to eliminate *M. tb* infection (Cunha *et al*., 1992; Fiorentino *et al*., 1991; Giacomini *et al*., 2001; Gong *et al*., 1996). Furthermore, several studies have indicated that the absence of IL-10 during *Mtb* infection elicit phagosome maturation and bacterial clearance (Murray and Young, 1999; Via *et al*., 1998).

TGF-β, like IL-10 decreases the activation of macrophages and the production of proinflammatory cytokines (Aung *et al*., 2000; Toossi and Ellner, 1998). The excessive production of TGF-β is coupled with the progression of *M. tb* infection to TB. TGF-β was reported to be present in the granulomas of TB patients (Aung *et al*., 2000; Toossi *et al*., 1995).

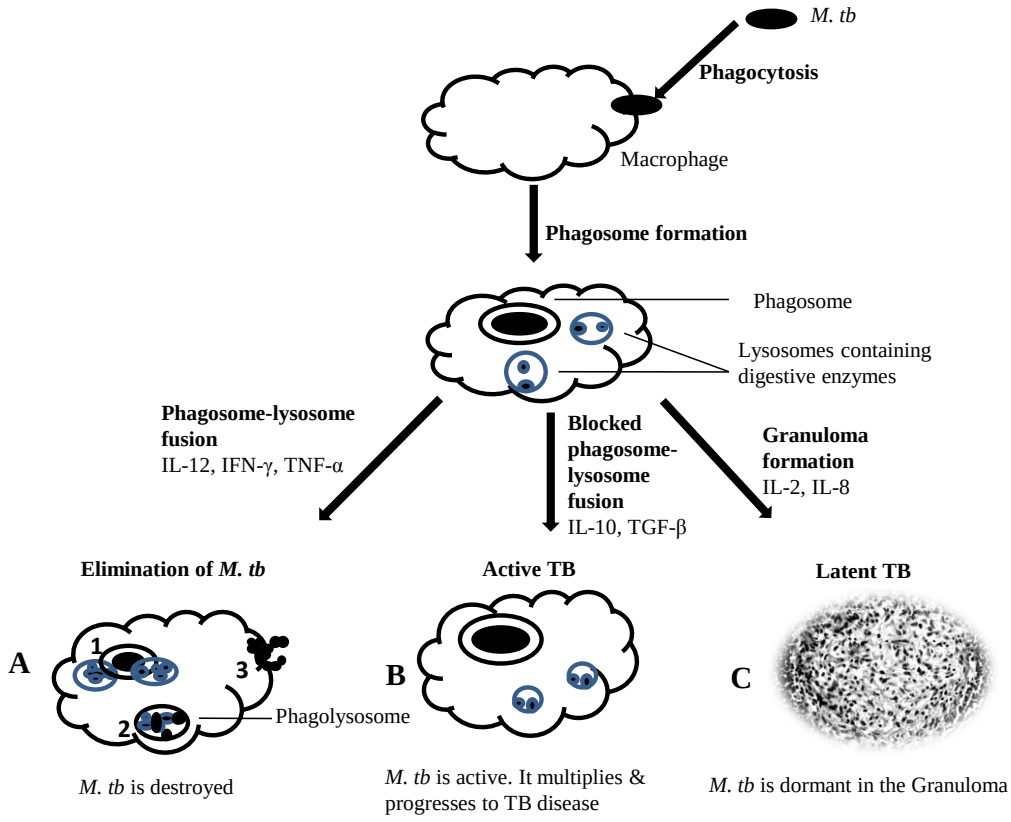

Figure 1.1 Different outcomes of *M. tb* infection and primary cytokines involved. A: High levels of proinflammatory cytokines and fusion of phagosomes with lysosomes lead to *M. tb* elimination. A: 1- phagosome-lysosome fusion, 2- enzymatic digestion and destruction of *M. tb* inside phagolysosome, 3- *M. tb* elimination. B: High levels of immunosuppressive cytokines and inhibition of phagosome-lysosome fusion lead to active TB. C: During latent TB, IL-2 and IL-8 are present in high levels and *M. tb* is dormant in the granuloma.

1.4 Selection rationale for the 12 genes studied

1.4.1 Toll-like receptors (TLRs)

TLR7 and TLR9 are members of the TLR family. They are endosomal PRRs found on cell surfaces (Avunje *et al*., 2011; Brown *et al*., 2011; Delgado *et al*., 2008). As PRRs, their primary role is to recognise invading pathogens and activate innate immunity in order to eliminate the invading pathogens (Crevel *et al*., 2002; De Meyer *et al*., 2012; Delgado *et al*., 2008). TLR9 is nucleotide-sensing, it binds the pathogen's DNA, leading to cell activation and proinflammatory cytokine secretion, via MyD88 (Chiang *et al*., 2012; Crevel *et al*., 2002). TLR7 ligands such as imiquimod induce autophagy (De Meyer *et al*., 2012; Delgado *et al*., 2008), a defence mechanism of the innate immunity, aimed at eliminating intracellular pathogens through ingestion into autophagosomes for subsequent degradation by lysosomal hydrolytic enzymes (Delgado *et al*., 2008; Levine and Klionsky, 2004; Mizushima *et al*., 2002).

1.4.2 Cytokines

IL-12 and IL-10 are classified as interleukins and are essential during *M. tb* infection (see sections 1.3.2.1 and 1.3.2.2). Cytokines are characterised as either proinflammatory or immunosuppressive. IL-12 is a proinflammatory cytokine and therefore plays a fundamental role in the immune response against invading pathogens, whereas, IL-10 is a potent immunosuppressive cytokine and functions to inhibit the immune response. IL-12 and IL-10 have an antagonistic effect on one another. IL-10 inhibits the expression of IL-12, resulting in decreased IFN-ϒ synthesis and thereby failure to destroy *M. tb*. They are both reported to be among the most predominant cytokines in *M. tb* infection (Giacomini *et al*., 2001).

1.4.3 SNAREs

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are transmembrane protein molecules (Chen and Scheller, 2001). SNAREs were previously categorised as v-SNAREs and t-SNAREs based on their localisation on either vesicle membrane or target membrane, respectively. However, they have recently been classified as R-SNAREs (arginine-containing) or Q-SNAREs (glutamine-containing), based on their structural features (Fasshauer *et al*., 1998; Söllner *et al*., 1993). These proteins have been

implicated in the fusion events of all intracellular membranes, except for mitochondrial fusions (Chen and Scheller, 2001). VAMP7 is a v-SNARE or an R-SNARE and a primary inducer of late endosome-lysosome fusion (J Paul Luzio, 2009; Pryor and Luzio, 2009; Verderio *et al*., 2012). It is involved in heterotypic late endosome-lysosome fusion in alveolar macrophages (Advani *et al*., 1999; Pryor and Luzio, 2009; Ward *et al*., 2000). STX7 is a t-SNARE or a Q-SNARE that is involved in late endosome-lysosome fusion, as well as homotypic lysosome fusion *in vitro* (Achuthan *et al*., 2008; Mashima *et al*., 2008; Ward *et al*., 2000). It was observed that the lack of transmembrane domain on STX7 and VAMP7 blocks these fusions from occurring, suggesting that the transmembrane domains are essential in the induction of the endosome-lysosome fusion (Ward *et al*., 2000). Furthermore, the presence of an antibody against STX7 blocks lysosome fusion in vitro (Ward *et al*., 2000). STX4 is a t-SNARE induced by the activation of macrophages. It is involved in exocytic pathways and secretion of TNF-α, a proinflammatory cytokine (Pagan *et al*., 2003). The deletion of its transmembrane domain interferes with membrane trafficking and inhibits the production of TNF-α by macrophages, whereas its overexpression enhances the production of this cytokine (Pagan *et al*., 2003). Furthermore, STX4 has also been localised on the phagosomal membranes in macrophages (Hackam *et al*., 1996).

1.4.4 Chemokines

CCL1 is a chemokine that plays a role in the regulation of the immune system and inflammatory processes. It recruits monocytes to the site of infection, thereby resulting in the formation of granulomas. CCL1 is implicated in the regulation of genes involved in apoptosis, oxidative stress and chemotaxis in humans. In accordance with the latter, CCL1 was identified to stimulate the expression of CCR8 (its receptor) and CCL2, a potent chemoattractant of macrophages involved in the invasion in many disease states. Furthermore, knockdown of CCR8 by siRNA was observed to inhibit the expression of CCL2 (Tal *et al*., 2007).

1.4.5 GANC

GANC is a glycosyl hydrolase enzyme that plays a fundamental role in the metabolism of glycogen, a large alpha-glucan (α-glucan) molecule that serves as carbon/ energy storage in bacteria and enhances cell survival (Chandra *et al*., 2011). α-glucan is synthesised in the

GlgE pathway, which was recently discovered in mycobacteria (*M. tb* and *M. smegmatis*) and has been genetically validated as target for anti-TB drugs (Kalscheuer *et al*., 2010; Sambou *et al*., 2008). Furthermore, glycogen-like α-glucan molecule was identified in the mycobacterial capsule and has been implicated in immune evasion, rather than in energy storage (Gagliardi *et al*., 2007; Sambou *et al*., 2008).

1.4.6 PKC-α

PKC-α is a member of the protein kinase C (PKC) family, a family of serine- and threonine-specific protein kinases. PKC-α is involved in signal transduction (Meisel *et al*., 2013; Teicher, 2006). It is implicated in phagocytosis of mycobacteria by macrophages and inhibits their subsequent intracellular survival (Chaurasiya and Srivastava, 2009, 2008). An infection of macrophages with *M. tb* or *M. bovis* BCG exhibited downregulation of PKC-α, whereas an infection with *M. smegmatis* leads to upregulation of this gene (Chaurasiya and Srivastava, 2009, 2008). These observations concur with the report that *M. tb* and *M. bovis* BCG are less efficiently phagocytosed by macrophages, when compared to *M. smegmatis*. Moreover, PKnG, a protein involved in *M. tb* intracellular survival, was implicated in this downregulation of PKC-α, since it is found in both *M. tb* and *M. bovis* BCG, but not in *M. smegmatis* (Swartz *et al*., 1988). Furthermore, PKC-α plays a role in phagolysosome biogenesis (Chaurasiya and Srivastava, 2009).

1.4.7 CLIC4

CLIC4 is a protein involved in the channel of chloride (an electrolyte involved in pH balance) in the endoplasmic reticulum (ER), mitochondria and nucleus (Zhong *et al*., 2012). Although its role is still unclear, it is implicated in many biological processes such as signal transduction, cell differentiation and apoptosis (Shiio *et al*., 2006; Zhong *et al*., 2012). Zhong *et al* (2012) suggested CLIC4 as potentially indirectly involved in autophagy, based on the observations that, under imitated nutrient-free conditions autophagy was induced and CLIC4 was upregulated. They further reported that autophagy was enhanced when CLIC4 was silenced via siRNA and apoptosis was triggered, under starvation conditions (Zhong *et al*., 2012). Autophagy and apoptosis are the most common forms of programmed cell death induced under stressful conditions, and they are central to *M. tb* infection (Conradt, 2009; Elliott and Reiners, 2008).

1.4.8 YWHAZ

YWHAZ, also known as 14-3-3ζ is a binding protein that interacts with proteins involved in cell surface regulation, such as human vacuolar protein sorting 34 (hVps34) (Pozuelo-Rubio, 2011). hVps34, the class III phosphatidylinositol-3-kinase, mediates processes involved in vesicle trafficking such as endocytosis and autophagy, and it is the key initiator of autophagy (Pozuelo-Rubio, 2012, 2011). The binding of 14-3-3 proteins to hVps34 implicates 14-3-3 proteins in the regulation of autophagosome formation (Pozuelo-Rubio, 2012). Pozuelo-Rubio (2012) reported the role of 14-3-3 proteins as negative regulators of autophagy, based on the observations that the forced expression of 14-3-3ζ decreased autophagy induced by C2-ceramide, whereas the reduction of 14-3-3ζ expression lead to increased autophagy. Further observations were that, the interaction of 14-3-3 proteins with hVps34 under physiological conditions inactivates hVps34, whereas under nutrient-free conditions 14-3-3/hVps34 complex dissociates, leading to increased activation of hVps34 lipid kinase (Pozuelo-Rubio, 2012, 2011). Furthermore, 14-3-3 proteins are well-known inhibitors of apoptosis (Masters *et al*., 2002).

1.5 Pathogenic versus non-pathogenic mycobacterial species

The main difference between pathogenic and non-pathogenic mycobacteria is the ability of the pathogenic mycobacterial species to avoid or withstand the hostile, acidic macrophage environments, thereby allowing their intracellular survival and promoting their virulence (Anes *et al*., 2006; McDonough *et al*., 1993; Schlesinger, 1996; Simeone *et al*., 2012). The intracellular killing of non-pathogenic *M. smegmatis* and their inability to cause disease even in immuno-compromised individuals, may be linked to their attribute of strongly stimulating the innate immune response (Anes *et al*., 2006, Bohsali *et al*., 2010). Phagocytosis of microbes is normally followed by phagosomal trafficking to lysosomes for subsequent phagosome-lysosome fusion, an important mechanism used by macrophages to eliminate intracellular bacteria. However, pathogenic mycobacterial species such as *M. tb* can either prevent maturation, of phagosomes, thereby blocking their fusion with lysosomes (Clemens and Horwitz, 1995; Koul *et al*., 2004; Xu *et al*., 1994), or escape the phagolysosomes to multiply in non-fused phagosomes as well as in the cytoplasm (McDonough *et al*., 1993). In contrast, non-pathogenic mycobacterial species fail to block or escape phagosome-lysosome

fusion and therefore get digested by hydrolytic enzymes of lysosomes for subsequent degradation (Anes *et al*., 2006; McDonough *et al*., 1993).

Infection of macrophages with pathogenic and non-pathogenic mycobacterial species induce different outcomes (McDonough *et al*., 1993; Simeone *et al*., 2012; Spira *et al*., 2003). Non-pathogenic mycobacteria promote TNF-α-induced apoptosis, an immune response during which macrophages destroy the invading bacteria. The role of apoptosis in mycobacteria infection is still controversial as its outcome depends on the nature of the infecting stimulus (Divangahi *et al*., 2009; Keane *et al*., 2000; Lee *et al*., 2011, 2006; Spira *et al*., 2003) Pathogenic mycobacteria infecting macrophages can either induce apoptosis as a survival mechanism or evade apoptosis and induce necrosis, depending on the multiplicity of infection (MOI) and strain virulence (Keane *et al*., 2000; Spira *et al*., 2003). Infection with a high MOI and/ or a highly virulent strain results in necrosis, a process of cell death during which cell membrane lyses, allowing the pathogen to escape into the surrounding tissues and spread the infection. Necrosis is harmful to macrophages but beneficial to pathogens. Furthermore pathogenic mycobacteria induce lower production of cytokines in macrophages, as compared to non-pathogenic mycobacteria (Yadav *et al*., 2006).

