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The role of heparin binding haemagglutinin adhesin and curli pili on the pathogenicity of *Mycobacterium tuberculosis*

by

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Doctor of Philosophy

Medical Microbiology

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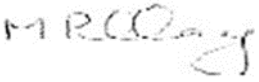

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DECLARATION 2: MANUSCRIPTS

This thesis consists of 3 manuscripts that are ready for submission. The contribution that my co-authors and I made to each of the manuscripts are presented here.

Manuscript 1:

Moodley S, Mvubu N.E, Pillay B, Christoffels A and Pillay M. *M. tuberculosis* heparin binding haemagglutin adhesin (HBHA) and curli pili (MTP) promote adhesion and invasion non-synergistically, and modulate cell signalling canonical pathways in macrophages. To be submitted to "*Functional & Integrative Genomics*".

Authors' contributions

I planned, executed the experimental design, analysed the data, conceptualized and prepared the manuscript. Dr Mvubu provided bioinformatics training and valuable insight in conceptualizing the manuscript. Prof M Pillay conceptualized the study, and provided scientific review of manuscript. Prof A Christoffels and Prof M Pillay funded the study. Prof B Pillay provided valuable insight in conceptualizing the manuscript and technical infrastructure.

Manuscript 2:

Moodley S, Mvubu N.E, Pillay B, Christoffels A and Pillay M. *Mycobacterium tuberculosis* heparin-binding haemagglutinin adhesin and curli pili modulate cytokine responses through inflammasome, NF-κB, toll-like receptor, MAPK and PI3-K/AKT pathways during infection of macrophages. To be submitted to "*Journal of Interferon & Cytokine Research*".

Authors contributions:

I conducted the experimental work, analysed data generated, designed and wrote the manuscript. Dr Mvubu was involved in the design and offered valuable scientific review of manuscript. Prof M Pillay designed the study, and revised the manuscript. Prof A Christoffels and Prof M. Pillay provided technical and financial support. Prof B Pillay provided access to the bioplex system and assisted in review of manuscript.

Manuscript 3:

Moodley S, Mvubu N.E, Muniram S, Pillay B, Christoffels A and Pillay M. *Mycobacterium tuberculosis* HBHA and MTP modulate the host immune response to facilitate intracellular replication in macrophages. To be submitted to "*Developmental and Comparative Immunology*".

Author contributions:

I conducted the experiments, analysed data, designed and composed the manuscript. Dr Mvubu was involved in the critical review of manuscript. Miss Muniram assisted with real time PCR experiments. Prof M. Pillay provided technical and financial support, conceived the study and critically reviewed the

manuscript. Prof A Christoffels offered project running expenses. Prof B Pillay provided equipment for real time PCR and offered valuable scientific review of manuscript.

PRESENTATIONS EMANATING FROM THIS THESIS

I. **Moodley S**, Mvubu N.E, Pillay B, Christoffels A and Pillay M (4 August 2017) Deletion of *Mycobacterium tuberculosis* HBHA and MTP enriches unique canonical pathways during early infection in THP-1 differentiated macrophages. School of Laboratory Medicine and Medical Sciences Research Day, Durban, South Africa. Oral presentation.

II. **Moodley S**, Mvubu N.E, Pillay B, Christoffels A and Pillay M (5-6 October 2017) Deletion of *Mycobacterium tuberculosis* HBHA and MTP enriches unique canonical pathways during early infection in THP-1 differentiated macrophages. College of Health Sciences Research Symposium, Durban, South Africa. Oral presentation.

III. **Moodley S**, Mvubu N.E, Pillay B, Christoffels A and Pillay M (9-11 November 2017) *Mycobacterium tuberculosis hbhA* and *mtp* deletion elicits unique canonical pathways during early infection in THP-1 differentiated macrophages. 7th FIDSSA Congress, Cape Town, South Africa. Poster presentation.

IV. **Moodley S**, Mvubu N.E, Pillay B, Christoffels A and Pillay M (20 – 23 February 2018) *Mycobacterium tuberculosis hbhA* and *mtp* deletion elicits unique canonical pathways during early infection in THP-1 differentiated macrophages. 5th Global Forum on TB Vaccines, New Delhi, India. Poster presentation.

V. **Moodley S**, Mvubu N.E, Pillay B, Christoffels A and Pillay M (12 – 15 June 2018) *Mycobacterium tuberculosis* Heparin-binding haemagglutinin adhesin and curli pili promote adhesion, invasion and cytokine production in THP-1 differentiated macrophages. 5th SA TB Conference, Durban, South Africa. Poster presentation.

DEDICATION

To my beloved parents and sister

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ABBREVIATIONS

Abbreviation	Abbreviated term
ANOVA	One-way analysis of variance
Apa	Alanine- and proline rich antigenic
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
BCG	Bacillus Calmette-Guerin
CAGE	Cap analysis gene expression
CARD	Caspase activation and recruitment domain
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CR	Complement receptors
CT	Cholera toxin
DAP12	DNAX-activating protein of 12 kDa
DCs	Dendritic cells
DC-SIGN	Dendritic cell specific intercellular adhesion molecule grabbing nonintegrin
DLX3	Distal-less homeobox 3
DM	Δ*hbhA-mtp* double knockout mutant
DNA	Deoxyribonucleic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
H	Δ*hbhA* single mutant
HBHA	Heparin-binding haemagglutinin adhesin
HC	hbhA complement
HIV	Human Immunodeficiency virus
IFN	Interferon
IFN-γ	Interferon gamma
IL	Interleukin
IPA	Ingenuity Pathway Analysis
IRAK	IL-1 receptor-associated kinases
kDa	Kilo-dalton
KZN	KwaZulu-Natal
LM	Lipomannan

M	Δ*mtp* single mutant
Man-LAM	Mannose-capped lipoarabinomannan
MAPK	Mitogen-activated protein kinase
MC	Mtp complement
MCP	Monocyte chemoattractant protein
MDR-TB	Multi-drug-resistant TB
MeV	Multi Experiment Viewer
MHC	Major Histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1 alpha
MR	Mannose receptors
mL	Millilitre
MOI	Multiplicity of infection
MTBC	*Mycobacterium tuberculosis* complex
MTP	*M. tuberculosis* curli pili
MyD88	Molecule myeloid differentiation primary response protein 88
ng/mL	Nanogram/millilitre
NF	Nuclear transcription factor
NK	Natural killer cells
NLRP3	Nucleotide oligomerization domain like receptor family, pyrin domain containing 3
NLRs	Nucleotide oligomerization domain like receptor
NOD	Nucleotide oligomerization domain
OD	Optical density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase reaction chain
PI3-K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pg/mL	Picograms/millilitre
PIMs	Phosphalidyl-myo-inositol mannosides
qRT-PCR	Quantitative real-time polymerase chain reaction
RANTES	Regulated upon Activation, Normal T cells Expressed and Secreted
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640
RT-PCR	Reverse transcription polymerase chain reaction
SDEGs	Significantly differential expressed genes
SEM	Standard error of mean

SP-A	Surfactant protein A
STAT	Signal transducer and activator of transcription
TB	Tuberculosis
TDR	Totally drug resistant
TGFβ	Transforming growth factor beta
TGM5	Transglutaminase 5
Th	T helper cell
TIR	Toll/IL-1R
TLR	Toll-like receptors
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cells
TRIF	Toll/IL-1R domain containing adapter inducing interferon
UCSC	University of California Santa Cruz
µg/mL	Microgram/millilitre
µL	Microlitre
µM	Micromolar
v/v	Volume/volume
WHO	World Health Organization
WT	Wildtype
XDR-TB	Extensively-drug-resistant TB
°C	Degree Celsius

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ABSTRACT

Background: Phagocytic host cells drive both the innate and adaptive arms of the host immune response during *Mycobacterium tuberculosis* (*M. tuberculosis*) infection. *M. tuberculosis* modulates the host immune responses and is able to proliferate in macrophages. The structures that mediate *M. tuberculosis* adherence (Adhesins) to macrophages are of particular interest for therapeutic development due to their cell surface localisation and immunogenic characteristics. *M. tuberculosis* produces numerous antigens that display adhesin functionality, including heparin-binding haemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) that are critical for adherence to host cells. Recently, the independent elucidation of the immunogenic potential of each suggested that HBHA and MTP may represent a novel combination as a biomarker for future therapeutic development. This study aimed to elucidate the effect of HBHA and MTP in combination on adhesion, invasion, replication, cytokine production and transcription regulation of macrophages infected with HBHA and MTP proficient and deficient strains in an attempt to assess their immunogenic capacity.

Materials and methods: THP-1 monocytic cells were differentiated into macrophages and infected at a multiplicity of infection of 5 with single mutants (Δ*hbhA* and Δ*mtp*), single complements of double mutant (hbhA comp and mtp comp), MTP and HBHA deficient double mutant Δ*hbhA-mtp* (DM) and MTP and HBHA proficient wild-type (WT) strain. The relative percentage adhesion/ invasion of the mutant and complemented strains was calculated at 1 h and 2 h post-infection respectively and compared to wild-type. Intracellular replication was quantified by colony forming units at 4 h, day 3 and day 6 post-infection.