1.6 Selection rationale for the infection parameters

The THP-1 cell line was chosen for its ability to resemble the morphology and mimic the response of human primary macrophages, when differentiated. Their homogenous genetic background minimizes the degree of variability in the cell phenotype and therefore produces reliable results. Since THP-1 cells are a human derived cell line, the results would be more relevant to compare to *in vivo* human infection (Qin, 2012).

M. tb is a pathogen that exhibits similar attributes to *M. bovis* BCG in that they both have a slow growth rate (24 h doubling time) (Bettencourt *et al*., 2010) and are able to survive inside macrophages (McGarvey *et al*., 2004; Walburger et al., 2004). However, *M. bovis* BCG is regarded as non-pathogenic due to the absence of the RD1 region that was found to be conserved in all pathogenic mycobacteria (Lewis *et al*., 2003; Majlessi *et al*., 2005). However, if the infected individual is immunocompromised, *M. bovis* BCG can cause disease (Hesseling *et al*., 2003; Zhou *et al*., 1999). *M. smegmatis* is a non-pathogenic mycobacterial

species with a rapid growth rate (3 h doubling time) (Bettencourt *et al*., 2010) and is killed by macrophages (Anes *et al*., 2006). Therefore, the rationale for selection of these 3 species was based on their killing or survival inside macrophages and they were considered suitable to distinguish if the transcriptional response is specific to macrophages.

An MOI of 1 was considered more feasible for this study since it is one of the commonly used MOIs in literature, due to its ability to stimulate gene expression without causing damage or cell death to the macrophages. Moreover, this MOI was more suited for this study, since about 90% of mycobacteria cultured without Tween 80 (to an OD_{600} of 0.3) were present in clumps, which were subsequently removed by filtration in processing for infection, resulting in low CFU counts (see Figures 3.1-3.3).

1.7 The effect of Tween 80 in mycobacterial cell wall

Mycobacteria are known for their unique cell wall, which resembles that of Gram positive bacteria due to the lack of an outer membrane, yet consist of a lipid bilayer as observed in Gram negative bacteria (Etienne *et al*., 2002; Masaki *et al*., 1991; Sani *et al*., 2010). The complexity of the extracellular capsule of the mycobacterial cell wall is associated with its low permeability to many toxic compounds such as antibiotics, which therefore contributes to the virulence of mycobacteria (Ehrt and Schnappinger, 2009; Hoffmann *et al*., 2008; Nguyen and Pieters, 2009; Sani *et al*., 2010). Mycolic acids are the major lipids present in mycobacterial cell wall and they are implicated in pathogenesis of mycobacteria and evasion of immune response by mycobacteria (Takayama *et al*., 2005). Moreover, the lipid constituents of the mycobacterial cell wall are responsible for the excessive clumping of the mycobacterial cells in liquid culture. The absence of clumps is important for macrophage infections, since these are rapidly trafficked to phago-lysosomes in macrophages, in contrast to phagosomes containing single mycobacteria (Figure 1.2) (Bettencourt *et al*., 2010). Furthermore, the host macrophage response to mycobacterial infection depends on the bacterial load (MOI) and the percentage of infected macrophages. Infection with clumped mycobacteria result in a relatively higher variability in the number of mycobacteria present in each macrophage: some macrophages may contain excessive mycobacterial load, whereas others may not contain any bacilli (Bettencourt *et al*., 2010). In order to minimize excessive clumping, the growth medium for mycobacterial cultures is normally supplemented with

Tween 80 (0.05%) detergent (Daffé and Etienne, 1999; Sattler and Youmans, 1948).

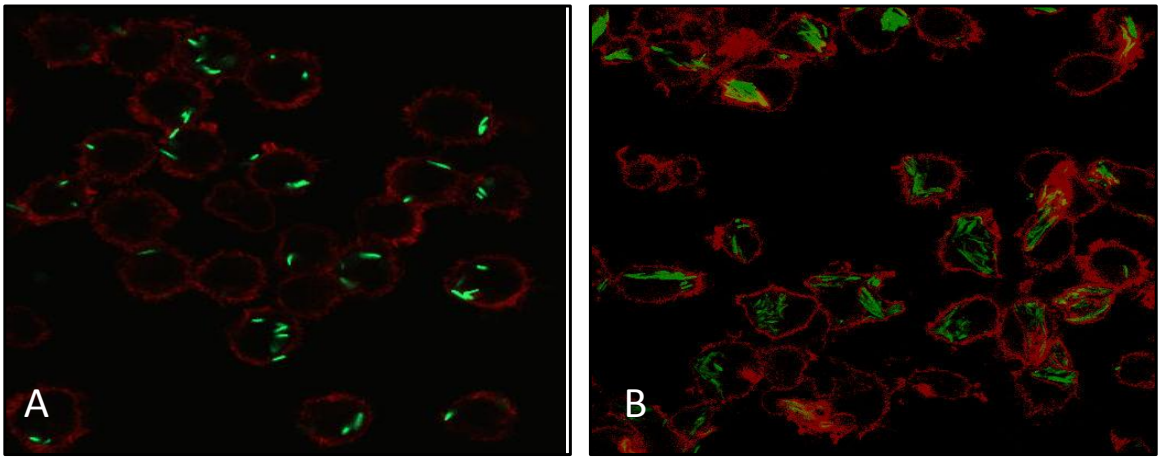

Figure 1.2: Confocal microscopy image of J774 macrophages infected with *M. smegmatis*–GFP after 1h uptake. *Red: actin labeled* using Rhodamine-Phalloidine staining; Green – Green Fluorescent Protein expressing bacteria. A: round-shaped macrophages containing unicellular *M. smegmatis* B: irregular-shaped macrophages containing clumps of *M. smegmatis*. Clumped bacteria triggered recruitment of F-actin and Hck at the phagosomes, events associated with lysosome fusion. Adapted from Bettencourt *et al*., 2010.

Although Tween 80 aids in reducing clumping in mycobacterial cultures, it has been observed to solubilise the mycobacterial extracellular capsule (see examples in Figures 1.3 and 1.4), resulting in the loss of the cell wall lipids that are implicated in mycobaterial virulence (Józefowski *et al*., 2008; Sani *et al*., 2010). To support this, a study by Van Boxtel *et al* (1990) reported that the *Myobacterium paratuberculosis* (*M. paratuberculosis*) strain was able to grow optimally in a drug-containing medium without Tween 80, but was unable to grow in the presence of Tween 80. The authors speculated that Tween 80 compromises the resistance of the *M. paratuberculosis* strain to antimicrobial agents (Van Boxtel *et al*., 1990). A similar observation was shared by other antimicrobial susceptibility studies with *M. avium* and *M. intracellulare*, wherein it was suggested that the increased permeability of drugs into the mycobacteria was due to the perturbed mycobacterial cell wall by Tween 80 (Naik *et al*., 1988).

Furthermore, it was revealed that Tween 80 supplemented in liquid culture medium was responsible for the structural changes observed in Mycobacterium avium complex **(**MAC). It was observed that in the presence of Tween 80 the cells of the S-139 *M. avium* strain were

elongated and the fibrillar material present in the L1 layer, a sheath that is thought to protect *M. avium* inside macrophages, disappeared. However, when the same cells were re-cultured without Tween 80, their size returned to normal and the fibrillar material was present in the L1 layer (Masaki *et al*., 1991).

Consequently, the inclusion of Tween 80 in mycobacterial cultures remains dubious regarding whether the *ex vivo* interaction between macrophages and mycobacteria mimics the *in vivo* infection state. Based on the known damaging effect of Tween 80 on the mycobacterial cell wall, all the strains were cultured without Tween 80 to ensure that the cell wall remains intact (Masaki *et al*., 1991; Sani *et al*., 2010; Van Boxtel *et al*., 1990). Despite this, most studies still make use of Tween 80 for culturing mycobacteria, therefore the strains were also cultured with Tween 80 to serve as reference and for comparison purposes to determine if Tween 80 perturbations on the mycobacterial cell wall affect the gene expression profile of macrophages. With the above said, the current study intended to investigate gene expression in differentiated THP-1 cells in response to mycobacterial species cultured in the presence or absence of Tween 80.

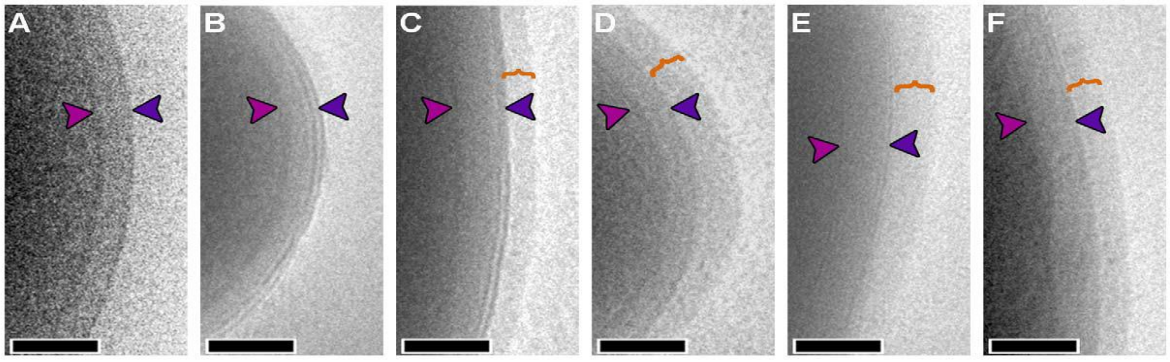

Figure 1.3 Visualization of the capsule in its native state. Cryo electron micrographs of intact Gram- negative bacterium S. flexneri cultured without Tween 80 and plunge frozen (A) depicts the typical cell envelope profile using this method of sample preparation. S flexneri is used as a control to illustrate the absence of the capsule that is of mycobacterial origin. (B) *M. smegmatis* cells cultured with both Tween 80 and agitation show a cell envelope with morphology similar to *S. flexneri*. (C) *M. smegmatis*, (D) *M. tb*, (E) *M. marinum* and (F) *M. bovis* BCG cells cultured without Tween 80 (before freezing) show the presence of an extra layer (bracket) surrounding the mycomembrane. Adapted from Sani *et al*., 2010.

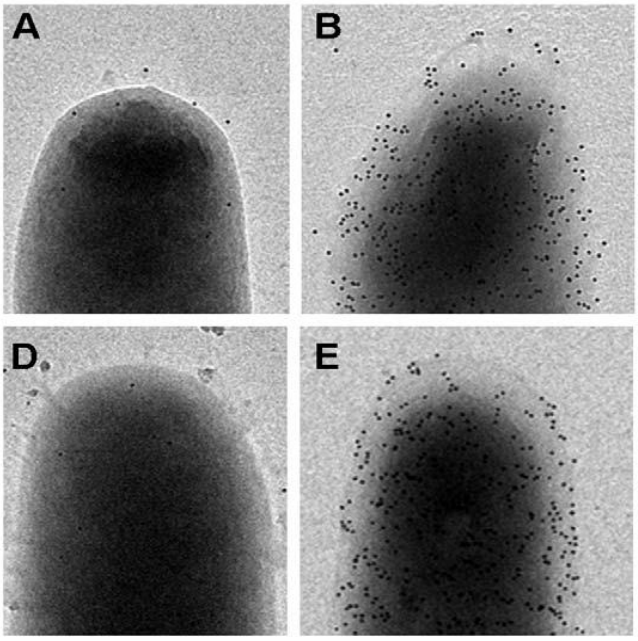

Figure 1.4 Effect of detergent on the localization of capsular components and the detection of ESX-1 proteins. *M. smegmatis* (A and B) and *M. tuberculosis* (D and E) were cultured with (A, D) and without (B, E) Tween 80, fixed and probed with anti-a-glucan (A–B) and anti PIM/capLAM (D–E) to demonstrate that this extra layer was of capsular origin. They used antibodies that recognize α-glucan and PIMs, components associated with the capsule. Surface of mycobacteria cultured without Tween 80 (B, E) were distinctly labelled with these antibodies and surface of mycobacteria cultured with Tween 80 (A, D) showed weak or no labelling. Adapted from Sani *et al*., 2010.

1.8 Problem Statement and Motivation

The TB epidemic remains a global burden and this is partly fuelled by the current anti-TB treatment that is failing due to the emergence of drug resistant strains (Fakruddin, 2013; WHO, 2010; Yokobori *et al*., 2013). The existing anti-TB drugs target mycobacterial enzymes and pathways (De La Iglesia and Morbidoni, 2006; Mdluli and Ma, 2007; Timmins and Deretic, 2006; Walsh, 2000) and in response, the mycobacteria have developed resistance against the drugs (WHO, 2010). A novel approach is therefore required to develop drugs that will have an indirect bactericidal effect thereby limiting the possibility of the development of drug resistance. Host directed anti-tubercular therapy might be a solution, as no drug resistance is likely to develop when the drugs are directed to host factors. In order to apply this approach, knowledge of the host factors that are involved in the intracellular survival of *M. tb* is necessary (Jayaswal et al., 2010; Li *et al*., 2006).

1.9 Hypothesis

Host macrophage genes that are differentially expressed by pathogenic and non-pathogenic mycobacteria potentially affect the survival of *M. tb* inside macrophages. Furthermore, mycobacteria cultured in the presence of Tween 80 induce different transcriptional macrophage responses from mycobacteria cultured without Tween 80.

1.10 Aim

To generate single cells from mycobacteria cultured without Tween 80 and determine if perturbations in mycobacterial cell wall caused by Tween 80 will affect transcriptional macrophage response. Furthermore, to determine the differential expression of a subset of macrophage genes and identify the ones that potentially affect *M. tb* survival in macrophages.

1.11 Objectives

1. Culture mycobacteria in liquid medium with and without Tween 80

Mycobacteria tend to form clumps when grown in liquid culture due to its high lipid constituents in the cell wall (Bettencourt *et al*., 2010). Therefore, detergent such as Tween 80 is used to minimize the formation of major clumps. However, there is considerable literature showing that Tween 80 has an adverse effect on mycobacterial cell wall integrity, thereby compromising its virulence (Sani *et al*., 2010; Van Boxtel *et al*., 1990). Thus, mycobacterial strains will be cultured in the presence or absence of Tween 80.

2. Infect differentiated THP-1 cells with *M. tb*, *M. bovis* BCG and *M. smegmatis*

M. tb and *M. bovis* BCG have the ability to survive inside macrophages, whereas *M. smegmatis* lacks this ability and is killed. In addition, *M. tb* is a pathogenic mycobacterial species, whereas *M. bovis* BCG and *M. smegmatis* are non-pathogenic (Anes *et al*., 2006; McGarvey *et al*., 2004; Walburger *et al*., 2004; McDonough *et al*., 1993). Therefore, infecting differentiated THP-1 cells with these 3 strains will enable us to determine the macrophage responses that potentially affect the intracellular survival of *M. tb*.