To assess host transcriptomic changes elicited during early infection of THP-1 differentiated macrophages by WT and DM, RNA was extracted from host cells at 4 h post-infection. For the biological adhesion data set, raw data were filtered for genes in common with the Gene Ontology biological adhesion dataset sourced from EntrezGeneIds using the molecular signatures database with a False Discovery Rate q-value <1 (Chapter 1). Significantly differentially expressed genes with a p value <0.05 were used for further enrichment analysis (Chapter 2 and 3). Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA) upstream regulator, canonical pathway and biofunctions enrichment analysis were used to further investigate the differential regulation of molecular signatures by MTP and HBHA proficient and deficient strains. Macrophage cytokine/chemokine production was quantified at 24, 48 and 72 h post-infection using the Bio-Plex Pro Human Cytokine Multi-Plex Panel (Bio-Rad). Real-time quantitative RT-PCR was used to validate RNA sequencing findings and investigate

transcriptional regulation of HBHA and MTP of following genes: *CD80, DLX3, NLRP3, TGM5* and *TLR2* at 1 h, 2 h and 4 h post-infection

Results: During adhesion, DM induced a similar decrease in percentage adhesion (33.16%) to Δ*mtp* (39.4%), Δ*hbhA* (22.78%), mtp comp (24.72%), but statistically lower decrease in percentage adhesion than hbhA comp (53.85%). During invasion, DM displayed a significant decrease in percentage invasion (36.49%) compared to Δ*mtp* (61.49%) and hbhA comp (53.85%); and significantly higher decrease in percentage invasion than Δ*hbhA* (22.29%) and mtp comp (24.72%). Δ*mtp* demonstrated a 39.4% and 61.49% decrease in percentage adhesion and invasion compared to WT respectively. The HBHA-MTP proficient strain induced greater transcriptional changes resulting in enhanced adhesion to phagocytes and invasion of cells. Furthermore, the HBHA-MTP proficient strain displayed the sole ability to induce activation of phagocytosis. Further investigation of canonical pathway differential regulation by HBHA-MTP proficient strain demonstrated greater induction of canonical pathways. The most differentially regulated pathway was Gαq signalling canonical pathway, which is vital for migration of phagocytes. In addition, the HBHA-MTP proficient strain also enhanced activation of the acute phase response, role of pattern recognition receptors in recognition of bacteria and viruses, and production of nitric oxide and reactive oxygen species in macrophages canonical pathways.

RNA sequencing analysis showed that the *M. tuberculosis* adhesins, HBHA and MTP, elicited differential transcriptional regulation in macrophages, and demonstrated that predicted upstream regulators were associated with cytokine production. Further investigation of canonical pathways associated with these upstream regulators and cytokine quantification revealed that HBHA and MTP activate NF-κB, toll-like receptor, p38 MAPK and PI3-K/AKT canonical signalling pathways. HBHA and MTP elicited greater production of IL-4 and IL-10 at 24 h; G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 48 h and G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 72 h respectively, compared to DM infection. IL-1β, IL-2, IL-6, IL-12(p70), IL-17, TNF-α, IFN-γ, colony-stimulating factors G-CSF, GM-CSF and chemokines MCP-1 and MIP-1β were produced in higher concentrations by *M. tuberculosis* infection than anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13.

The bacillary load of M was significantly less than WT at all time intervals and similar to DM. The decreased replication ability of the HBHA-MTP mutant was attributed to MTP and not HBHA, suggesting that MTP facilitates replication during infection of macrophages. A transcriptional response common to both WT and DM, independent of HBHA-MTP, as well as unique responses induced by HBHA-MTP presence and deficiency were observed. The common transcriptional pattern exhibited the most enrichment for granulocyte adhesion and diapedesis canonical pathway, TNF upstream regulation and migration of

cells biological function. The HBHA-MTP uniquely induced transcripts were associated with the most significant enrichment of the Adipogenesis pathway, whilst HBHA-MTP deficiency induced the most significant enrichment of T helper cell differentiation. Unique transcripts elicited by HBHA-MTP deficiency induced less enrichment of NF-κB upstream regulator and were associated with migration of cells. The top 10 canonical pathways enriched by all transcripts were similar between both infections, but differed in molecules involved and their significance. HBHA-MTP enriched the TREM1 signalling pathway to a greater degree than HBHA-MTP deficiency in macrophages. HBHA-MTP deficiency, but not presence, enriched Th1 and Th2 Activation, Th1, Th2, Melatonin degradation, Sumoylation, Methylglyoxal degradation III, Granzyme A signalling, PCP pathways.

Discussion and conclusion: MTP played a greater role in adhesion and invasion during independent knockout and complementation in the double knockout strain than HBHA. HBHA and MTP together induced transcriptional changes that favour adhesion and invasion of macrophages. In addition, these 2 adhesins serve as pathogen-associated molecular patterns that enable host immune recognition during early infection of macrophages. HBHA and MTP activate intracellular signalling pathways that result in the longitudinal enhancement of a pro-inflammatory response during *M. tuberculosis* infection of macrophages. HBHA and MTP predominately induced a pro-inflammatory cytokine profile instead of an anti-inflammatory cytokine profile. This suggests that HBHA and MTP play a role in protective immunity and immunopathology as a consequence of pro-inflammatory cytokines such as TNF-α and minimal anti-inflammatory cytokines during *M. tuberculosis* infection. HBHA and MTP deficiency led to advanced immune activation and decreased intracellular growth. This suggests in the absence of HBHA and MTP, the presence of multiple, alternate antigens stimulate the intracellular signalling and transcriptional regulation *in vitro*. This advanced immune activation would potentially be detrimental to *M. tuberculosis* establishing a successful infection and would suggest that HBHA and MTP play a role in host immune response modulation as a protective measure during initial infection. Further investigation into the identity of these antigens would possibly result in a more successful, novel therapeutic target combination in addition to HBHA and MTP.

CHAPTER 1: Introduction and Literature Review

1.1. Introduction

Tuberculosis (TB) is still the leading cause of death from a single infectious agent including Human Immunodeficiency virus (HIV). The TB incidence is still alarmingly high with 10.4 million cases reported in 2016 (WHO, 2017). HIV severely impacts the TB burden as indicated by the high TB-HIV co-infection rates (74%) in Africa, and the 25% contribution to the global TB burden by the WHO African region (WHO, 2017). Drug resistance continues to be a persisting issue confounding TB control, with an estimated 600 000 new cases with rifampicin resistance, of which 490 000 were multi-drug-resistant TB (MDR-TB) (WHO, 2017). In 2016, 123 WHO member states reported extensively-drug-resistant TB (XDR-TB) cases and 6.2% of MDR-TB cases were attributed to XDR-TB (WHO, 2017). In addition, the first totally drug resistant TB case was identified in Italy in 2007 (Migliori *et al*., 2007) and since then, has been reported in South Africa (Klopper *et al*., 2013) and India (Udwadia, 2016).

Currently, TB mortality and incidence rates are decreasing by an estimated 3% and 2% yearly respectively, but these rates need to improve drastically in order to reach the first milestone of the End TB Strategy (WHO, 2017). TB control is seriously hampered by many factors, including the lack of rapid point of care diagnostic tests for timeous detection and initiation of treatment (McNerney and Daley, 2011; Dheda *et al*., 2013). The emergence of drug resistant strains has rendered the available treatment regimen virtually ineffective, while the extended treatment duration and drug toxicity has increased patient non-compliance, leading to increased TB transmission (Mittal and Gupta, 2011; Adane *et al*., 2013; Dela *et al*., 2017). Moreover, TB control is also challenged by the lack of an effective vaccine to provide adequate protection against TB in adult (Mangtani *et al*., 2014). Novel biomarkers for improved, new diagnostic tests, and therapeutic targets are urgently required to initiate earlier case detection and effective treatment, thereby decreasing TB morbidity and mortality.

The search for novel biomarkers is reliant on new knowledge of *Mycobacterium tuberculosis* pathogenesis. Attachment and entrance into host cell types including macrophages, facilitates intracellular replication and survival of *M. tuberculosis* in the host (Kline *et al*., 2009). Alveolar macrophages together with dendritic cells drive the induction of the cytokine and chemokine response to the stimulus of *M. tuberculosis* phagocytosis. These cytokine/chemokines activate anti-microbial responses and recruitment of leukocytes culminating in the formation of the granuloma (Guirado *et al*., 2013). Mycobacterial antigens have previously been shown to modulate the macrophage response by inducing pro-inflammatory cytokine secretion (Kim *et al*., 2011). The interactions between *M. tuberculosis* antigens and host immune response

signalling pathways and receptors are essential for protective host immune response initiation during *M. tuberculosis* infection (Jo *et al*., 2007).

Adhesion of pathogens to host cells is an important virulence characteristic of bacteria. Bacterial adhesins specifically target molecules or receptors on host cells, and are often present on the cell surface, thus making them attractive diagnostic and therapeutic targets (Kline *et al*., 2009). *M. tuberculosis* possesses a plethora of adhesins such as alanine- and proline rich antigenic Apa (Pasula *et al*., 1997), 19 kDa lipoprotein (Neyrolles *et al*., 2001), heparin binding haemagglutinin adhesin (Pethe *et al*., 2001), laminin binding protein (Pethe *et al*., 2002), Cpn60.2 (Stokes *et al*., 2004), malate synthase (Kinhikar *et al*., 2006), curli pili (Alteri *et al*., 2007), membrane protein, N-acetylmuramoyl-L-alanine amidase, and L,D-transpeptidase (Kumar *et al*., 2013).