3. Identify genes that are differentially expressed by *M. tb*, *M. smegmatis* and *M. bovis* BCG.

M. tb is an intracellular mycobacterial parasite that depends on host factors for survival inside macrophages. Knowledge of the host macrophage genes that are upregulated or downregulated by *M. tb* but not by *M. smegmatis* and to a lesser degree by *M. bovis* BCG is necessary for identifying the host factors that potentially affect *M. tb* survival inside macrophages. Furthermore, this knowledge will contribute to the development of host directed anti-tubercular therapeutics (Jayaswal *et al*., 2010; Li *et al*., 2006, p. 200).

2

Materials and Methods

2.1 Culturing of Mycobacteria

2.1.1 Culturing of *M. tb* and *M. bovis* BCG in Tween 80-enriched medium

M. tb Beijing R179 (drug resistant clinical strain) and *M. bovis* BCG Pasteur were obtained from our departmental strain bank. The mycobacteria were cultured in T25 flasks (NUNC, Germany) by inoculating a 1 ml frozen stock in 9ml of Middlebrook 7H9 (BD, France) medium enriched with oleic acid, albumin, dextrose and catalase (OADC; BD, France) (Growth medium), supplemented with 0.05% Tween 80 (Sigma Aldrich, UK) and incubated at 37°C and 5% CO_2. When the cultures reached an Optical Density (OD_{600}) of 0.8, a 1 ml was inoculated in T75 flask (NUNC, Germany) containing 49 ml growth medium supplemented with 0.05% Tween (which will henceforth be referred to as growth medium^{T}) and incubated at 37°C until an $OD_{600} \approx 0.8$. One ml aliquots containing 15% glycerol were stored at - 80°C until use. The culturing of *M. bovis* BCG and *M. smegmatis* was conducted inside the Biohazard class II hood; whereas *M. tb* was cultured under Biosafety level 3 conditions, due to its high level of pathogenicity. Culturing method adapted from (Sani *et al*., 2010).

2.1.2 Culturing of *M. tb* and *M. bovis* BCG in growth medium without Tween 80

This methodology was developed in order to culture mycobacteria without compromising the integrity of their cell wall, as a result of the presence of detergents such as Tween 80 in the culture medium. A 1 ml frozen stock of *M. bovis* BCG or *M. tb* was split equally and inoculated into two T25 flasks containing 9 ml of growth medium without Tween 80 (henceforth, will be referred to as growth medium^{NT}) and grown to an $OD_{600} \approx 0.3$ at 37°C. Subcultures were prepared by splitting each T25 flask into 5 flasks containing 8 ml growth medium^{NT}. At $OD_{600} \approx 0.3$ each flask was split into 2 flasks, such that each contained 5 ml bacterial culture and 5 ml growth medium^{NT}, where after they were subsequently re-

incubated at 37°C until $OD_{600} \approx 0.3$. The flasks were then combined in 4 separate 50 ml tubes and left to stand for 30min at room temperature in order to sediment the major mycobacterial clumps. The top 45 ml of each tube was transferred to a new 50 ml tube, centrifuged at 450 xg for 5 min and each pellet was resuspended in 5 ml growth medium^{NT}. The bacterial suspensions were combined, mixed and left to stand for another 15 min, at room temperature. The top 17 ml was transferred to a new 50 ml tube and 1 ml aliquots containing 15% glycerol were stored at -80°C until use.

2.1.3 Culturing of *M. smegmatis* in growth medium^{T}

M. smegmatis mc^2 155 was kindly donated by a colleague (Sao Emani *et al.*, 2013). The frozen stock of *M. smegmatis* was inoculated in a 100 ml Erlenmeyer flask containing 10 ml growth medium^{T}, to starting OD_{600} of 0.0025. The culture was covered with foil and incubated at 37 °C with shaking (200 rpm). At $OD_{600} \approx 0.8$, the culture was inoculated in a 500 ml Erlenmeyer flask containing 50 ml growth medium^{T} to starting OD_{600} of 0.0025, incubated at 37 °C until it reached an $OD_{600} \approx 0.8$. 1 ml aliquots containing 15% glycerol were stored at -80 °C until use.

2.1.4 Culturing of *M. smegmatis* in growth medium^{NT}

M. smegmatis was cultured in growth medium^{NT} in a similar fashion as in growth medium^{T} (section 2.1.3). However, the starting culture and the subculture were grown to an OD_{600} of 0.3, due to excessive clumping observed beyond this OD_{600}.

Please note: henceforth, all mycobacteria cultured with Tween 80 will be annotated with (^{T}) and the ones cultured without Tween 80 will be annotated with (^{NT}). When referring to mycobacteria culture with or without Tween 80, will be annotated with ($^{T/NT}$).

2.1.5 Contamination assessment of mycobacteria$^{T/NT}$

Blood agar plating and Ziehl-Neelsen (ZN) staining were performed on mycobacterial cultures as well as on stocks to assess the presence/ absence of contamination. ZN stains were also used to examine the morphology and the level of clumpiness of the mycobacterial cells.

2.1.5.1 ZN staining

The ZN staining was performed according to the standard protocol (Bishop and Neumann, 1970). An aliquot (50 µl) of mycobacterial culture was smeared and fixed on a glass slide by heating at 80°C for 2 hours or overnight. Formalin (10%) (BD, France) was used as a fixative for *M. tb* cultures in order to ensure proper fixation. Mycobacteria were stained with carbol fuchsin (BD, France), heated until steam appeared and allowed to stand for 5 min before rinsing with water. The bacteria were decolourised with acid-alcohol, left to stand for 2 min and then rinsed with water. Methylene blue (BD, France) was then added and left to stand for 2 min to counterstain. Next, the slide was air-dried and examined microscopically under oil immersion (100X magnification). The staining method was performed according to (Bishop and Neumann, 1970).

2.1.5.2 Blood Agar plates

An aliquot (40 µl) of *M. bovis* BCG and *M. tb* cultures were plated on blood agar (BD, France) and incubated at 37°C for 48 hours, in order to assess the sterility of the cultured mycobacteria. In addition, 7H9 (BD, France) medium was plated on the same plates to serve as a control, thereby confirming the sterility of the plates. *M. tb* and *M. bovis* BCG do not grow on blood agar within 48 hours. Therefore, any growth observed within 48 hours is considered as contamination. In contrast, *M. smegmatis* has a rapid growth rate and was therefore not examined with this method.

2.1.6 Determining the mycobacterial^{T} titre

For determination of colony forming units (CFUs) in mycobacteria^{T}, three frozen stock vials of each of the mycobacterial strains (*M. tb*, *M. bovis* BCG and *M. smegmatis*) were thawed and passed 15X through a 25G needle connected to a 1 ml syringe as means of breaking up the clumps. Serial dilutions (10^{-1} to 10^{-8}) were prepared with growth medium^{T} for each of the stock vials and 100 µl from each of the 10^{-5} to 10^{-8} dilutions were cultured on Middlebrook 7H11 (BD, France) agar plates, followed by incubation at 37°C until colonies were visible. CFUs were calculated using the 10^{-5} dilution plates, on which between 30 and 300 colonies were observed.

2.1.7 Determining the mycobacterial^{NT} titre

For determination of CFUs in mycobacteria^{NT}, three frozen stock vials of each of the mycobacterial strains were thawed, syringed 15X with a 25G needle and left to stand for 30 min to allow the major clumps to sediment. The top 750 µl of each stock vial was diluted to 5 ml with mycobacterial growth medium, mixed with a pipette and subsequently filtered through a 5 µm pore sized filter (Satorius, Germany) into a new tube. Serial dilutions of 10^{-1} to 10^{-6} were prepared from the filtered bacterial suspension and the 10^{-3} to 10^{-5} dilutions were plated out on Middlebrook 7H11 agar plates and incubated at 37°C until colonies were observed. The 10^{-3} dilution plates were used to calculate the CFUs, since they showed colony counts between 30 and 300.

2.2 Tissue culture

2.2.1 Growth of THP-1 cells

A frozen stock vial of THP-1 cells (ATCC 88081201) was thawed, washed with 20 ml RPMI 1640 (Sigma Aldrich, USA) to remove Dimethyl Sulfoxide (DMSO) and subsequently cultured in complete medium (RPMI 1640 enriched with 10% Heat-inactivated Fetal calf serum (FCS; Biochrome, Germany) supplemented with 100 U/ml Penicillin and 100 µg/ml Streptomycin (Pen-Strep) (Sigma Aldrich, USA), to limit bacterial contamination. The cells were incubated at 37°C under 5% CO_2. The medium was changed every 2nd to 3rd day and the cells were passaged or split when a confluence of 70-80% was reached. Medium change and passaging or splitting of the cells was established by centrifugation followed by re-suspension in complete medium supplemented with Pen-Strep. The culturing method was performed according to (Park et al., 2007) with some modifications.

2.2.2 Cell counting using haemocytometer

To prepare for counting, the THP-1 cell culture was transferred to a 50 ml tube and centrifuged at 240 xg for 3 min. After the supernatant was discarded, the pellet was resuspended in 10 ml mycobacterial growth medium. Twenty five microlitres of the cell suspension was mixed with 75µl of Trypan blue (Thermo Scientific, USA) (1:4 dilution) in a 1.5 ml tube and 20 µl of the mixture was transferred to a haemocytometer (Marienfield, Germany) for counting. The cells were visualised and counted under an inverted light

microscope (Nikon). The number of live cells (shiny) was counted against the number of dead cells (blue) to determine cell viability.

2.2.3 Differentiation of THP-1 cells

Between the 3rd and 5th split, cells were resuspended in complete medium, seeded at $3X10^6$ cells per well in 6-well plates (Nunc, Germany) and differentiated into macrophage-like cells with Phorbol 12-Myristate 13-Acetate (PMA; Sigma Aldrich, USA) at a final concentration of 100 nM. After 48 hours at 37°C under 5% CO_2, the cells were washed with warm RPMI 1640 and reincubated for another 24 hours in complete medium supplemented with PMA at final concentration of 100 nM, to ensure complete differentiation. Henceforth, PMA-differentiated THP-1 cells will be referred to as dTHP-1 cells. The differentiation method was performed according to (Park *et al*., 2007) with some modifications.

2.3 Infection of dTHP-1 cells with mycobacteria$^{T/NT}$

2.3.1 Processing of mycobacteria^{T} for infection

A frozen stock vial of mycobacteria^{T} was thawed in a 37°C water bath and subsequently passed 15X through a 25G needle connected to a 1 ml syringe in order to disrupt the clumps (our standard laboratory procedure). The required amount of mycobacteria (based on the Multiplicity of Infection (MOI) and number of cells to be infected) was transferred to a new 1.5 ml tube and centrifuged at 14 000 x g for 5 min, in order to remove the Tween 80 the mycobacteria was cultured in. The pellet was resuspended in 1 ml growth medium and passed 10X through a 25G needle connected to a 1 ml syringe and diluted in growth medium to the required volume which was then passed through a 5 µm pore sized filter (Sartorius, Germany); according to Sani *et al*., (2010), with modifications, to remove any remaining clumps.

2.3.2 Processing of mycobacteria^{NT} for infection

Frozen stocks of mycobacteria^{NT} were prepared for infection in the same fashion as mycobacteria^{T} (see section 2.3.1). However after syringing 10X, the pellet resuspension was diluted to 5 ml with complete medium to prevent filter blockage due to clumps. The required amount of the diluted bacterial suspension was topped up with complete medium to the final

volume required for infection.

2.3.3 Infection procedure

The dTHP-1 cells were infected with pathogenic and non-pathogenic mycobacteria$^{T/NT}$ (cultured with or without Tween 80) at an MOI of 1 (1 bacillus per dTHP-1 cell). The cells were allowed to phagocytose the bacilli for 3 h after which they were washed 4X with ice-cold Phosphate Buffer Saline (PBS; Sigma Aldrich, USA) to remove extracellular mycobacteria, and re-incubated at 37°C under 5% CO_2 for further time points (8 h, 24 h and 48 h). The experiment was conducted in biological duplicates and technical duplicates.

2.4 RNA extraction

RNA was extracted at 3hr, 8hr, 24hr and 48hr time points from the biological duplicates and technical duplicates of dTHP-1 cells infected with the pathogenic and non-pathogenic mycobacterial strains cultured with and without Tween 80, as well as from the uninfected cells which served as the control, using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, the cell culture medium was completely aspirated from the cells and the cells were washed 3X with ice-cold PBS in order to completely remove any traces of the medium. Cells were lysed by the addition of RLT buffer containing 10 µl/ml β-mercaptoethanol (Sigma Aldrich, USA) and homogenised by transferring the lysate to QIAshredder spin columns which were vortexed and centrifuged for 2 min at maximum speed in a microcentrifuge. The lysate was transferred to the genomic DNA (gDNA) Eliminator spin columns, vortexed and centrifuged for 1 min at 8 000 xg, to remove gDNA. 350 µl of 70% ethanol was added to the flow through, mixed and transferred to RNeasy spin columns and centrifuged for 30s at 6 000 xg to precipitate RNA onto the spin column membrane. The flow through was discarded and the RNA bound to the membrane of the RNeasy spin column was washed with RW1 buffer and twice with RPE buffer to remove the contaminants. The RNeasy spin columns were placed in new 2 ml collection tubes and centrifuged for 1 min at maximum speed in order to remove the residual buffer. The RNeasy spin columns were placed in new 1.5 ml tubes and the total RNA was eluted by the addition of 30 µl RNase-free water directly to the spin column membrane and centrifuged for 1 min at 8 000 xg. This step was repeated with the same eluate. The RNA was stored at - 80°C.

2.5 RNA quality control

2.5.1 Assessment of gDNA contamination

Polymerase chain reaction (PCR) (Table 2.2) using the HotStart polymerase (Qiagen, Germany) was performed on the RNA samples and the reaction products were electrophoresed on a 1% agarose gel for 1 h at 100 volts. Given the fact that PCR amplifies DNA only, the extracted RNA was used as the template for the PCR amplification in order to verify the absence of gDNA traces. The positive and negative controls were included for verification of positive amplification and purity of the PCR reagents, respectively. ACTB primer set (Table 2.1) was used for PCR amplification. The gel was visualised under UV light. Since PCR amplifies DNA only, any bands observed on the lanes of the negative control and RNA samples are considered as gDNA contamination or primer dimers (which are visible after Gel electrophoresis of the PCR product, about 30-50 bp or smear which has a distinguishable intensity from the band of the target sequence).

2.5.2 Assessment of RNA integrity

The Agilent 2100 Bioanalyzer (version 2.6) (Agilent Technologies, USA) is an automated gel electrophoresis system that calculates the integrity of RNA to provide a quantitative measure of the RNA quality. Good quality (intact) RNA is indicated by the 28S/18S ratio of 2, 28S bands with double intensity and size compared to the 18S bands, absence of heavy smearing around the bands, height of the 28S peaks that is double that of the 18S, sharp peaks and RNA integrity number (RIN) that is between 8 and 10. RIN calculates a numerical value from 1 to 10 with a score of 1 indicating the most degraded RNA and 10 indicating the most intact RNA (Nolan *et al*., 2006)

2.6 cDNA synthesis

For all the samples 1 µg of RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Germany). 2 µl gDNA 'Wipeout Buffer' and water were added to each RNA sample to make up a total volume of 14 µl and then incubated at 42°C for 2 min in order to eliminate gDNA contamination in the RNA samples. To convert RNA to cDNA, 6 µl of the master mix (1 µl RT enzyme, 4 µl RT buffer and 1 µl Primer mix) was added to make up 20 µl reactions, which were incubated at 42°C for 20 min and at 95°C for another 3 min.

The cDNA samples were stored at -20°C until use.

2.7 Quantitative Real Time-PCR

The Quantitative Real Time-PCR (qRT-PCR) (Table 2.3) was performed using the LightCycler® 480 Faststart SYBR Green 1 Master kit (Roche, Germany). The efficiencies of all the validated QuantiTect primer sets (Table 2.1) were assessed by conducting serial dilutions (10^{-1} to 10^{-4}) from the cocktail of all the cDNA samples and 10 µl reactions containing 7 µl H_2O, 1 µl primer set, 1 µl SYBR Green mix and 1 µl cDNA template, were prepared. Primer efficiency testing was performed to ensure that the primer sets will work optimally as well as to determine what concentration of cDNA was required to use in the experiment to identify the changes in gene expression. Subsequent to primer efficiency verification, the expressions of the 12 target genes and 3 reference genes (Table 2.1) were analysed. The LC480 results were analysed further using the REST programme, in which the expression of each of the 12 target genes was normalised relative to the expression of the reference gene (ACTB, was used for normalisation of RNA levels because it had the most consistent expression across different samples), according to the $R = \frac{(E_{target})^{\Delta CPtarget(control-sample)}}{(E_{ref})^{\Delta CPref(control-sample)}}$ formula, whereby the infected dTHP-1 cells (sample) are compared to the uninfected dTHP-1 cells (control). REST is a statistical programme in which a ***p-value* ≤ 0.05 is considered as significant.** The statistical test behind REST programme is a Randomised Pair Wise test, where two groups (control versus sample) are compared (Pfaffl *et al*., 2002).

Table 2.1 List of validated QuantiTect primer sets (forward and reverse primers; Qiagen, Germany). Bioinformatically validated for use in SYBR Green-based qRT-PCR on any cycler. Primer sequences were not provided by the manufacturer since these are validated primers (property rights).

	Gene	QT number	Amplicon size
Target genes	Syntaxin-7 (STX7)	QT00091917	146 bp
	Toll-like receptor 9 (TLR9)	QT00015183	77 bp
	Glucosidase, alpha; neutral C (GANC)	QT01001105	105 bp
	Interleukin-10 (IL-10)	QT00041685	113 bp
	Syntaxin 4 (STX4)	QT00014679	126 bp
	Toll-like receptor 7 (TLR7)	QT00030030	79 bp
	Chemokine ligand 1(CCL1)	QT00203154	174 bp
	Vesicle-associated membrane protein 7 (VAMP7)	QT00006664	101 bp
	Chloride intracellular channel 4 (CLIC4)	QT00058170	97 bp
	Protein kinase C, alpha (PRKCA)	QT00095746	97 bp
	Tyrosine-3-monooxygenase/tryptophan(YWHAZ)	QT00087962	147 bp
	Interleukin-12 beta (IL-12β)	QT00000364	119 bp
Reference genes	Beta actin (ACTB)	QT00095431	146 bp
	Beta-2- Microglobulin (B2M)	QT00088935	98 bp
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	QT00079247	122 bp

Table 2.2 PCR parameters

Reaction	Temperature (°C)	Time	Cycle no.
Enzyme (Taq) activation	95	15 min	1
Template denaturation	94	30s	40
Annealing of primers	60	30s	
Extension	72	1 min	
Proof reading	72	10 min	1
Hold	4	∞	

Table 2.3 qRT-PCR parameters

Reaction	**Temperature (°C)**	**Time**	**Cycle no.**
Enzyme (Taq) activation	95	15 min	1
PCR: Template denaturation Annealing of primers Extension	 95 59 95	 15s 30s 30s	40
Cooling	4	10s	1

Table 2.4 Primer efficiencies

Primer	Efficiency value
ACTB	2
B2M	2
GAPDH	2
STX7	1.93
GANC	1.96
STX4	2
CLIC4	1.96
PRKCA	1.94
YWHAZ	1.95
VAMP7	2.09
CCL1	2
TLR9	2
IL-10	2.08
TLR7	1.95
IL-12β	2

Table 2.5 ACTB consistent expression across uninfected and *M. tb* infected samples. This table is a representative example of ACTB expression values for *M. tb*, *M. bovis* BCG and *M. smegmatis*.

	***M. tb*^{T}**			***M. tb*^{NT}**		
	3 h	**24 h**	**48 h**	**3 h**	**24 h**	**48 h**
Control (Uninfected cells)	11.38	11.98	11.5	11.98	11.98	11.5
	11.39	11.92	11.14	11.93	11.92	11.28
	11.35	11.98	11.52	11.92	11.98	11.52
	11.58	11.82	11.13	11.82	11.82	11.27
	11.62	11.93	11.52	11.98	11.93	11.52
	11.63	11.88	11.13	11.88	11.88	11.28
Sample (Infected cells)	12.54	11.56	12.68	11.49	11.26	11.56
	11.47	11.53	13.55	11.28	11.22	12.36
	12.21	11.54	12.71	11.23	11.28	11.57
	11.47	12.38	13.58	11.22	11.39	12.38
	12.24	12.4	12.72	11.41	11.41	11.54
	11.48	12.41	13.57	11.26	11.37	12.37

3

Results and Discussion

3.1 Results

In the present study a total of 12 genes (Table 2.1) of the dTHP-1 cells were investigated for differential expression in response to infection with *M. tb* (3h, 24h and 48h), *M. bovis* BCG (3h, 24h and 48h) and *M. smegmatis* (3h and 8h), cultured with and without Tween 80, using the LightCycler® 480 (LC480) version 1 (Roche, Germany). (see Table 2.3 for PCR conditions). The results were analysed further on the REST programme to determine differential gene expression.

ZN staining was conducted to assess possible contamination as well as the uniformity of mycobacteria cultured with or without Tween 80. The acid-fast bacteria (AFB) retained the pink colour of carbol fuschin and appear as pink-bright red rods on a blue background (due to counterstaining with methylene blue), whereas non-AFB are destained and pick up the blue colour of the methylene blue (Figures 3.1 and 3.3). *M. smegmatis* was observed to have an exception of staining both pink and blue (Figure 3.2). It was speculated that this may be because *M. smegmatis* is less acid-fast. However, no supporting studies could be found in the literature. To rule out contamination, the morphology (shape and size) of the cells was closely assessed and it was observed to be the same for both the pink and the blue rods, indicating that it is the same species (*M. smegmatis*). This verification was supported by the Southern blot results obtained in a previous study, in which the right size of an expected band was observed (Sao Emani *et al*., 2013).

3.1.1 Evaluation of ZN slides to determine uniformity of mycobacteria

The mycobacterial cultures formed tighter clumps in growth medium^{NT}, but loose and smaller clumps in growth medium^{T} (Figures 3.1-3.3).

3.1.1.1 ZN slides of frozen stocks of *M. tb*$^{T/NT}$

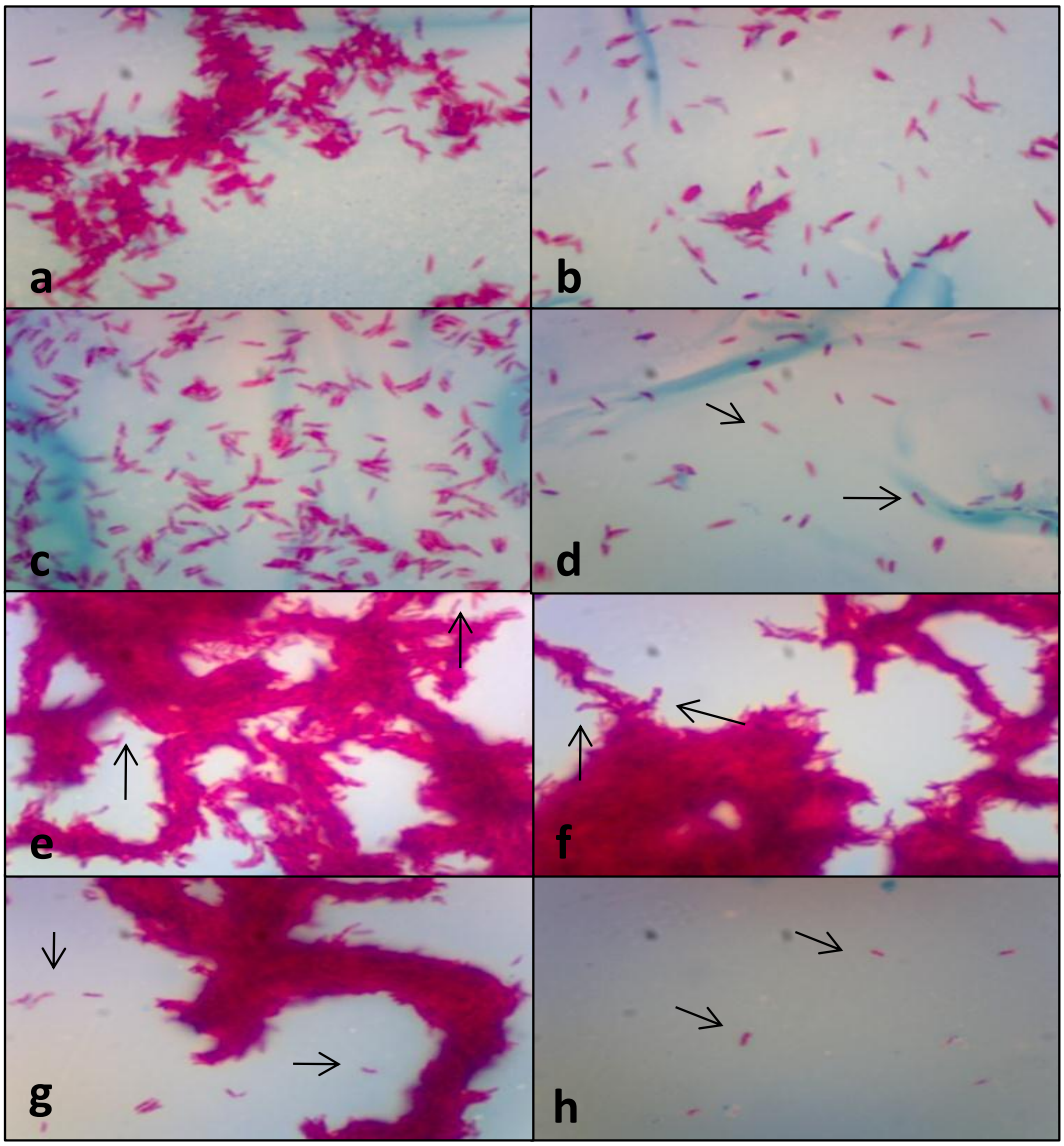

Figure 3.1 ZN slides of the processing of *M. tb* for infection of dTHP-1 cells under oil immersion (100X objective X 10X ocular magnification). a-d: *M. tb*^{T} and e-h: *M. tb*^{NT} a & e: raw, b & f: syringed 15X, c & g: centrifuged and syringed 10X (following the 15X syringing), d & h: Diluted 5X and then filtered through a 5 µm pore filter. The arrows depict single mycobacterial cells. The blue background is Methylene blue counter-stain.

3.1.1.2 ZN slides of frozen stocks of *M. smegmatis*$^{T/NT}$

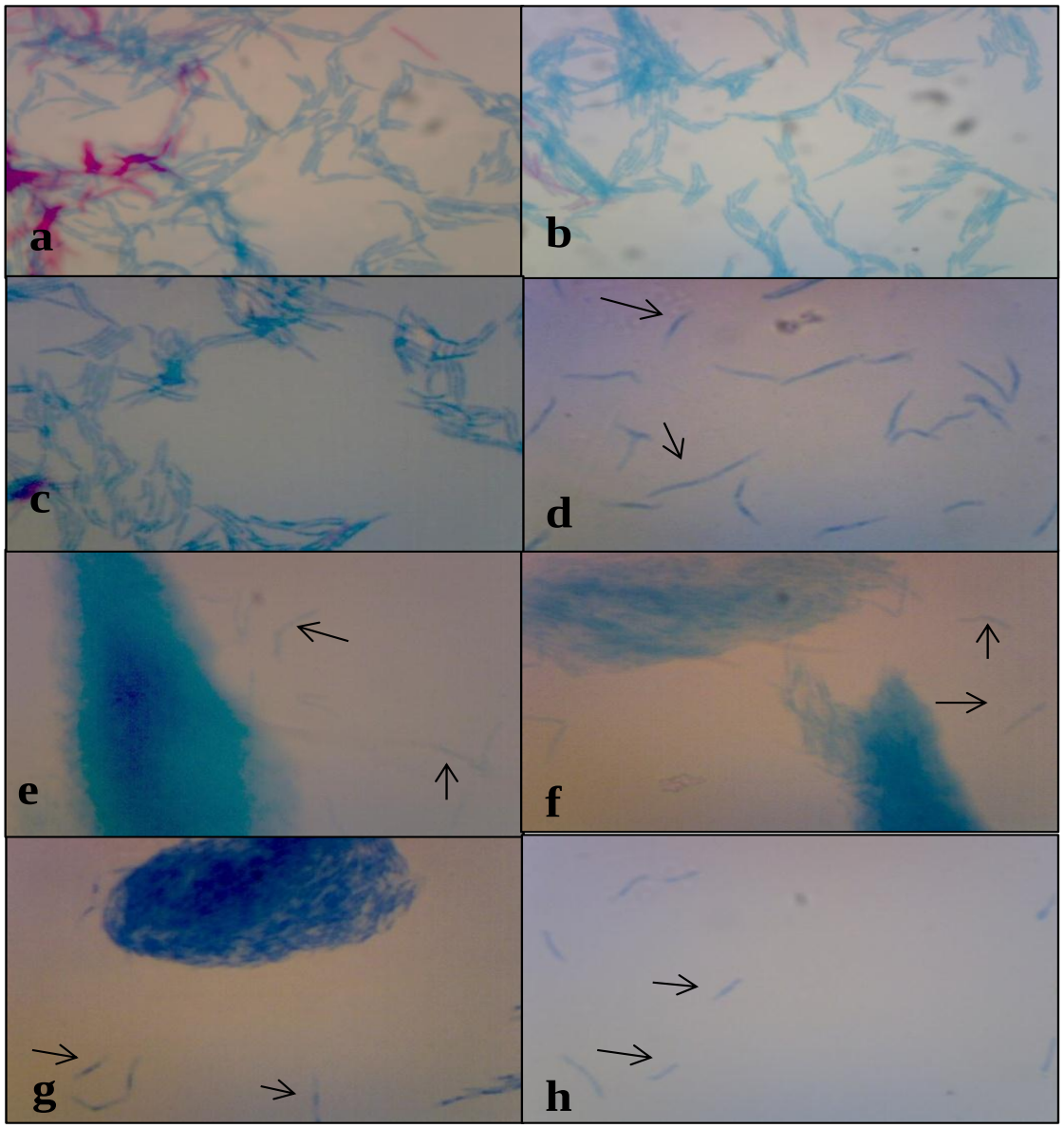

Figure 3.2 ZN slides of the preparation of *M. smegmatis* for infection of THP-1 macrophage-like cells under oil immersion (100X objective X 10X ocular magnification). a-d: *M. smegmatis*^{T} and e-h: *M. smegmatis*^{NT} a & e: raw, b & f: syringed 15X, c & g: centrifuged and syringed 10X (following the 15X), d & h: Diluted 5X and then filtered through a 5 µm pore filter. The arrows depict single mycobacterial cells.