Heparin-binding haemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) are antigens that are critical for adherence to host cells. HBHA is a 28kDA, cell surface exposed antigen with the ability to enhance mycobacterial auto-aggregation and mediate interaction of *M. tuberculosis* and epithelial cells (Menozzi *et al*., 1996). In addition to its role as an adhesin of epithelial cells, HBHA promotes dissemination during *M. tuberculosis* infection (Pethe *et al*., 2001; Menozzi *et al*., 2006; Esposito *et al*., 2011). Gene expression studies demonstrated that *hbhA* is differentially regulated during *M. tuberculosis* infection and *M. tuberculosis* infecting bone marrow-derived macrophages exhibit low expression of *hbhA* (Delogu *et al*., 2006). HBHA has also been implicated in apoptosis (Sohn *et al*., 2011; Choi *et al*., 2013) and explored as a possible biomarker for latent TB infection (Hougardy *et al*., 2007). Multiple studies have explored the potential of HBHA as a booster to vaccines (Kohama *et al*., 2008; Rouanet *et al*., 2009; Guerrero *et al*., 2010; Guerrero and Locht 2011), and most recently, as part of multi-antigen vaccine boosters (Teimourpour *et al*., 2017; Copland *et al*., 2018). In addition, HBHA has demonstrated immunogenic characteristics against *M. tuberculosis* infection (Menozzi *et al*., 1996; Masungi *et al*., 2002; Temmerman *et al*., 2004; Shin *et al*., 2006; Kim *et al*., 2011). Despite extensive advances in the development of HBHA as a vaccine candidate, there has been limited success suggesting that a possible multi-antigen booster may improve efficacy and immunogenicity.

It was believed that *M. tuberculosis* did not produce pili, until Alteri, *et al*., (2007) showed that mycobacteria produce fine, aggregative, comprising protein subunits and two distinct morphotypes (Type IV and MTP) (Alteri *et al*., 2007). MTP binds to the extracellular matrix protein laminin *in vitro* and is recognised by IgG in sera of patients with active TB (Alteri *et al*., 2007). Recently, MTP's role in biofilm formation and the essentiality of *Rv3312A* (*mtp)* gene for piliation (Ramsugit *et al*., 2013) was described using a *mtp* deletion mutant and complemented strain. The *mtp* gene was absent in non-tuberculous

mycobacteria and other respiratory bacteria suggesting that the pilin subunit protein of *M. tuberculosis* may be a suitable diagnostic marker (Naidoo *et al*., 2014). MTP has been described to be involved in adhesion and invasion to macrophages (Ramsugit and Pillay, 2014) and epithelial cells (Ramsugit *et al*., 2016). MTP has been demonstrated to be a strong immunogen in mice and epithelial cell models (unpublished), despite a previous study showing minimal influence on cytokine production (Ramsugit *et al*., 2016). These studies together provided evidence of the utility of MTP as a possible therapeutic and vaccine candidate biomarker, however, this has not yet been evaluated. The significant role of HBHA and MTP in pathogenesis was established independently of each other in the studies described above. The current study aimed to assess the combined potential of HBHA and MTP as possible multi-immunogen for further vaccine and drug development. This was achieved by functional genomics and global transcriptomics, using a double *hbhA-mtp* gene knockout and complemented strains to elucidate the role of the two adhesins in adhesion, invasion, replication and cytokine quantification in THP-1 macrophages.

1.2. Epidemiology of TB

Tuberculosis caused by *Mycobacterium tuberculosis*, one of the world's deadliest airborne diseases, was responsible for an estimated 10.4 million new cases and 1.4 million deaths in 2016 (WHO, 2017). TB is currently the ninth leading cause of death worldwide, resulting in approximately 1.3 million TB deaths among HIV-negative people and 374 000 deaths among HIV-positive in 2016 (WHO, 2017). South Africa was recently identified as one of the 30 high TB burden countries (WHO, 2017). The TB incidence in 2016 was 781 per 1000 000 population of which 461 per 1000 000 population was attributed to HIV and TB co-infected patients (WHO, 2017). TB was the leading underlying cause of death in nearly half of South African 52 districts that combined form the 9 provinces of South Africa (Statistics South Africa, 2016). Mpumalanga, Eastern Cape and KwaZulu-Natal provinces reported the highest number of deaths due to TB in 2016 (Statistics South Africa, 2016). Drug-resistance and HIV co- infection continue to be threats to TB control.

1.2.1. TB drug resistance

Drug-resistant strains include multidrug-resistant (MDR) which are resistant to rifampicin and isoniazid, extremely drug-resistant (XDR) strains that are MDR, in addition to any aminoglycoside and fluoroquinolones (WHO, 2017) and totally drug resistant (TDR), resistant to all first- and second-line drugs (Velayati *et al*., 2009). TDR cases have been documented in Italy (Migliori *et al*., 2007), India (Udwadia, 2016) and South Africa (Klopper *et al*., 2013). There were 600 000 new cases of rifampicin resistance and 490 000 cases displayed multi-drug-resistance in 2016 (WHO, 2017). In South Africa, 3.4% of new cases

and 7.1% of previously treated cases were infected with MDR or rifampicin resistant strains (WHO, 2017). MDR TB cases were more prevalent than previously surmised in Msinga sub-district of KwaZulu-Natal, South Africa (Gandhi *et al*., 2006). In a cohort of 475 culture confirmed TB cases, 39% were attributed to MDR and 6% to XDR TB (Gandhi *et al*., 2006). In South Africa, there were an estimated 8200 MDR/rifampicin resistant cases among identified pulmonary TB cases (WHO, 2017). Furthermore, XDR TB cases were widespread in KwaZulu-Natal (Moodley *et al*., 2011). In a cohort of 20858 culture confirmed TB cases, 20% and 11% were MDR- and XDR- TB cases respectively (Moodley *et al*., 2011). Additionally, XDR TB cases were associated with transmission to HIV co-infected population and high mortality rates, indicating that control of TB drug resistance and treatment is further complicated by HIV co-infection (Gandhi *et al*., 2006).

1.2.2. HIV co-infection

TB greatly impacts HIV associated morbidity and mortality in sub-Saharan Africa (Mukadi *et al*., 2001). In 2016, 74% and 59% of new and relapsed TB cases were HIV-positive in Africa and South Africa respectively (WHO, 2017). HIV co-infection interferes with the host pathogen interactions, consequentially increasing bacillary loads, active disease and poor immune control (Lawn *et al*., 2011). Therefore, TB and HIV are major public health concern, in South Africa and worldwide (Karim *et al*., 2010).

1.3. General characteristics of *M. tuberculosis*

Mycobacteria are acid-fast bacilli due to the presence of mycolic acids in the cell wall. *M. tuberculosis* bacilli are slightly curved, rod-shaped, of an approximate 1 to 4 mm length and 0.3 to 0.6 mm width. *M. tuberculosis* belongs to the *Mycobacterium tuberculosis* complex (MTBC) and is the causative agent of TB in humans. *M. tuberculosis* exhibits slow growth, is nonsporulating and non-motile (Sakamoto, 2012). The MTBC is made up of various species including *M. africanum*, *M. canettii*, *M. caprae*, *M. bovis*, *M. microti*, *M. mungi*, *M. orygis*, *M. pinnipedii* and *M. suricattae*, that are the causative agents of TB in various hosts (Alexander *et al*., 2010).

1.3.1. Mycobacterial cell wall

The mycolic acid layer impairs nutrient entry, thus accounting for the slow growth of mycobacteria and aids in resistance against lysosomal enzyme degradation (Sakamoto, 2012). The external layer consists mainly of mycolic acids and mannose-capped lipoarabinomannan (Man-LAM), the related lipomannan (LM), and mannoglycoproteins (Torrelles and Schlesinger, 2011). The inner layer of cell wall consists of

arabinogalactan, phosphalidyl-myo-inositol mannosides (PIMs) and peptidoglycans. The outer capsule and surface is composed of Mannan and arabinomannan (Torrelles and Schlesinger, 2011).

1.3.2. Strain families

The MTBC has been classified into 7 lineages based on whole genome sequencing analyses and these lineages have associated with specific geographical regions. Lineages 1 to 7 are also known as Indo-Oceanic, East-Asian, East-African-Indian, Euro-American, West-Africa (5 and 6) and Ethiopia respectively (Coscolla and Gagneux, 2014). Lineages 2 and 4 are more broadly distributed, and lineage 2 includes the Beijing strain family which has been found in East and central Asia, Russia and South Africa (De Jong *et al*., 2010; Gehre *et al*., 2013; Coscolla and Gagneux, 2014). *M. tuberculosis* strain families found in South Africa include W/Beijing, S, T and F15/LAM4/KZN (Chihota *et al*., 2012; Pillay and Sturm, 2007). Drug-susceptible TB cases were most often attributed to W/Beijing, S and T strain families (Gandhi *et al*., 2014), whereas the W/Beijing strain family was related to MDR and XDR TB cases in the Western and Eastern Cape (Chihota *et al*., 2012). In KwaZulu-Natal, the F15/LAM4/KZN strain family was responsible for the MDR and XDR TB cases (Pillay and Sturm, 2007; Chihota *et al*., 2012; Gandhi *et al*., 2014).