3.1.1.3 ZN slides of frozen stocks of *M. bovis* BCG$^{T/NT}$

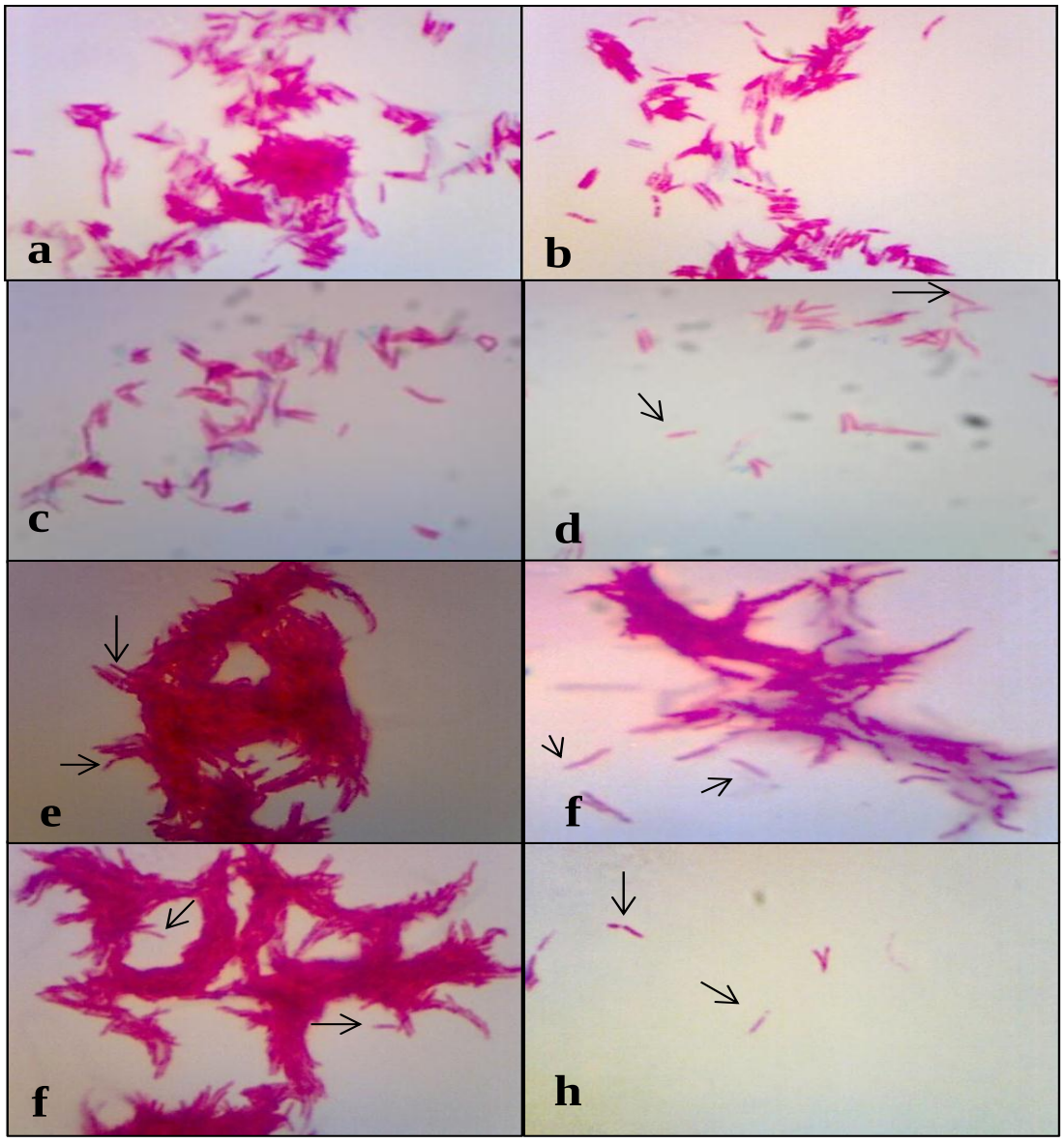

Figure 3.3 ZN slides of the preparation of *M. bovis* BCG for infection of THP-1 macrophage-like cells under oil immersion (100X objective X 10X ocular magnification). a-d: *M. bovis* BCG^{T} and e-h: *M. bovis* BCG^{NT} a & e: raw, b & f: syringed 15X, c & g: centrifuged and syringed 10X (following the 15X), d & h: Diluted 5X and then filtered through a 5 µm pore filter. The arrows depict single mycobacterial cells.

In order to obtain a single cell (clump-free) mycobacterial suspension in preparation for the infection of dTHP-1 cells, a mycobacterial frozen stock was syringed twice (15X and then 10X subsequent to centrifugation) with a 1 ml 25G needle in order to break up the clumps. Following syringing, the required volume of mycobacteria was mixed with RPMI 1640 plus 10% FCS and the suspension was passed through a 5 µm pore filter to eliminate the remaining clumps and allow single mycobacterial cells to pass through. The filtration process was performed for both mycobacteria cultured with and without Tween 80 to ensure a clump-free mycobacterial suspension. Even though the mycobacteria^{T} had relatively minor clumps and more single cells, the filtration step was performed to ensure uniformity between the treatment protocols for mycobacteria cultured with and without Tween 80.

The ZN slides (Figures 3.1-3.3) confirmed that the mycobacteria^{T} have relatively more single cells and minor clumps (Figures 3.1-3.3: **a**). Although minor clumps were formed despite the presence of Tween 80 in the culture medium, the clumped cells separated as mycobacteria were syringed 15X (Figures 3.1-3.3 **b**). Following centrifugation and 10X syringing, the clumps disappeared almost completely and the number of single cells increased (Figures 3.1-3.3: **c**), confirming the effectiveness of Tween 80 in reducing the formation of mycobacterial clumps. A clump-free mycobacterial suspension was obtained through filtration with a 5 µm pore filter (Figures 3.1-3.3: **d**).

Approximately 90% of the mycobacteria cultured without Tween 80 were present in clumps (Figures 3.1-3.3: **e**). Although the clumps were in the majority, there were some mycobacteria that were in single form. Subsequent to syringing twice, i.e 15X (Figures 3.1-3.3 **f**) and then 10X, following centrifugation (Figures 3.1-3.3 **g**), excessive major clumps remained. This observation suggests that mycobacteria form tighter clumps when cultured in liquid medium without Tween 80. Hence, it was necessary to filter the mycobacterial suspension in order to get rid of the clumps. Following filtration, all the major clumps were removed and only mycobacteria in single form and small clumps (containing 2 to 3 bacteria) were remained (Figures 3.1-3.3 **h**).

3.1.2 Quality and quantity assessment of total RNA

RNA was extracted from the biological and technical duplicates of infected and uninfected macrophage-like cells, at 3 h, 24 h and 48 h time points for *M. tb* and *M. bovis* BCG infections and 3 h and 8 h for *M. smegmatis* infections. The quality and quantity of total RNA was assessed using the Bioanalyzer and Gel electrophoresis of PCR products. The extracted total RNA was intact and free of gDNA contamination and other impurities.

3.1.2.1 Assessment of gDNA contamination in extracted RNA by gel electrophoresis

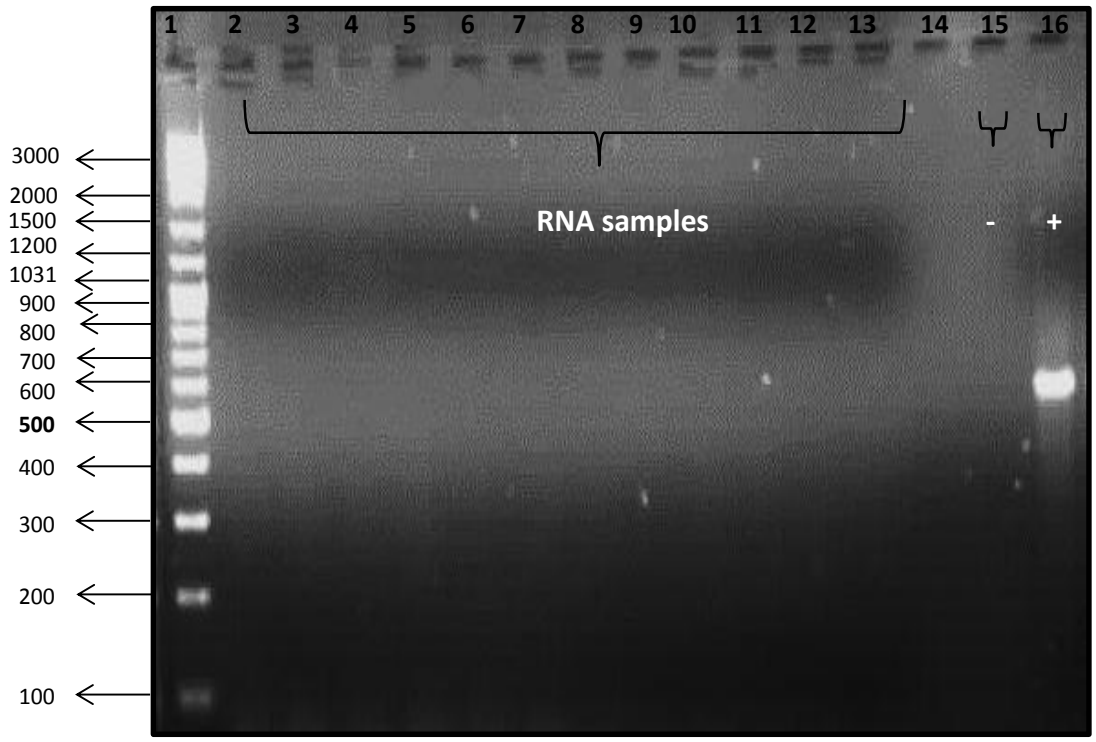

Figure 3.4 Gel Electrophoresis of the PCR products. Lane 1: Marker (GeneRuler^{TM} 100bp DNA ladder plus), Lane 2-14: RNA samples, Lane 15: negative control (template = water) and Lane 16: positive control (template = DNA). Lanes 2-7 consist of *M. tb* experiment*:* 2&3 are controls, 4&5 are samples infected with *M. tb* ^{NT} and 6& 7 are samples infected with *M. tb*^{T}. Lanes 8-13 consist of *M. smegmatis* experiment: 8& 9 are controls, 10& 11 are samples infected with *M. smegmatis*^{NT} and 12& 13 are samples infected with *M. smegmatis*^{T}. ACTB primer set was used for amplification.

In order to assess the purity of the extracted RNA, the agarose gel was visualised under UV light (see section 2.5.1 for details). As expected, a band was observed for the positive control and not for the negative control or the RNA samples (Figure 3.4), since PCR amplifies DNA only. These results indicate that the PCR reagents and the extracted RNA were free of gDNA contamination.

3.1.2.2 Assessment of RNA integrity and quantity using the Agilent 2100 Bioanalyzer

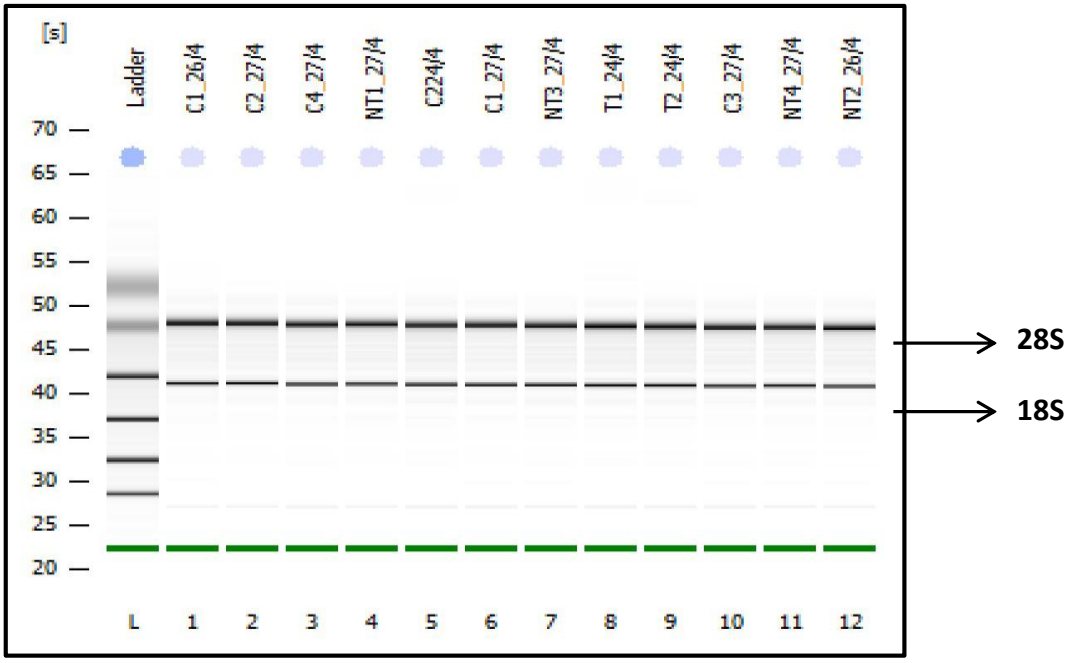

Figure 3.5 Virtual gel generated by the Agilent Bioanalyzer 2100, illustrating the Integrity of the extracted total RNA. This image is a representative example of the quality of all the RNA samples. RNA was extracted from the infected and uninfected cells (control) at 3 h, 24 h and 48 h post infection with *M. tb*. C= uninfected, NT= samples infected with *M. tb*^{NT} and T= samples infected with *M. tb*^{T}.