1.4. TB pathogenesis

M. tuberculosis infection can be described as three related but separated stages (Cooper, 2009). The first stage involves the transmission of *M. tuberculosis* via aerosol exchange between infected and healthy individuals (Cooper, 2009). Following inhalation of infectious droplets, *M. tuberculosis* infects and multiplies within alveolar macrophages and dendritic cells (DCs). Initial infection control relies on the host and *M. tuberculosis* strain genetics (Cooper, 2009). During primary infection, macrophages fail to adequately function due to *M. tuberculosis* evasion strategies such as reduction of acidification of phagosomes, reactive nitrogen and oxygen species and production of anti-inflammatory cytokines (Cooper, 2009). This supports *M. tuberculosis* proliferation and induces mild inflammatory response (Cooper, 2009). The second stage is the cell-mediated immunity and granuloma formation (Cooper, 2009). Once *M. tuberculosis* is able to proliferate within the macrophages, macrophage death attracts monocytes and inflammatory cells such as neutrophils from blood (Cooper, 2009). Monocytes differentiate into macrophages and DCs that phagocytose *M. tuberculosis* but fail to control *M. tuberculosis* proliferation. During this stage, *M. tuberculosis* proliferates with minimum tissue damage (Cooper, 2009). Approximately 6-8 weeks post-infection, T lymphocytes are differentiated and recruited in lymph nodes by antigen presenting DCs, and migrate to site of infection resulting in initiation of granuloma formation

(Cooper, 2009). Sustained T cell activation results in granuloma formation leading to the persistence stage of infection, during which 90% of individuals are asymptomatic, however, *M. tuberculosis* are possibly still present in alveolar macrophages (Cooper, 2009). The final stage is reactivation of persistent *M. tuberculosis*, due to changes in host immunity or failure to sustain immune response resulting in pulmonary disease and lung cavitation (Sasindran and Torrelles, 2011).

1.5. Immune response to TB

The innate immune system is supported by macrophages, DCs, neutrophils and natural killer cells (NK) (Sia *et al*., 2015). Phagocytic cells are responsible for the activation of adaptive T-cell immunity due to the presentation of mycobacterial antigens and the resultant secretion of chemokines and cytokines (Cooper *et al*., 2011). *M. tuberculosis* infected DCs have been shown to be less efficient at activating antigen-specific T cells compared to uninfected DCs (Wolf *et al*., 2007), as a possible immune evasion strategy by *M. tuberculosis*.

The adaptive immune response is responsible for generating immunological memory that assists pathogen elimination during the late phases of infection (Gupta *et al*., 2011). The adaptive immunity involves both humoral immunity which is facilitated by B lymphocytes and cell-mediated immunity which is facilitated by T lymphocytes. Naïve T and B cells are activated by professional antigen presenting cells that process and present antigens on the cell-surface of major Histocompatibility complex (MHC) molecules (Gupta *et al*., 2011). Activated T cells clonally expand and differentiate into CD4+ effector cells and migrate to sites of infection from lymph nodes stimulated by chemokines (Gupta *et al*., 2011).

Cytokines are an important part of the adaptive immune response. CD4+ T cell cytokine expression pattern is responsible for the th1-like response that is associated with control of *M. tuberculosis* infection (Cooper *et al*., 2011).

1.5.1. Macrophage and *M. tuberculosis* interaction

Macrophages are an important part of the innate immune system and are regarded to be one of the first cells encountered by inhaled bacilli (Pieters, 2008). Once phagocytosis has taken place, the bacilli form a phagosome which fuses with a lysosome to form a phagolysosome which favours degradation (Pieters, 2008). However, some bacilli are able to escape this environment by delaying phagosome maturation or escaping to cytosol and survive within the macrophages (Pieters, 2008). This induces a cascade of signalling pathways that result in the ultimate control of intracellular growth by the production of innate immune and antimicrobial mediators (Guirado *et al*., 2013).

Many macrophage models have been used to study *M. tuberculosis*-macrophage interactions including mouse macrophage cell lines, human monocyte derived macrophages and THP-1 monocytic cell line. The reproducibility of data from the human monocyte derived macrophage models is questionable due to genetic variability from donor to donor. The transcriptional profile for intracellular *M. tuberculosis* in the THP-1 model did not respond to a low-iron environment as observed for intracellular *M. tuberculosis* in the mouse macrophage model suggesting variability between mouse and human macrophage model results (Fontán *et al*., 2008). The THP-1 monocytic cell lines is differentiated into mature macrophages using phorbol-myristate acetate and displays similar characteristics to monocyte-derived macrophages after infection with *M. tuberculosis* such as low levels of oxygen radicals and nitric oxide (Sly *et al*., 2001). Transcriptional analysis of intracellular *M. tuberculosis* in the THP-1 model has shown that the major transcriptional changes were related to the metabolism of lipids, cell envelope, PE/PPE, the ESAT-6-like families of proteins and TAT system of secretion suggesting that secretion and expression of surface components and cell wall structure are altered in intracellular *M. tuberculosis* (Fontán *et al*., 2008).

1.6. Pattern-recognition receptors

Pattern-recognition receptors found on various cells of the innate immune system enable pathogen recognition by identifying the microbe through pathogen associated molecular patterns (PAMPs) (O'Garra *et al*., 2013). *M. tuberculosis* components interact with various pattern-recognition receptors on the cell surface of phagocytes (Hossain and Norazmi, 2013) and airway epithelial cells (Li *et al*., 2012) including Toll-like receptors, nucleotide-binding-oligomerization-domain (NOD)-like receptors, complement receptors, mannose receptors, surfactant protein A, scavenger receptors and the dendritic cell specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) (O'Garra *et al*., 2013).

1.6.1. Toll-like receptors

Toll-like receptors (TLR) have been shown in mammals to comprise 12 forms (Kawasaki and Kawai, 2014). TLRs are found on the cell membrane surface or on endocytic vesicle membranes of immune cells such as DCs and macrophages. Initial interaction of *M. tuberculosis* and TLRs has been shown to lead to activation, but not ingestion of the bacillus (Kawasaki and Kawai, 2014). Following this activation, TGFβ-activated protein kinase 1, IL-1 receptor-associated kinases (IRAK), mitogen-activated protein kinase (MAPK) and TNF receptor-associated factor 6 are employed via a signalling cascade, resulting in activation of transcriptional factors like nuclear transcription factor (NF)-κB (Kawasaki and Kawai, 2014). This results in production of nitric oxide and pro-inflammatory cytokines such as TNF, IL1β, and IL-12 which play vital roles in the innate immune response (Kawasaki and Kawai, 2014). Fremond *et al*., (2007)

demonstrated that signalling pathways involving adaptor molecule myeloid differentiation primary response protein 88 (MyD88) knockout mice were more susceptible to *M. tuberculosis* infection compared to wildtype mice, thus highlighting the vital role of MyD88 in innate immune activation during *M. tuberculosis* infection. TLR4 has previously been demonstrated to elicit signalling via an alternate pathway, which utilizes the adaptor molecule Toll/IL-1R domain containing adapter inducing interferon (IFN)-β (TRIF), leading to autophagy induced by lipopolysaccharides produced by gram negative bacteria (Xu *et al*., 2007). *M. tuberculosis* recognition has been reported for the following TLRs; TLR2, TLR4, TLR9 and probably TLR8 (Means *et al*., 1999, 2001; Tapping and Tobias, 2003; Bafica *et al*., 2005; Davila *et al*., 2008). Various mycobacterial proteins and lipids (Table 1.1) are associated with TLR signalling.

Table 1.1: TLR recognition of mycobacterial components (Jo *et al*., 2007). List of literature describing known *M. tuberculosis* TLR ligands.

Mycobacterial components	TLR usage	Species	Reference
19 kDa lipoprotein (LpqH)	TLR2	*M. tuberculosis, M. bovis*	Brightbill *et al.* (1999); Noss *et al.* (2001)
27 kDa lipoprotein	TLR2	*M. tuberculosis*	Hovav *et al.* (2004)
33 kDa lipoprotein	TLR2	*M. leprae*	Krutzik *et al.* (2003)
38 kDa glycolipoprotein	TLR2, TLR4	*M. tuberculosis*	Jung *et al.* (2006)
Ara-LAM	TLR2	*M. smegmatis*	Wieland *et al.* (2004)
GPL	TLR2	*M. avium*	Sweet and Schorey (2006)
HSP65	TLR4	*M. tuberculosis*	Bulut *et al.* (2005)
HSP70	TLR2, TLR4	*M. tuberculosis*	Bulut *et al.* (2005)
Lipomannan	TLR2	*M. tuberculosis, M. bovis*	Quesniaux *et al.* (2004b)
LprA lipoprotein	TLR2	*M. tuberculosis, M. bovis*	Pecora *et al.* (2006)
LprG lipoprotein	TLR2	*M. tuberculosis, M. bovis*	Gehring *et al.* (2004)
Man-LAM	–	*M. tuberculosis, M. bovis, M. kansasii*	Quesniaux *et al.* (2004b)
PE_PGRS33	TLR2	*M. tuberculosis*	Basu *et al.* (2007)
PIM 2, PIM 6	TLR2	*M. tuberculosis, M. bovis, M. smegmatis*	Gilleron *et al.* (2003)
PILAM	TLR2	*M. smegmatis*	Heldwein and Fenton (2002)
Soluble tuberculosis factor	TLR2	*M. tuberculosis*	Means *et al.* (1999)

TLR2 was reported to induce tumour necrosis factor alpha (TNF-α) when stimulated with whole *M. tuberculosis*, thus identifying TLR2 as a major pro-inflammatory signal inducer (Underhill *et al*., 1999) and contributor to host protection against infection with this pathogen. This was supported by mice deficient in TLR2 displaying host resistance and neutrophil impairment in response to a virulent strain of *Mycobacterium avium* (Feng *et al*., 2003) and inadequate granuloma formation in response to high-doses of *M. tuberculosis* (Reiling *et al*., 2002; Drennan *et al*., 2004). In addition, TLR2 stimulation is required for IL-12 production by macrophages and not in DCs (Pompei *et al*., 2007).

TLR4 has also been described to be involved in host protection against *M. tuberculosis* infection. In murine TB models, non-functional TLR4 resulted in increased bacillary load and mortality (Abel *et al*., 2002; Branger *et al*., 2004). However in *M. avium* infection, TLR4 deficiency did not play any role in overall progression of disease (Feng *et al*., 2003). TLR4 plays a role in *M. tuberculosis* recognition by murine macrophages and Chinese hamster ovary cells (Means *et al*., 1999; Means *et al*., 2001). Murine

macrophages isolated from TLR4-deficient mice were reported to reduce but not completely eliminate TNF-α production (Reiling *et al*., 2002). TLR9 identifies bacterial DNA, specifically the unmethylated CpG motifs. IL-12 production by DCs was observed to be TLR9-dependent (Bafica *et al*., 2005; Pompei *et al*., 2007). TLR9 deficient mice were characterized by advanced disease progression (Bafica *et al*., 2005). TLR8 was able to identify single-stranded RNA, present in pathogens such as RNA viruses, however, it was also shown to be upregulated in macrophages infected with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) (Davila *et al*., 2008). Recently, genetic polymorphisms in TLR7 and TLR8 have been correlated to susceptibility to *M. tuberculosis* infection due to impaired phagocytosis and TLR signalling (Lai *et al*., 2016).

1.6.2. NOD-Like Receptors

Mammalian nucleotide oligomerization domain (NOD) like receptors (NLRs) consist of more than twenty variation receptors that are characterized by conserved structures (Lu *et al*., 2014). The NLRs are composed of central nucleotide binding domain (NACHT) or NOD (Lu *et al*., 2014). Leucine rich repeats form the C-terminal and are considered the domain that identify the PAMPs and result in activation of the receptors (Lu *et al*., 2014). The N-terminal consists of an effector molecule which is either a caspase activation and recruitment domain (CARD), pyrin or baculovirus inhibitor of apoptosis repeat domain (Proell *et al*., 2008). NLRs that consist of CARD (NOD1 and NOD2), form oligomers subsequently resulting in recruitment of receptor-interacting protein 2 (or CARD containing kinase) via CARD-CARD association, which ultimately results in NF-κB recruitment (Lu *et al*., 2014).

The inflammasome represents one of the signalling pathways important for the host antimycobacterial defence, which is activated by caspase-1 leading to the processing of IL-1 procytokines into either IL-1β or IL-18. Pyrin-domain containing NLRs also possess the potential to form different forms of the inflammasome which consist of either NLRP1, NLRP3 or NLRC4 (Jo *et al*., 2016) and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Nguyen and Nguyen, 2014). NOD2 and TLR/TLR6 receptors have been implicated in inducing IL-1β production during *M. tuberculosis* infection (Kleinnijenhuis *et al*., 2009). Contradictory data obtained by (Master *et al*., 2008) suggested that *M. tuberculosis* infection results in inflammasome inhibition and IL-1β production. These differences could be due to different models of infection used in both studies. NOD2 identifies bacterial peptidoglycans (Girardin *et al*., 2003), and pro-inflammatory cytokines and nitric oxide production were reduced during *M. tuberculosis* infection of mice deficient in this receptor, but the susceptibility to the infection was variable (Divangahi *et al*., 2008).

1.6.3. C-Type Lectin

C-type lectins are pathogen recognition receptors associated with polysaccharide recognition. Mannose receptors (MR) are composed of one cysteine-rich and eight carbohydrate recognition domains, and are expressed on alveolar macrophages (Kleinnijenhuis *et al*., 2011). During *M. tuberculosis* infection, MR stimulation induces production of IL-4 and IL-13, decreased IL-12 production and decreased activation of oxidative responses (Kleinnijenhuis *et al*., 2011). MR has the ability to bind to Man-LAM and *M. tuberculosis* cell wall components such as PIMs resulting in induction of phagocytosis, but limited phagosome-lysosome fusion (Vergne *et al*., 2003; Hmama *et al*., 2004; Kang *et al*., 2005).

1.6.4. Complement, scavenger, surfactant protein A and DC-SIGN receptors

Complement receptors (CR) are expressed on cell surface of phagocytic cells and interact with complement components. CR1, CR3 and CR4 involvement in *M. tuberculosis* internalization has been previously described (Ferguson *et al*., 2004). CR facilitate uptake of both virulent and avirulent *M. tuberculosis* strains and CR blocking results in inhibition of *M. tuberculosis* phagocytosis (O'Garra *et al*., 2013). The interaction of CR3 and *M. tuberculosis* has been noted to inhibit the formation of respiratory bursts and production of inflammatory response (Kleinnijenhuis *et al*., 2011).

Upon CRS inhibition, scavenger receptor class A may facilitate *M. tuberculosis* entry by phagocytosis (Zimmerli *et al*., 1996). In addition, scavenger receptor B class 1 facilitates *M. tuberculosis* recognition, but is minimally involved in host immune response to *M. tuberculosis* infection (Schäfer *et al*., 2009). Scavenger receptor class A deficiency delayed progression of disease by enhancing formation of multinucleated giant cells and subsequent *M. tuberculosis* containment but did not influence early formation of granulomas (Sever-Chroneos *et al*., 2011).

DCs express DC-SIGN, which functions as a recognition receptor for carbohydrates that mediates adhesion and the interaction between DCs and T-cells (Kleinnijenhuis *et al*., 2011). DC-SIGN recognition of *M. tuberculosis* Man-LAM and lipomannans results in production of IL-10 (Geijtenbeek *et al*., 2003).

Surfactant protein A (SP-A) enhances phagocytosis of *M. tuberculosis* by macrophages (Gaynor *et al*., 1995; Pasula *et al*., 1997) and decreases production of reactive nitrogen intermediates (Pasula *et al*., 1999) thus facilitating intracellular survival of *M. tuberculosis*. HIV-*M. tuberculosis* co-infected patients exhibit

enhanced expression of SP-A in their lungs and this correlated with higher risk of active disease (Downing *et al*., 1995).

1.7. CD4+ T-cells

CD4+ T-cells are divided into groups according to the cytokine profiles which are dependent on antigen presenting cells. Naïve CD4+ T-cells differentiate into Th1, Th2, Th9, Th17, Th22 and T-follicular effector cells (Akdis *et al*., 2011). Th1, Th2, Th17 and T regulator cells have been described to play vital roles in *M. tuberculosis* infection (Romero-Adrian *et al*., 2015).

1.7.1. Th1 cells

Th1 cells are induced by infection of intracellular pathogens, produce interferon-gamma (IFN-γ) and activate macrophages, thereby facilitating protection during infection (Wei *et al*., 2010; Evans and Jenner, 2013; Zhu *et al*., 2013). Th1 inducing cytokines are mainly IL-12 and IFN- γ. IL-12 is mostly produced by antigen-presenting cells and induces the transcription factor signal transducer and activator of transcription (STAT) 4 when interacting with receptors on T cells, thereby inducing transcriptional factor T-bet and IFN-γ production (Wei *et al*., 2010; Evans and Jenner, 2013; Zhu *et al*., 2013). T-bet is an important regulator of Th1 cells and inhibits the differentiation of Th2 and TH17 cells by negative regulation of the expression of their specific genes (Wei *et al*., 2010; Evans and Jenner, 2013; Zhu *et al*., 2013). IFN-γ induces STAT1 which synergizes with STAT4, thereby activating Th1 gene activation (Lighvani *et al*., 2001; Mikhak *et al*., 2006). Multiple studies have demonstrated that Th1 cells are central to protection against TB by initiation of IFN-γ production, resulting in activation of macrophage activities (Flynn *et al*., 2011; Weiss and Schaible, 2015) and increased susceptibility to TB has been associated with mutations in gene encoding Th1 cytokines (Cooke *et al*., 2006; Ding *et al*., 2008; Stein *et al*., 2008). TB patients with good clinical outcomes have been associated with greater Th1/Th2 ratio than patients with poor outcomes (Lienhardt *et al*., 2002).