The Agilent 2100 Bioanalyzer is an automated gel electrophoresis system that was used to calculate the integrity of RNA to give a quantitative measure of the RNA quality (Nolan et al., 2006). The virtual gel generated from the Bioanalyzer system illustrates the intensity and the size of the 28S and 18S rRNA bands and is used to assess the integrity of the RNA (Figure 3.5). The size and the intensity of the 28S bands were detected to be double that of the 18S bands, indicating intact RNA. In order to verify the calculated integrity of the RNA, a

28S/18S ratio, as well as the RIN value (Table 3.1) was used. The RIN value is considered to be more accurate for the evaluation of RNA integrity as compared to the 28S/18S ratio (Nolan et al., 2006). All the RIN values were above 9.5, confirming intact RNA; even though the 28S/18S ratios were ≥ 1.5 (Table 3.1).

Table 3.1 Summary of the results generated by the Agilent 2100 Bioanalyzer. This data is from one of the experiments conducted and it is a representative example of all the RIN values, 28S/18S ratios and RNA concentration of extracted RNA. Samples 1-6 represent *M. tb* experiment and 7-12 represent *M. smegmatis* experiment. The experiments were conducted in technical duplicates. Samples 1, 2, 7 and 8 are controls; samples 3, 4, 9 and 10 are infected with mycobacteria^{NT} and samples 5, 6, 11 and 12 are infected with mycobacteria^{T}.

Sample	Conc. (ng/µl)	28S/18S ratio	RIN
1	753	1.8	9.7
2	789	1.9	9.7
3	778	1.8	9.7
4	599	1.7	9.6
5	901	1.8	9.6
6	795	1.8	9.7
7	599	2.0	9.7
8	984	1.8	9.6
9	1 000	1.8	9.6
10	492	1.5	9.7
11	511	1.6	9.7
12	1 344	1.8	9.6

3.1.3 Gene expression analysis

The 12 genes (Table 2.1) that were assessed were selected based on their roles in the phagocytic pathway and autophagy (section1.6), since these pathways are central to *M. tb* infection. qRT-PCR was performed to determine the differential expression levels of the selected genes in response to *M. tb*, *M. bovis* BCG and *M. smegmatis*, cultured with and without Tween 80. Statistically significant (***p-values*** **≤ 0.05**) differential gene expression was observed in dTHP-1 cells infected with mycobacteria^{NT}.

Gene expression was measured at different time periods during infection, in order to determine whether the trends of expression were maintained over the infection period. The expression was measured at 3 h, 24 h and 48 h from *M. tb*- and *M. bovis* BCG-infected dTHP-1 cells and at 3 h and 8 h in *M. smegmatis*- infected dTHP-1 cells; relative to the uninfected cells across the same time points. The 24 h was included because it is a common time point reported in macrophage infection studies (Delgado *et al*., 2008; Herbst *et al*., 2011; McGarvey *et al*., 2004; Simeone *et al*., 2012). The 3 h and the 48 h time points were included to assess the early and the late response of the dTHP-1 cells, respectively. The 3 h time period for *M. smegmatis*-infected dTHP-1 cells was used to assess the early response. The 8 h time point instead of the 24 h and 48 h was selected to assess the late response of *M. smegmatis*-infected dTHP-1 cells. Since *M. smegmatis* is a fast grower, the problem of extracellular growth was observed at time periods later than 8 h infection, while *M. smegmatis* is killed inside macrophages (Anes *et al*., 2006).

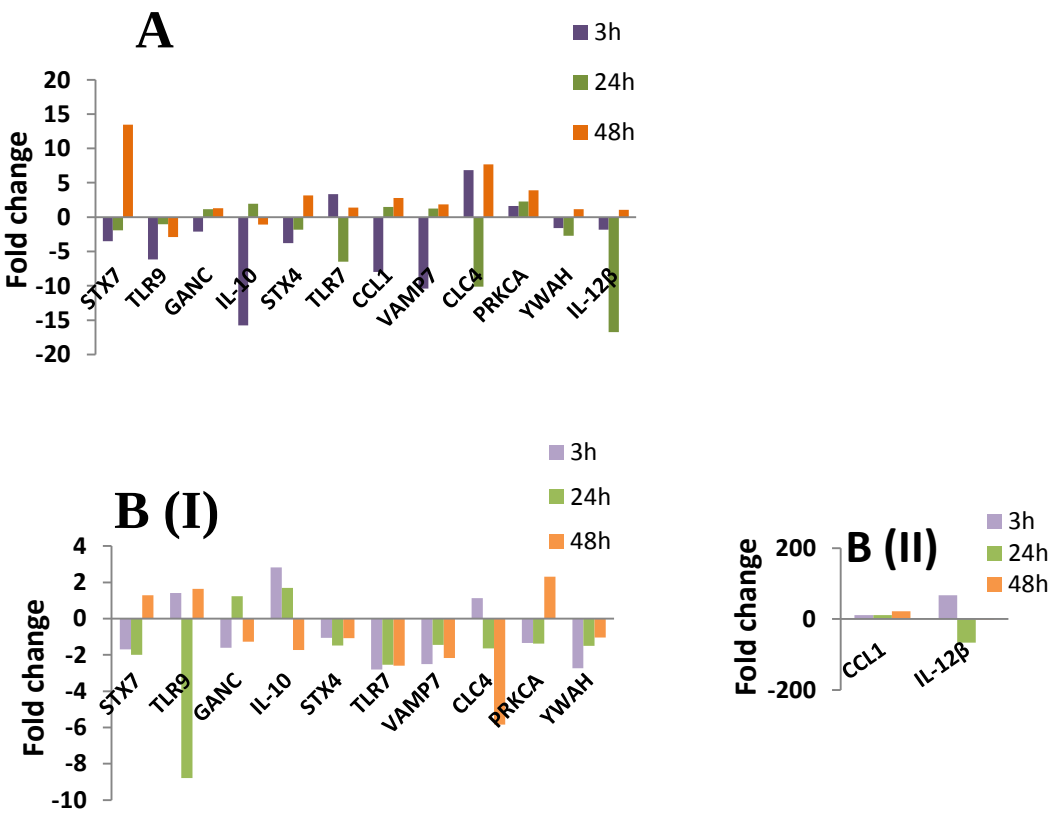

Figure 3.6 Gene expression of dTHP-1 cells in response to *M. tb* infection. A. Infection with *M. tb*^{T}. **B.** Infection with *M. tb*^{NT}. The inserted graph Figure **B** (II) depicts the two genes (CCL1 and IL-12β) that were highly expressed by *M. tb*^{NT} in all 3 time periods. CCL1 and IL-12β were excluded from Figure **B** (I) due to their large fold changes and for better visual display of the fold changes of the 9 other genes. The upregulation and downregulation of the target genes was measured relative to the uninfected controls, using the randomised pair wise statistical test of the REST programme. See Table 3.2 for statistical significant results.

The *M. tb* results are illustrated in Figure 3.6 and summarised (Fold changes and *p-values*) in Table 3.2. Following 3 h infection with *M. tb*^{NT}, IL-10, CCL1 and IL-12 were significantly upregulated and VAMP7, TLR7 and YWAH were downregulated. During the same time period in response to *M. tb*^{T}, TLR9, IL-10, STX4 and VAMP7 were downregulated; while TLR7, CLIC4 and PRKCA were upregulated. A significant downregulation of STX7, TLR7, CLIC4, YWAH, IL-12 and upregulation of IL-10 were observed 24 h post infection with *M. tb*^{T}, while upregulation of CCL1 and downregulation of STX7, TLR9, TLR7 and IL-12 were observed for *M. tb*^{NT} infection. In response to 48 h infection; STX7, CLIC4 and PRKCA were significantly upregulated by *M. tb*^{T} and PRKCA and CCL1 were upregulated by *M. tb*^{NT}, while VAMP7 and CLIC4 were downregulated.

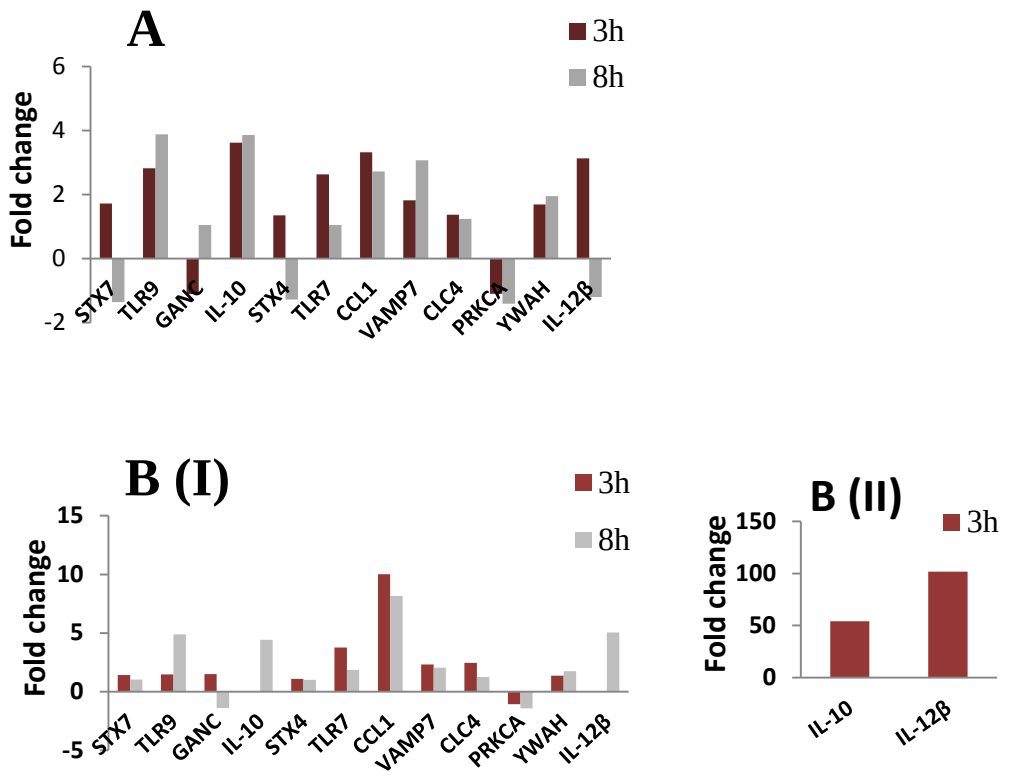

Figure 3.7 Gene expression of dTHP-1 cells in response to *M. smegmatis* infection. A. Infection with *M. smeg*^{T}. **B.** Infection with *M. smeg*^{NT}. The inserted graph, Figure **B** (II) depicts the two genes (IL-10 and IL-12β) that were highly expressed by *M. tb*^{NT} at 3h time period. The 3h time point of IL-10 and IL-12β was excluded from Figure **B** (I) due to their large fold changes and for better visual display of the fold changes of the 9 other genes. The upregulation and downregulation of the target genes was measured relative to the uninfected controls, using the randomised pair wise statistical test of the REST programme. See Table 3.3 for statistical significant results.

The *M. smegmatis* results are illustrated in Figure 3.7 and summarised (Fold changes and *p-values*) in Table 3.3. IL-10, TLR7, CCL1 and IL-12 were significantly upregulated by both *M. smegmatis*^{T} and *M. smegmatis*^{NT} 3 h post-infection, TLR9 was upregulated by *M. smegmatis*^{T} and VAMP7 and CLIC4 by *M. smegmatis*^{NT}. During the 8 h time period TLR9, IL-10, CCL1, VAMP7 and YWAH were significantly upregulated by both *M. smegmatis*^{T} and *M. smegmatis*^{NT}, while TLR7 and IL-12β were upregulated by only *M. smegmatis*^{NT}.

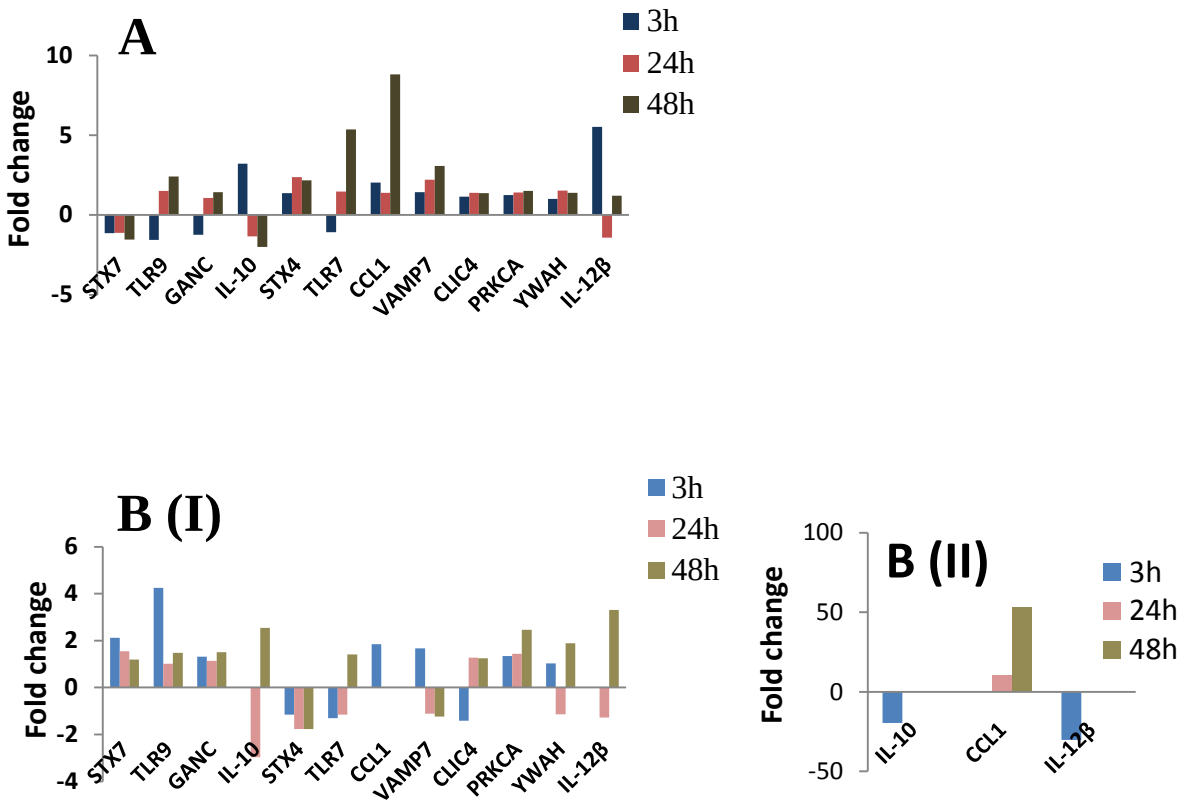

Figure 3.8 Gene expression of dTHP-1 cells in response to BCG infection. A. Infection with BCG^{T} **B.** Infection with BCG^{NT}. The inserted graph (Figure **B** (II)) depicts the three genes (IL-10, CCL1 and IL-12β) that were highly expressed by *M. tb*^{NT} at 3h, 24h and 48h time periods. IL-10, CCL1 and IL-12β were excluded from Figure **B** (I) due to their large fold changes and for better visual display of the fold change of the 8 other genes. The upregulation and downregulation of the target genes was measured relative to the uninfected controls, using the randomised pair wise statistical test of the REST programme. See Table 3.4 for statistical significant results.

The *M. bovis* BCG results are illustrated in Figure 3.8 and summarised (Fold changes and *p-values*) in Table 3.4. In response to 3h infection with *M. bovis* BCG, STX7 and TLR9 were significantly downregulated by *M. bovis* BCG^{T} while IL-10, CLIC4 and IL-12 were upregulated. In contrast, STX7 and TLR9 were upregulated by *M. bovis* BCG^{NT} while IL-10, CLIC4 and IL-12 were downregulated. In addition, VAMP7 was upregulated by both *M. bovis* BCG^{T} and *M. bovis* BCG^{NT}, while STX4 and CCL1 were upregulated by only *M. bovis* BCG^{T} and PRKCA by *M. bovis* BCG^{NT}**.** Furthermore, GANC and TLR7 were downregulated by *M. bovis* BCG^{T} and *M. bovis* BCG^{NT}, respectively. When dTHP-1 cells were infected with

M. bovis BCG^{T} for 24 h IL-10 was downregulated, while STX4 and VAMP7 were upregulated. When infected with *M. bovis* BCG^{NT} for the same time period CCL1 was upregulated. CCL1 and PRKCA were significantly upregulated in response to both *M. bovis* BCG^{T} and *M. bovis* BCG^{NT} 48 h post infection and STX4 was upregulated by *M. bovis* BCG^{T} but downregulated by *M. bovis* BCG^{NT}. In addition, TLR9, TLR7 and VAMP7 were significantly upregulated by *M. bovis* BCG^{T}, whereas IL-10, YWAH and IL-12β were upregulated by *M. bovis* BCG^{NT}.

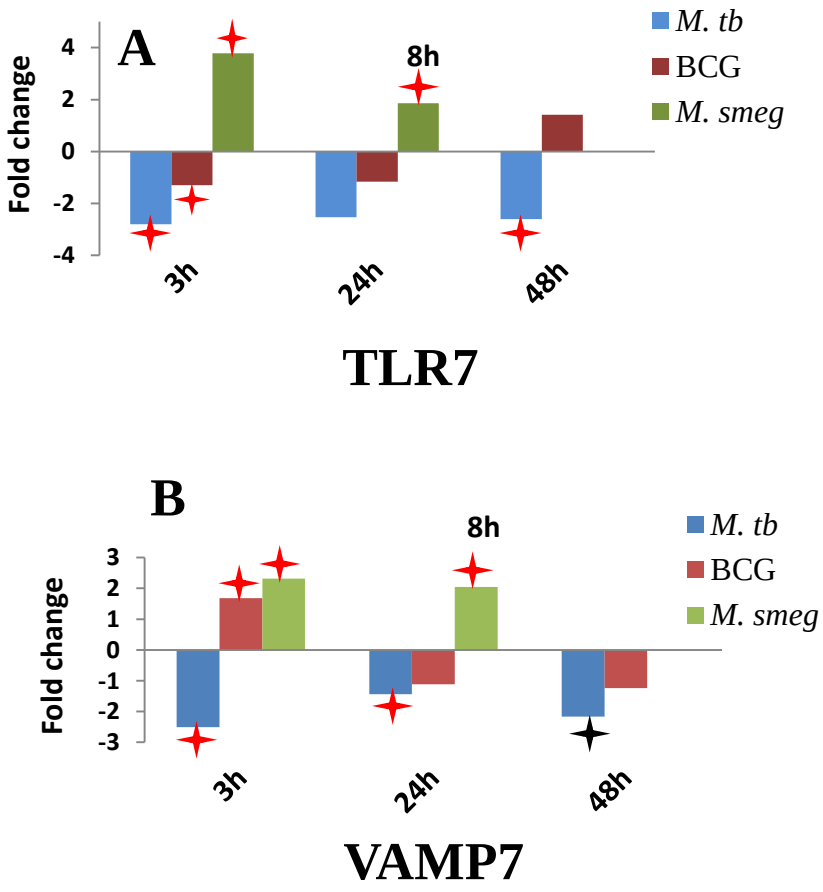

Figure 3.9 Differential expression of dTHP-1 cells infected with pathogenic and non-pathogenic mycobacteria. TLR7 (**A**) and VAMP7 (**B**) showed difference in expression by *M. tb*^{NT} and *M. smegmatis*^{NT}. The upregulation and downregulation of the target genes was measured relative to the uninfected controls, using the randomised pair wise statistical test of the REST programme. Statistical significant (*p-values* ≤ 0.05) results are indicated by red stars and the black star indicates a *p-value* close to 0.05 (0.008).

Of the 12 genes that were studied, a significant differential expression in response to *M. tb* and *M. smegmatis* was detected for TLR7 and VAMP7 (Figure 3.9). dTHP-1 cells exhibited a consistent downregulation of TLR7 and VAMP7 when infected with *M. tb*^{NT} and upregulation when infected with *M. smegmatis*^{NT} during all time periods. However, the 24 h expression of VAMP7 and the 48 h expression of TLR7 were not significantly different (*p-values* of 0.547 and 0.064, respectively) (Tables 3.2 and 3.3). In response to BCG^{NT} infection, there was no consistent trend of expression detected for VAMP7 or TLR7; however, a significant upregulation of VAMP7 in response to BCG^{T} was observed at all 3 time periods (Table 3.4). Results illustrated in Figure 3.9 and summarised (Fold changes and *p-values*) in Tables 3.2-3.4.

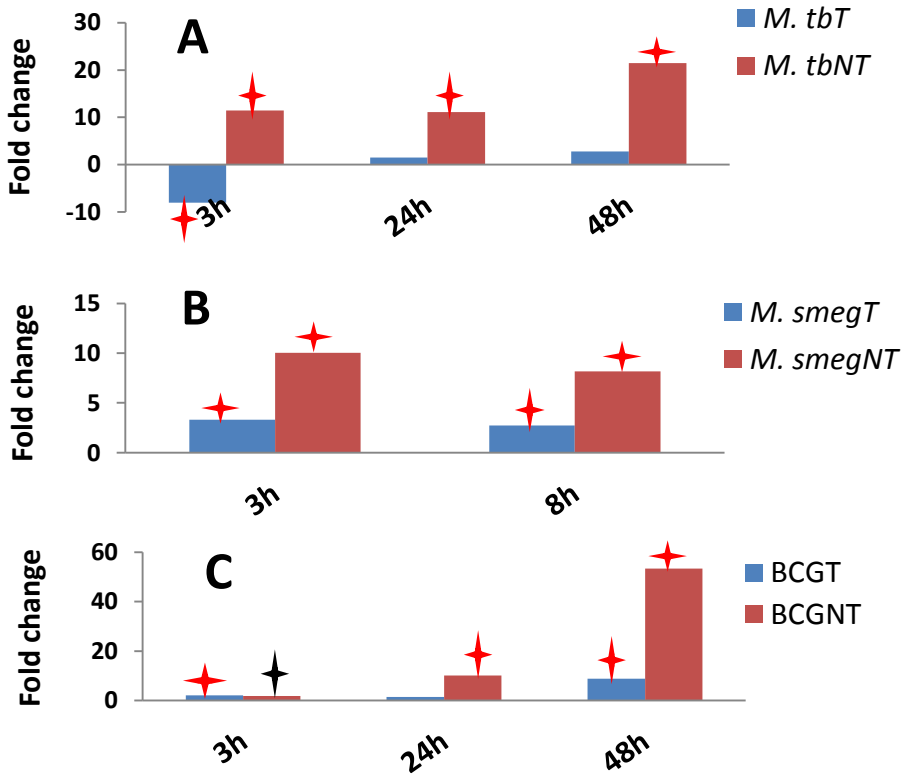

Figure 3.10 Differential expression of CCL1 in response to Mycobacteria cultured with and without Tween 80. A. CCL1 response to *M. tb* infection. **B.** CCL1 response *M. smegmatis* infection. **C.** CCL1 response to BCG infection. The significant difference in CCL1 expression levels in response to infection with mycobacteria$^{T/NT}$ suggests that the presence of Tween 80 in the culture medium of mycobacteria seems to inhibit the expression of CCL1. The upregulation and downregulation of the target genes was measured relative to the uninfected controls, using the randomised pair wise statistical test of the REST programme. Red stars indicate statistically significant results (*p-value* ≤ 0.05) and the black star indicates *p-value* close to 0.05 (0.074).

CCL1 showed a similar expression trend in response to *M. tb*, *M. bovis* BCG and *M. smegmatis* infections. This gene was upregulated in response to all 3 strains, cultured with or without Tween 80. Furthermore, the expression of CCL1 was significantly higher in response to mycobacteria^{NT} as compared to mycobacteria^{T}. Results are illustrated in Figure 3.10 and summarised (Fold changes and *p-values*) in Tables 3.2-3.4.

Table 3.2 Summary of results generated by the REST programme for *M. tb* infection. Differential gene expression of dTHP-1 cells in response to 3h, 24h and 48h infection with *M. tb*$^{T/NT}$.

M. tb												
	3h				**24h**				**48h**			
	T		**NT**		**T**		**NT**		**T**		**NT**	
	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*
STX7	-3.52	0.073	-1.70	0.074	-1.92	**0.001**	-2.00	**0.024**	13.47	**0.001**	1.30	0.368
TLR9	-6.15	**0.01**	1.41	0.682	-1.01	0.979	-8.78	**0.011**	-2.91	0.46	1.64	0.437
GANC	-2.11	0.083	-1.61	0.252	1.13	0.767	1.24	0.761	1.28	0.915	-1.26	0.726
IL-10	-15.76	**0.001**	2.83	**0.027**	1.94	**0.001**	1.69	0.311	-1.10	0.978	-1.73	0.533
STX4	-3.77	**0.014**	-1.05	0.951	-1.82	0.111	-1.48	0.57	3.17	0.16	-1.07	0.91
TLR7	3.32	**0.02**	-2.80	**0.041**	-6.49	**0.001**	-2.53	**0.015**	1.39	1	-2.60	0.064
CCL1	-8.00	**0.006**	11.43	**0.001**	1.48	0.483	11.13	**0.028**	2.81	0.385	21.48	**0.001**
VAMP7	-10.41	**0.025**	-2.51	**0.032**	1.25	0.98	-1.44	0.547	1.87	0.996	-2.17	0.008
CLIC4	6.82	**0.001**	1.14	0.835	-10.11	**0.001**	-1.64	0.466	7.70	**0.001**	-5.83	**0.001**
PRKCA	1.61	**0.001**	-1.34	0.154	2.29	0.102	-1.37	0.987	3.90	**0.005**	2.31	**0.02**
YWAH	-1.60	0.215	-2.74	**0.001**	-2.71	**0.001**	-1.51	0.465	1.15	1	-1.05	0.9
IL-12	-1.82	0.233	66.95	**0.001**	-16.74	**0.001**	-66.03	**0.001**	1.08	1	-1.95	0.101

T = Cultured with Tween 80, NT = Cultured without Tween 80, *p-values* highlighted in red represent a significant response

Table 3.3 Summary of results generated by the REST programme for *M. smegmatis* $^{T/NT}$

Differential gene expression of dTHP-1 cells in response to the 3h and 8h infection with *M. smegmatis*$^{T/NT}$.

M. smegmatis								
	3h				**8h**			
	T		**NT**		**T**		**NT**	
	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*
STX7	1.72	0.161	1.42	0.563	-1.37	0.354	1.04	0.917
TLR9	2.82	**0.001**	1.47	0.457	3.88	**0.001**	4.88	**0.001**
GANC	-1.11	0.938	1.50	0.488	1.05	0.549	-1.40	0.208
IL-10	3.62	**0.001**	54.22	**0.001**	3.86	**0.001**	4.42	**0.003**
STX4	1.35	0.841	1.09	0.998	-1.28	0.496	1.02	1
TLR7	2.63	**0.019**	3.78	**0.001**	1.05	0.914	1.86	**0.014**
CCL1	3.32	**0.001**	10.03	**0.001**	2.72	**0.001**	8.17	**0.001**
VAMP7	1.82	0.101	2.32	**0.017**	3.07	**0.001**	2.05	**0.001**
CLIC4	1.37	0.75	2.45	**0.001**	1.23	0.608	1.25	0.843
PRKCA	-1.11	0.987	-1.06	0.995	-1.41	0.272	-1.42	0.364
YWAH	1.68	0.204	1.36	0.695	1.95	**0.001**	1.74	**0.012**
IL-12β	3.13	**0.001**	101.83	**0.001**	-1.20	0.473	5.06	**0.001**

T = Cultured with Tween 80, NT = Cultured without Tween 80, *p-values* highlighted in red represent a significant response

Table 3.4 Summary of results generated by the REST programme for *M. bovis* BCG. Differential gene expression of dTHP-1 cells in response to 3h, 24h and 48h infection with *M. bovis* BCG$^{T/NT}$.

***M. bovis* BCG**												
	3h				**24h**				**48h**			
	T		**NT**		**T**		**NT**		**T**		**NT**	
	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*	Fold change	*p-value*
STX7	-1.14	**0.001**	2.12	**0.001**	-1.12	0.899	1.55	0.746	-1.53	0.289	1.19	0.339
TLR9	-1.55	**0.026**	4.26	**0.001**	1.51	0.297	1.01	1	2.42	**0.018**	1.49	0.218
GANC	-1.23	**0.03**	1.32	0.103	1.07	0.981	1.15	0.992	1.44	0.291	1.51	0.071
IL-10	3.22	**0.001**	-19.56	**0.001**	-1.34	**0.001**	-2.97	0.128	-2.00	0.068	2.54	**0.044**
STX4	1.36	**0.015**	-1.15	0.201	2.37	**0.008**	-1.77	0.444	2.17	**0.019**	-1.76	**0.012**
TLR7	-1.08	0.537	-1.30	**0.018**	1.47	0.353	-1.16	0.968	5.36	**0.001**	1.42	0.074
CCL1	2.03	**0.001**	1.85	0.074	1.39	0.47	10.13	**0.001**	8.82	**0.001**	53.38	**0.001**
VAMP7	1.44	**0.004**	1.68	**0.001**	2.20	**0.001**	-1.12	0.968	3.07	**0.001**	-1.24	0.323
CLIC4	1.15	**0.044**	-1.41	**0.03**	1.38	0.431	1.28	0.995	1.37	0.353	1.25	0.187
PRKCA	1.26	0.245	1.35	**0.001**	1.40	0.133	1.44	0.863	1.52	**0.034**	2.47	**0.001**
YWAH	1.00	0.959	1.03	0.893	1.53	0.214	-1.14	0.979	1.40	0.38	1.89	**0.003**
IL-12	5.52	**0.001**	-30.20	**0.001**	-1.41	0.414	-1.28	0.822	1.22	0.671	3.31	**0.001**

T = Cultured with Tween 80, NT = Cultured without Tween 80, *p-values* highlighted in red represent a significant response

3.2 Discussion

The current anti-TB drugs have had success in decreasing the deaths caused by TB (WHO, 2013), however this success has been compromised by the emergence of drug resistant strains (Fakruddin, 2013; Merza *et al*., 2011; Wang *et al*., 2006). The mechanism of action of the anti-TB drugs (target mycobacterial enzymes and pathways) is a major contributing factor to drug resistance (De La Iglesia and Morbidoni, 2006; Mdluli and Ma, 2007; Timmins and Deretic, 2006; Walsh, 2000). A different approach involves developing drugs that will target host enzymes, rather than mycbacterial enzymes (Jayaswal *et al*., 2010; Schwegmann and Brombacher, 2008) and it requires knowledge of the host factors that affect the intracellular survival of the pathogen (Huang *et al*., 2010; Jayaswal *et al*., 2010; Li *et al*., 2006). Therefore, this study focused on identification of genes of dTHP-1 cells that are differentially expressed by different mycobacterial species

A subset of 12 eukaryotic genes was selected for this study based on their known involvement in the phagocytic pathway and autophagy, as well as for their roles in immune response, in general (see section 1.6). Of these, VAMP7 and TLR7 were significantly differentially expressed by mycobacteria^{NT}, but not by mycobacteria^{T}. Therefore, only their responses to mycobacteria^{NT} will be discussed. Additionally, CCL1 was highly expressed by mycobacteria^{NT} as compared to mycobacteria^{T} and will also be discussed further.