1.7.2. Th2 cells

Production of IL-4, IL-5, IL-9, IL-13 (Akdis *et al*., 2011) induces a Th2 and inflammatory response, which in turn, results in production of IL-25, IL-31 and IL-33 (Kang *et al*., 2005; Bilsborough *et al*., 2006; Kakkar and Lee, 2008). Th2 cytokines counteract the host protective immune response by inhibiting IFN-γ production and macrophage activation. TB patients have previously shown high levels of Th2 cytokines (Barnes *et al*., 1993a). Effective clearance of *M. tuberculosis* requires both Th1 and Th2 responses, but the

balance of both is important for effective immune response and thus, the outcome of the disease (Infante-Duarto and Kamradt, 1999).

1.7.3. Th17 cells

Th17 cells are regulated by transcription factor RORγt and are independent of Th1 and Th2 transcription factors (Ivanov *et al*., 2006). IL-17 is the most important effector cytokine but IL-22, IL-26 and GM-CSF also play a role in Th17 differentiation (Ling *et al*., 2013; Zambrano-Zaragoza *et al*., 2014). The role of Th17 cells during primary infection has been controversial. In the mice model, IL-17 was deemed to play an unimportant role in primary immunity (Khader *et al*., 2005), in contrast to other studies that have reported increased levels of IL-17/Th17 cells in mice infected with HN878. IL-17 deficient mice were impaired in induction of chemokines, co-localization of macrophages and T-lymphocytes, leading to elevated bacillary loads in lungs (Gopal *et al*., 2014). In support of this, Th17 cells have also previously shown partial inhibition of *M. tuberculosis* growth (Gallegos *et al*., 2011).

1.7.4. T regulator cells

Regulatory T (Treg) cells are differentiated by transforming growth factor β (TGF-β) and influence *M. tuberculosis* infection by producing IL-10 and TGF- β (Romero-Adrian *et al*., 2015). These cells have been found in larger amounts in peripheral blood of TB patients with active disease compared to previously BCG vaccinated individuals (Scott-Browne *et al*., 2007) and are capable of suppressing the immune response. Decreased numbers of Treg cells were reported in peripheral blood during TB treatment (Ribeiro-Rodrigues *et al*., 2006).

1.8. Cytokine production upon *M. tuberculosis* infection

Cytokines are important mediators of the host immune response and are produced by a plethora of immune cells. Cytokines possess regulatory functions and participate in inflammatory, immune and tissue repair responses (Gupta *et al*., 2011). Various bacterial cell components and mycobacterial components (Table 1.1) are able to induce cytokine production (Jo *et al*., 2007; Rakotosamimanana *et al*., 2010). During TB infection, immune and non-immune cells generate specific cytokines and chemokines (Manca *et al*., 2004; Lee *et al*., 2009; Carmona *et al*., 2013). The cytokine responses to *M. tuberculosis* infection can be broken down into pro- and anti-inflammatory responses. The generation of pro-inflammatory cytokines induce an inflammatory response via a cascade of pathways and networks that are involved in the regulation. Interleukin-1 (IL-1), IL-7, IL-12, IL-15, IL-18, IL-23 and IL-27 have been identified as important for

effective control of TB (Cavalcanti *et al*., 2012). Post-inhalation, *M. tuberculosis* bacilli are ingested by macrophages and other antigen presenting cells such as DCs. These cells together initiate production of pro-inflammatory cytokines; interleukin (IL)-12, IL-1β and TNF-α (Flynn *et al*., 2011).

1.8.1. Pro-inflammatory cytokines involved in *M. tuberculosis* infection

Macrophages and epithelial cells contribute to the local inflammatory response by producing pro-inflammatory cytokines (Lee *et al*., 2009). Multiple studies have described mycobacterial antigens that induce a pro-inflammatory response including 38-kDa glycolipoprotein (Jung *et al*., 2006), 19 kDa Lipoprotein (Rao *et al*., 2005), FMN binding nitroreductase domain containing protein (Peddireddy *et al*., 2016), macrophage-activating protein, *Rv2882c* (Choi *et al*., 2016) and HBHA (Kim *et al*., 2011).

1.8.1.1. Interleukin 1-β (IL-1β)

IL-1β is a pro-inflammatory cytokine that is synthesised by macrophages, monocytes and DCs (Dahl *et al*., 1996) and is involved in activating mycobactericidal activity, promotes anti-microbial molecules, increases TNF-α and Tumour necrosis factor receptor-1 expression and activating caspase-3 (Jayaraman *et al*., 2013; Krishnan *et al*., 2013). IL-1 was implicated in protection against *M. tuberculosis* during early infection (Yamada *et al*., 2000). Deficiency in IL-1β and IL-1R resulted in defective granuloma formation and increased growth (Juffermans *et al*., 2000). IL-1β production during *M. tuberculosis* infection does not require caspsase-1 or TLR signalling (Mayer-Barber *et al*., 2010). IL-1β production by DCs and bone marrow-derived macrophages infected with *M. tuberculosis in vitro* is facilitated by NOD like receptor family, pyrin domain containing 3 (NLRP3)-inflammsome mediated activation of caspase-1 (Koo *et al*., 2008; TeKippe *et al*., 2010).

1.8.1.2. Interleukin 2 (IL-2)

IL-2 is produced most often by CD4+ and CD8+ T cells, DCs and natural killer cells stimulated by antigens (Akdis *et al*., 2011). IL-2 plays a role in the activation of T-cells and B-cells. High expression and production of IL-2 which demonstrates a synergistic relationship to IFN-γ was observed during early stages of *M. avium* infection in the mice model but the same effect was not seen during late stage infection (Mannering and Cheers, 2002). TB patients have reduced response to IL-2, due to a defective IL-2 receptor or polymorphisms in the IL-2 gene (Sivangala *et al*., 2014). IL-2 has been proposed to be used as a possible diagnostic marker to distinguish between active and latent TB cases (Biselli *et al*., 2010).

1.8.1.3. Interleukin 6 (IL-6)

IL-6 which is produced at the sites of early infection, possesses properties of both pro- and anti-inflammatory cytokines (Romero-Adrian *et al*., 2015). The lack of IL-6 resulted in increased bacterial burden and delay in IFN-γ production. However, it was reported to be not essential for the protective host response to *M. tuberculosis* infection (Saunders *et al*., 2000). IL-6 is possibly detrimental in *M. tuberculosis* infections due to its ability to inhibit TNF-α and IL-1β (Schindler *et al*., 1990).

1.8.1.4. Interleukin-7 (IL-7)

IL-7 produced by keratinocytes, DCs, B cells, epithelial cells and macrophages, is a lymphopoetic cytokine. It induces the synthesis of inflammatory cytokines in macrophages (Akdis *et al*., 2011) and prolongs survival of pre-T-cells by induction of *bcl-2* expression (Niu and Qin, 2013). Prolonged survival of IL-7 treated *M. tuberculosis*-infected mice was postulated to be due to IL-7 activating immune cells, thereby, enhancing the anti-mycobacterial activity in infected macrophages. Even though IL-7 promoted survival in mice, it can also exhibit a deleterious effect on disease. IL-7 also enhanced the concentrations of IL-4 and IL-10 and decreased IFN-γ during early infection of mice (Maeurer *et al*., 2000). Memory CD8+ cell proliferation was enhanced by IL-7 and IL-15, but these cytokines were shown to be not necessary for memory CD4+ cells (Tan *et al*., 2002). However, IL-7 and IL-15 treated mice displayed increased CD4+ and CD8+ T-cell memory response, Th1 cytokine production and production of *M. tuberculosis* specific memory T-cells (Singh *et al*., 2010).

1.8.1.5. Interleukin 12 (IL-12)

IL-12, which is produced by phagocytic cells, is an important cytokine that is involved in the host immune response to *M. tuberculosis* infection (O'Garra *et al*., 2013). IL-12 plays a vital role in inducing production of IFN-γ and thereby, influencing the adaptive immune response to *M. tuberculosis* infection (O'Neill and Greene, 1998). Patients with mutations in either *IL-12R* or *IL-12p40* genes demonstrated reduction in the produced IFN-γ and T-cell response resulting in enhanced susceptibility to *M. tuberculosis* infection (Raja, 2004). IL-12 plays an important role in $CD4^+$ T cell differentiation into Th1 cells (Hamza *et al*., 2010). Mice infected with CDC1551 *M. tuberculosis* strain elicited early production of IFN-γ and IL-12 ensuring control of *M. tuberculosis* growth and therefore prolonged survival (Manca *et al*., 1999).

1.8.1.6. Interleukin 15 (IL-15)

IL-15 displays similar biological characteristics to IL-2, but is synthesised primarily by macrophages and monocytes unlike IL-2. IL-15 expression has been shown to be associated with a Th1-mediated chronic

inflammatory disease of the lung (Muro *et al*., 2001). *M. tuberculosis* infected animals treated with IL-7 or IL-15 elicited increased TNF-α and decreased IFN-γ in spleen cells (Maeurer *et al*., 2000). IL-15 was shown to be required for induction of effector mechanisms dependant on lectin-like stimulatory receptor natural killer group 2D in CD8+ T cells (Rausch *et al*., 2006). BCG infected mice that over expressed IL-15 offered enhanced protection due to NK and T cytotoxic 1 response augmentation (Umemura *et al*., 2001). IL-15 was also reported to have the ability to differentiate Vγ9Vδ2 T cells via generation of effector memory and terminally differentiated cells (Meraviglia *et al*., 2010).

1.8.1.7. Interleukin 18 (IL-18)

IL-18 is a pro-inflammatory cytokine similar to IL-1 (Akdis *et al*., 2011) and has been shown to stimulate production of transcriptional factors, pro-inflammatory cytokines and chemokines (Schneider *et al*., 2010). IL-18 deficient mice demonstrated increased susceptibility to BCG and *M. tuberculosis* (Sugawara *et al*., 1999). *M. tuberculosis*-stimulated peripheral blood mononuclear cells from patients with active disease produced reduced levels of IL-18 and IFN-γ compared to healthy individuals (Vankayalapati *et al*., 2000). These studies suggest that IL-18 plays a protective role in *M. tuberculosis* infection.

1.8.1.8. Tumour necrosis factor alpha (TNF-α)

TNF-α belonging to the pro-inflammatory group of cytokines, functions in synergy with IFN-γ, and supports the function of macrophages by assisting the production of reactive nitrogen intermediates (Flynn *et al*., 1995; Scanga *et al*., 2000). In addition, TNF-α encourages immune cells to migrate to sites of infection, thus assisting in granuloma formation and disease control (Mohan *et al*., 2001). Both the TNF-α and the 55 kDa TNF receptor have been demonstrated to be essential for the protective immune response against *M. tuberculosis* in mice (Flynn *et al*., 1995). In the murine TB model, TNF-α neutralization resulted in reactivation of TB depicted by increase in bacillary burden and histopathological deterioration (Mohan *et al*., 2001). TNF-α was also found expressed during the latent infection in infected tissue (Flynn *et al*., 1998). Increased levels of TNF-α were observed in pulmonary TB patients when peripheral blood mononucleated cells were stimulated with mycobacterial antigens (Al-Attiyah *et al*., 2012). Muramyl dipeptide interactions with NOD2 in human alveolar macrophages resulted in production of IL-1β, IL-6, and TNF-α and improved control of intracellular growth of *M. tuberculosis* (Juárez *et al*., 2012). TNF-α also increased phagocytosis and apoptosis of macrophages, thereby leading to enhanced DCs presentation (Keane *et al*., 2000). Increased mortality was demonstrated during chronic infection of mice with *M. tuberculosis* and this was attributed to the role of TNF-α in granuloma formation and maintenance (Robinson *et al*., 2013). *M. tuberculosis* utilizes surface antigens to modulate the host immune response by

inducing production of IL-4 and IL-10 which counter the pro-inflammatory effects of TNF-α (Manca *et al*., 2004). IL-4 has been shown to exacerbate the tissue damage associated with TNF-α (Seah and Rook 2001). Recently, Cpn60.2 was demonstrated to induce TNF-α production in both mouse-derived bone marrow-derived macrophages and human THP-1 cells (Torres-Huerta *et al*., 2017).

1.8.1.9. Interferon gamma (IFN-γ)

Interferons (IFN) are divided into two major types: type I and type II. Type I IFN is secreted in response to viruses and type II IFN (IFN-γ) is secreted by T lymphocytes and NK cells in response to immune and inflammatory signals (Cavalcanti *et al*., 2012). In the murine TB model, IFN-γ knockout mice produced granulomas but not reactive nitrogen intermediates, leading to defective restriction of bacillary burden (Flynn *et al*., 1993). IFN- γ is integral in the protective immune response to *M. tuberculosis* infection and is synthesised by CD4, CD8 T lymphocytes and NK cells (Tavares *et al*., 2006; Cooper and Khader, 2008; Nienhaus *et al*., 2008; Costa *et al*., 2010). Patients with defective IFN-γ receptor genes have demonstrated susceptibility to *M. tuberculosis* infection (Newport *et al*., 1996) and a complete absence of IFN-γ receptor was characterised by advanced disease progression, defective granuloma formation and multibacillary lesions (Cavalcanti *et al*., 2012). Macrophage activation is the main function of IFN-γ, thus enabling their mycobactericidal activity. IFN-γ also enhances antigen presentation by inducing the expression of MHC and promoting CD4 T lymphocyte differentiation to Th1 cells (Cavalcanti *et al*., 2012). Exposure to IFN-γ results in transcription of greater than 200 genes in macrophages inclusive of genes involved in production of various antimicrobial factors which facilitate the eradication of *M. tuberculosis* infection (Cooper 2009). Mycobacterial antigens, for example, the 19 kDa lipoprotein, has moderated the macrophage response by altering the transcription of genes that respond to the IFN-γ signal (Gehring *et al*., 2003; Pai *et al*., 2003).

1.8.2. Anti-inflammatory cytokines involved in *M. tuberculosis* infection

Anti-inflammatory cytokines serve to antagonize the effects of pro-inflammatory cytokines, thereby preventing inflammatory diseases (Slight and Khader, 2013). Chemokines are also produced during *M. tuberculosis* infection and play a role in chemotactic response involving circulating granulocytes, T-cells, DCs and macrophages (Jang *et al*., 2008; Vesosky *et al*., 2010; Yu *et al*., 2012; Slight and Khader, 2013).

1.8.2.1. Interleukin 4 (IL-4)

IL-4 has been noted for its involvement in suppression of IFN-γ production and macrophage activity (Romero-Adrian *et al*., 2015). In the mouse model, increased production of IL-4 was found during progressive disease (Hernández-Pando *et al*., 1996) and reactivation (Howard and Zwilling, 1999). IL-4 deficiency led to normal susceptibility to mycobacteria (Erb *et al*., 1998; North, 1998). In contrast, IL-4

deficiency was related to increased granuloma size and mycobacterial burden (Sugawara *et al*., 2000). IL-4 has also been detected in some TB patients (Kaplan *et al*., 1993; Sanchez *et al*., 1994) but this finding has not been consistent (Barnes *et al*., 1993b; Hernández-Pando and Rook, 1994; Lin *et al*., 1996; Lai *et al*., 1997).

1.8.2.2. Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that is produced by monocytes, macrophages, T cells, NK cells, DCs and B cells (Deniz *et al*., 2008). Binding of mycobacterial lipoarabinomannan to macrophages has been shown to elicit IL-10 production (Dahl *et al*., 1996). Treatment with recombinant IL-10 reduced levels of IL-12, and the neutralization of IL-10 resulted in minor increases in IL-12 levels in human monocytes infected with *M. tuberculosis H37Ra* (Fulton *et al*., 1998). DCs were demonstrated to produce IL-10 that targeted IL-10Ra TH17 CD4 T cells specifically (Doz *et al*., 2013). In the mouse model infected with *M. tuberculosis*, deficiency of IL-10 permitted the formation of granulomas that were mature and fibrotic, suggesting that IL-10 is involved in prevention of protective immunity development (Cyktor *et al*., 2013). Deficiency of IL-10 in mice resulted in reduction of bacterial load due to enhanced IFN-γ responses, together with an increase of CD4+ T cells in the lung as well as cytokines and chemokines (Redford *et al*., 2010). IL-10 was also observed to negatively regulate macrophage functions, thereby decreasing the inflammatory response in mice (Murray and Young, 1999). Deficiency in IL-10 terminated the ability of DNAX-activating protein of 12 kDa (DAP12)-competent antigen presenting cells to supress activation of the Th1 cell response (Jeyanathan *et al*., 2014).

1.8.2.3. Interleukin-11 (IL-11)

IL-11 displays both pro- and anti-inflammatory characteristics and is produced by stromal cells, osteoblasts, fibroblasts, epithelial cells, tumour cell lines and synoviocytes (Akdis *et al*., 2011). IL-11 functions include epithelial cell protection and immunomodulation, suppressing pro-inflammatory production, neuronal development, bone marrow recovery and adipogenesis, platelet production and haematopoiesis (Paul and Schendel, 1992; Akdis *et al*., 2011). IL-11 neutralization with antibodies resulted in decreased lung pathology and cytokine production in *M. tuberculosis* infected mice (Kapina *et al*., 2011).

1.8.2.4. Interleukin 13 (IL-13)

IL-13 is an anti-inflammatory cytokine from the Th2 cytokine family and is produced by mast cells, T-cells, basophils and eosinophils (Akdis *et al*., 2011). IL-13 is generally involved in eosinophil and mast cell recruitment during parasite infection (Akdis *et al*., 2011). It can also play a role in B-cell regulation and functions by inhibiting macrophage activation and down-regulating TLR2 signalling pathway and nitric

oxide synthesis (Akdis *et al*., 2011). In murine and human macrophages, IL-13 negates autophagy-mediated killing of *M. tuberculosis* (Harris *et al*., 2007).