3.2.1. Generation of single cell from mycobacteria^{NT}

Several studies have emphasized the drawbacks of using Tween 80 as a supplement in mycobacterial cultures. Although it is essential in minimizing clumps, it has been identified to have adverse effects on the mycobacterial cell wall integrity, solubilising the capsule that is implicated in mycobacterial virulence (Sani *et al*., 2010; Van Boxtel *et al*., 1990; Wang *et al*., 2010). In order for an *in vitro* interaction to mimic the *in vivo* state, it is important that the mycobacteria are as close to their native state as possible. This has raised concern about whether the effect of Tween 80 on the mycobacterial cell wall interferes with the interaction of mycobacteria with macrophages. It is for this reason we decided to culture the 3 mycobacterial species in growth medium^{NT}

We observed that about 90% of mycobacteria cultured in growth medium^{NT} were present in clumps. Additionally, the older (high OD_{600}) the cultures got the more clumps were formed. We observed that beyond the OD_{600} of 0.3 more mycobacteria were present in clumps than in single form, and there were almost no mycobacteria present in the culture medium (explained by the clear colour of the culture medium and mycobacterial precipitates at the bottom of the 50 ml tubes, as well as the very low (close to Zero) OD_{600} readings. Since the cultures grown to an OD_{600} of 0.3 exhibited some (about 10%) mycobacteria cells present in single form (assessed by ZN slides under oil immersion, section 2.1.4.1), we therefore decided to culture mycobacteria^{NT} to a lower OD_{600} of 0.3, in order to increase the probability of generating mycobacterial cultures with cells present in a single form.

Although the frozen mycobacterial^{NT} stocks were syringed twice with a 25G needle (our standard lab protocol used to break up mycobacterial clumps) during processing for infection, we still observed the same amount of clumps following syringing. The elimination of the clumps was achieved through filtration process using the 5 µm pore filter (as described in section 2.3.2), adapted from (Sani *et al*., 2010). The 5 µm pores of the filter were big enough to allow single bacteria and small clumps (containing 2-3 bacteria) to pass through without compromising cell viability (assessed by CFUs). Even though most of the mycobacteria^{NT} were lost after filtration, we managed to generate single form mycobacterial suspension for infection, limiting the probability of mycobacterial phagosomes to be trafficked to lysosomes due to clumps.

3.2.2 Effect of Tween 80 on the mycobacterial cell wall affects gene expression in dTHP-1 cells

Our results have interestingly revealed differences between the macrophage responses to mycobacteria^{NT} and mycobacteria^{T}, wherein the expression of CCL1, a chemokine involved in the formation of granulomas (Tal *et al*., 2007), was more expressed in response to mycobacteria^{NT} compared to mycobacteria^{T}. The lowered expression of CCL1 due to the presence of Tween 80 in mycobacteria cultures indicates that the perturbations of Tween 80 on mycobacterial cell wall potentially affect the interaction of mycobacteria with macrophages. This data suggest that a high throughput screening method is warranted to analyse the global changes induced by mycobacteria^{T}. Consistent with our results, Thuong *et*

al (2008) demonstrated that CCL1 was upregulated by *M. tb*; additionally, they identified 6 SNPs near CCL1 that were associated with pulmonary TB (Thuong *et al*., 2008). Furthermore, CCL1 was reported to be upregulated in granulomas induced by *M. bovis* purified protein derivative (PPD) (Chiu *et al*., 2003; Hoshino *et al*., 2007).

In the present study it was observed that CCL1 was upregulated by both *M. tb* and *M. smegmatis* strains cultured with and without Tween 80. The upregulation of CCL1 leads to granuloma formation, which primarily acts to prevent the spread of infection (Hoshino *et al*., 2007; Thuong *et al*., 2008). However, *M. tb* is able to survive in a dormant state inside the granuloma and later reactivate and escape the granuloma to subsequently spread to the rest of the body and progress to active TB disease (Silva Miranda *et al*., 2012). Therefore, the upregulation of CCL1 upon infection with *M. tb* does not guarantee control of *M. tb* spread, since it can escape the granulomas. Although CCL1 was also upregulated by *M. smegmatis*, this response may not be important since *M. smegmatis* is killed inside macrophages (Anes *et al*., 2006); hence the infection does not progress to the point of granuloma formation.

3.2.3 Effect of different times of exposure on gene expression of mycobacteria^{NT}- infected dTHP-1 cells

M. tb and *M. bovis* BCG are distinctly characterised from *M. smegmatis* by their ability to survive inside macrophages (Anes *et al*., 2006; McDonough *et al*., 1993; McGarvey *et al*., 2004; Walburger *et al*., 2004) and this ability is associated with *M. tb* pathogenicity (McGarvey *et al*., 2004; Schlesinger, 1996). It is worth mentioning that the classification of BCG pathogenicity remains ambiguous. However, it is regarded as non-pathogenic due to the absence of the RD1 region that was found to be conserved in all pathogenic mycobacteria (Lewis *et al*., 2003; Majlessi *et al*., 2005; Simeone *et al*., 2012). Even though these mycobacteria species can tolerate microbicidal activities of macrophages, under certain conditions they can be killed (Cross *et al*., 1999; Herbst *et al*., 2011) and the killing mechanisms are poorly understood; however, phagosome/ endosome-lysosome fusion and autophagy are implicated (Anes et al., 2006; Gutierrez *et al*., 2004).

VAMP7 is a transmembrane protein predominantly found in late endosomes and it is involved in the transport and fusion of late endosomes with lysosomes for subsequent degradation of invading pathogens (Advani *et al*., 1999). Moreover, VAMP-7 has been indicated to play a role in the phagocytic pathway (Braun *et al*., 2004), however, no other studies (to our knowledge) are available to support this. TLR7 is predominantly expressed in lung macrophages (Delgado *et al*., 2008), which are primary cells of pulmonary *M. tb* infection (Reljic *et al*., 2010). The ligands of TLR7, especially imiquimod induce autophagy in macrophages (De Meyer *et al*., 2012; Delgado *et al*., 2008).

We observed that the infection of macrophages with *M. tb*^{NT} resulted in suppression of VAMP7 and TLR7 expression and this response was maintained over the infection period (defined by the constant fold changes observed throughout the time periods). Since both genes induce protective effects against invading pathogens (Advani *et al*., 1999; De Meyer *et al*., 2012; Delgado *et al*., 2008), their downregulation by *M. tb* concurs with the ability of *M. tb* to withstand the hostile environment of macrophages (Walburger *et al*., 2004). Hence; *M. tb* most likely suppressed the expression of these genes as a defence mechanism to avoid induction of endosome-lysosome fusion and autophagy, thereby promoting its intracellular survival. A similar observation at 24 h and 48 h was seen in an *in vivo* study by (Avunje *et al*., 2013, 2011) in which VHSV (Viral haemorrhagic septicaemis virus) infection caused suppression of TLR7 in Olive flounder reared at 15 °C (Avunje *et al*., 2013, 2011).

In contrast to *M. tb* and *M. bovis* BCG, the infection with *M. smegmatis*^{NT} led to induction of these genes, which was expected since *M. smegmatis* is killed inside macrophages (Anes *et al*., 2006). A pronounced decrease with time was observed for TLR7 (whereas the response of VAMP7 remained constant), wherein the 3 h infection period exhibited a two-fold higher expression than the 8 h. This difference could be attributed to the fact that TLR7 is a surface receptor (Delgado *et al*., 2008), and therefore may be more induced during the uptake of the bacteria (3 h) than downstream during the intracellular killing or survival of the mycobacteria (8 h). This explanation is supported by the general role of PRRs, which is to protect the host during early infection (Schiller *et al*., 2006) through recognition of invading pathogens and activation of innate immunity for subsequent elimination of the intracellular pathogens (De Meyer *et al*., 2012; Delgado *et al*., 2008).

3.2.4 Effect of different mycobacterial strains on macrophage gene expression

M. tb elicits greater resistance to the acidic macrophage environment as compared to BCG (McDonough *et al*., 1993; Simeone *et al*., 2012). This was evident at 3 h post-infection, in which a greater degree of suppression of TLR7 and VAMP7 expression by *M. tb*^{NT} was observed when compared to *M. bovis* BCG^{NT}. These results may potentially relate to the fact that *M. bovis* BCG has a relatively lower survival rate in macrophages (McDonough *et al*., 1993).

3.2.5 Potential therapeutic applications of TLR7 and VAMP7

Therapeutic applications relavant to this study results include targeting TLR7 and VAMP7 via RNA activation (RNAa). RNAa is a newly discovered phenomena that uses synthetic small double-stranded RNAs (dsRNAs), termed small activating RNAs (saRNAs) to induce sequence-specific gene expression activation by targeting specific regions in the gene promoter (Huang *et al*., 2010; Janowski *et al*., 2007; Li *et al*., 2006; Place *et al*., 2008). If the activation of TLR7 and VAMP7 by synthetic saRNAs in *M. tb*-infected macrophages lead to decreased growth of *M. tb* intracellularly, this may suggest that these 2 genes are feasible targets for RNAa-based TB therapeutics.

The therapeutic feasibility of the RNAa approach has been demonstrated by several studies. Yang *et a*l (2013) have successfully demonstrated that the saRNA-induced activation of PAWR gene, a cancer-selective target for cancer therapies, that is essential for apoptosis and for suppressing tumours, induces the growth inhibition and apoptosis of cancer cells. These findings suggested potential of PAWR as target gene in cancer therapy (Yang *et al*., 2013). Additionally, Li et al. (2006) verified the role of small ds-RNAs in activating gene expression and further identified their potential use in gene activation-based therapies (Li *et al*., 2006).

A further approach to anti-TB drug development research may include application of RNA interference (RNAi). The cellular process in which gene expression can be silenced transcriptionally by targeting the specific region in the gene promoter or post-transcription via cleavage and degradation of mRNA, using small interfering RNAs (siRNAs) (Bumcrot *et*

al., 2006; Burnett and Rossi, 2012; Pal-Bhadra *et al*., 2002; Sijen *et al*., 2001). However, this approach will be feasible for genes that are upregulated by *M. tb* (Jayaswal *et al*., 2010).

Since the current approach to drug development is challenged due to the emergence of drug resistant strains, RNA-based therapeutics (RNAa and RNAi) could be a solution to replace the drugs that are currently failing, based on their advantage to potentially limit drug resistance, since host enzymes are the direct targets (Jayaswal *et al*., 2010; König *et al*., 2008; Li *et al*., 2006). However, there are potential challenges such as optimazation of selectivity, stability, delivery and long term safety that will need to be closely considered. Although RNA-based therapeutics is still in its infancy, there is accumulating evidence supporting their feasibility (Melnikova, 2007).

4

Conclusions

This study has successfully generated single cells from mycobacteria^{NT} during processing for infection, using the filtration method (as described in section 2.3.1, 2.3.2) without compromising the viability of the cells (assessed by CFUs). According to our knowledge this is the first study in which mycobacteria was successfully cultured without chemical (Tween 80) and/or mechanical (agitation) perturbations and was still able to generate single cells for infection.

Both VAMP 7 and TLR 7 were downregulated by *M. tb*^{NT} and upregulated by *M. smegmatis*^{NT}. This differential gene expression induced by the pathogenic and non-pathogenic mycobacterial species suggest that these genes may be feasible to target via RNAa, in order to determine if their induction will result in decreased growth of intracellular mycobacteria. Since the results of BCG were not statistically significant at 24 h and 48 h (Table 3.4), we could not distinguish if the response of these genes was specific to macrophages. However, the *M. tb* and *M. smegmatis* results suggest that these genes may be potential targets for RNA-based TB-therapeutics

The consistent significant differential expression of TLR7 and VAMP7 was induced by mycobacteria^{NT} but not by mycobacteria^{T}, suggesting that the perturbations caused by Tween 80 on the mycobacterial cell wall might have altered the transcriptional response of macrophages

CCL1 was the only gene that showed difference in gene expression in relation to culturing mycobacteria with and without Tween 80. It expression was stronger in response to mycobacteria^{NT} when compared to mycobacteria^{T}. This is proof of concept that the

perturbations caused by Tween 80 in the mycobacterial cell wall potentially altered the macrophage response to mycobacterial infection. Furthermore, these findings are in accordance with the studies (Sani *et al*., 2010; Van Boxtel *et al*., 1990) that have emphasized the need for Tween 80 exclusion in mycobacterial cultures.

Despite all the statistically significant gene expression induced by mycobacteria^{T}, none of the genes showed consistent differential expression between pathogenic and non-pathogenic mycobacteria, when dTHP-1 cells were infected with mycobacteria^{T}.

For future studies, given the results obtained for CCL1, a follow up study using a high throughput screening method such as RNA sequencing, is necessary in order to determine the global changes in gene expression, in relation to the effect of Tween 80. In addition, it is necessary that Tween 80 be excluded from culture media for all mycobacterial species. In case of comparison studies, all mycobacterial species (cultured in the presence or absence of Tween 80) should be grown to the same. Since it was observed that the cultures became excessively clumpy beyond OD_{600} of 0.3, this OD was chosen as a feasible cut off. Furthermore, more and later time points should be included, in order to study the macrophage response at later stages of mycobacterial infection and to verify if the response will be maintained over the longer infection period.

5

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