1.8.2.5. Transforming growth factor beta (TGFβ)

TGFβ is induced by monocytes and DCs in response to mycobacterial products (Van Crevel *et al*., 2002), including lipoarabinomannan (Dahl *et al*., 1996). TGFβ has been described to suppress the cell mediated immunity, including T cell proliferation and IFN-γ production (Van Crevel *et al*., 2002). TGFβ and IL-10 were shown to be involved in the down-regulation of IFN-γ produced by T-cells during *M. tuberculosis* infection (Othieno *et al*., 1999). Fibrosis and tissue repair during drug treatment in active TB disease was shown to be related to the production of TGFβ (Difazio *et al*., 2016).

1.8.3. Chemokines

Chemokines and chemokine receptors have been shown to be involved in communication between various cell types, thereby facilitating immune cell trafficking to affected tissues and supporting immunity (Slight and Khader, 2013). The four chemokine subfamilies based on the position of cysteine residues: C, CC, CXC, and CX3C, bind to their corresponding receptors on various cell types (Zlotnik *et al*., 2006). The CC chemokine family generally bind to CCR receptors, and the CXC chemokine bind to CXCR receptors (Slight and Khader, 2013). XCL and CX3C chemokines bind to XCR and CX3CR receptors respectively (Zlotnik *et al*., 2006). Macrophages produce chemokines upon stimulation with *M. tuberculosis* antigens (Flynn *et al*., 2011). CCL2 (monocyte chemoattractant protein, MCP-1) production results in defective control of *M. tuberculosis* infection by increasing differentiation of $CD4^+$ T cells to Th2 phenotype (Hasan *et al*., 2009). In addition, CCL2 deficiency has been linked to inhibition of granuloma formation in murine models (Lu *et al*., 1998). CCL2 have been found to be present in serum during TB disease and treatment. CCL3 (macrophage inflammatory protein 1 alpha, MIP-1α) induces more Th1 than Th2 cells. CCL5 (Regulated upon Activation, Normal T cells Expressed and Secreted, RANTES) is involved in recruiting Th1 cells to form granulomas and plays a vital role in early responses of IFN-γ producing T-cells. CCL7 (monocyte chemoattractant protein-3, MCP-3) induces NK cells, phagocytic cells and T lymphocytes. IL-8 (CXCL8) is involved in chemoattraction of monocytes, T-cells and neutrophils (Zhang *et al*., 1995; Zlotnik and Yoshie 2000). Increased levels of CXCL8 were present in plasma and bronchoalveolar lavage fluids of TB patients (Nibbering *et al*., 1993; Meddows-Taylor *et al*., 1999).

1.9. The role of bacterial adhesins in pathogenesis

Adhesins are expressed by pathogenic and saprophytic organisms, highlighting their overall importance as a bacterial pathogenesis and ecological factor. Bacterial adhesins are cell surface molecules that are either monomeric or filamentous in structure, and display selectivity for specific target ligands that recognise various motifs. Attachment to mucosal surfaces and host cells are important steps in the pathogenicity of various bacterial groups (Kline *et al*., 2009). Many pathogens possess a plethora of adhesins that are expressed at various stages during the infection process and are vital for virulence. An example of a bacterial adhesin that functions as a virulence factor is the fimbrial adhesin (Bien *et al*., 2012). Most commensal isolates of *E. coli* and *Salmonella* express curli pili that bind and assemble the contact-phase system on the bacterial surface, which result in the release of bradykinin leading to fever, pain, and hypotension (Herwald *et al*., 1998). Curli pili produce an inflammatory response in the host. Infection with curli piliated *E. coli* resulted in a dramatic drop in blood pressure as compared to noncurliated mutants. Curli pili are also recognized by TLRs and activate the innate immune system, resulting in the production of IL-6, IL-8, and TNF-α (Bian *et al*., 2001). Disrupting this integral step in bacterial pathogenesis using specific antibodies that target the adhesin might prove to be successful vaccine or drug targets. But this approach has been confounded by the multitude of adhesins present in bacteria. Targeting several adhesins may offer a solution in these cases, as in the case of vaccines against Human enterotoxigenic *E. coli* (Wizemann *et al*., 1999).

1.10. *M. tuberculosis* adhesins

Various studies have described *M. tuberculosis* antigens that interact with receptors on mammalian host cells and therefore, have been identified as *M. tuberculosis* adhesins. Some of the known adhesins in *M. tuberculosis* include alanine- and proline rich antigenic (Apa) (Pasula *et al*., 1997), 19 kDa lipoprotein (Neyrolles *et al*., 2001), heparin binding hemagglutinin adhesin (Pethe *et al*., 2001), laminin binding protein (Pethe *et al*., 2002), Cpn60.2 (Stokes *et al*., 2004), malate synthase (Kinhikar *et al*., 2006), pili (Alteri *et al*., 2007), membrane protein, N-acetylmuramoyl-L-alanine amidase, and L,D-transpeptidase (Kumar *et al*., 2013). The potential of adhesins as biomarkers that can be targeted for the design of therapeutics was reviewed by Govender *et al*. 2014.

1.10.1. Heparin-binding hemagglutinin adhesin (HBHA)

Several respiratory pathogens including bacteria (Isaacs, 1994; Menozzi *et al*., 1994; Van Putten and Paul, 1995), parasites (Frevert, 1993; Love *et al*., 1993), mycoplasma (Krivan *et al*., 1989) and viruses (Nahmias

and Kibrick, 1964), have been shown to possess HBHA that bind specifically to sulphated glycosaminoglycan receptors present on cell surface or extracellular matrix of epithelial pneumocytes. Menozzi *et al* (1996) first characterised HBHA in mycobacteria as a 28kDa, surface exposed (Fig. 1.1) and secreted protein from *M. tuberculosis* and *M. bovis* BCG broth culture and cell wall extracts. Other mycobacteria such as *M. avium* and *Mycobacterium leprae* (Reddy and Kumar, 2000) express heparin-like proteins, while similar proteins were not observed in *M. smegmatis* (Menozzi *et al*., 1996; Pethe *et al*., 2002). HBHA was also described as an adhesive molecule that induces hemagglutination, bacterial-host cell- and auto-aggregation, thereby promoting communication between mycobacterial and host cells, suggesting that HBHA could possibly be an adhesin (Menozzi *et al*., 1996). Further studies corroborated hemagglutination activity and HBHA was further classified to be unique glycoprotein capable of glycosylation (Menozzi *et al*., 1998). HBHA was shown to comprise 3 major subunits, an α- helical coiled-coil region, transmembrane domain, and the methylated C-terminal Lys-Pro-Ala-rich region (Delogu and Brennan, 1999). The binding capacity of HBHA to sulphated glycoconjugates is facilitated by the methylated C-terminal Lys-Pro-Ala-rich region (Menozzi *et al*., 1996).

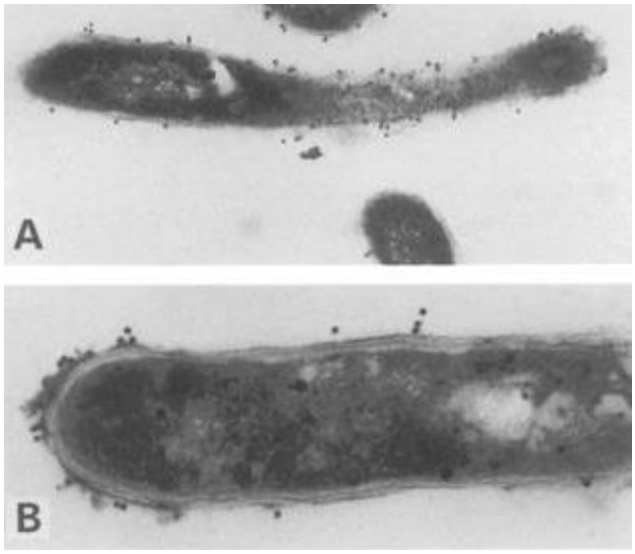

Fig. 1.1: Immuno-electron micrographs of *M. tuberculosis* labelled with monoclonal antibodies (mAbs) that specifically recognize HBHA. (A) *M. tuberculosis* H37Ra visualized (x29, 500) post incubation with mAb 3921E4; (B) *M. tuberculosis* H37Ra visualized (x61, 200) post incubation with mAb 4057D2 and gold-labelled (10 nm) goat anti-mouse immunoglobulin (Menozzi *et al*., 1996).

1.10.1.1. Adhesin potential of HBHA

A HBHA-deficient strain was reported to have a reduced ability to adhere to epithelial cells, but did not influence adherence to macrophages (Pethe *et al*., 2001). This study also demonstrated the role of HBHA in extrapulmonary dissemination by using a mouse infection model to show impaired dissemination of the HBHA-deficient strain to the spleen, but no influence on bacillary load in lungs. In addition, the use of antibodies targeting the C-terminal domain inhibited binding of HBHA to sulphated glycoconjugates receptors present on epithelial cells, suggesting that the HBHA induced humoral response could assist in protection against extra-pulmonary dissemination of *M. tuberculosis* (Pethe *et al*., 2001). HBHA was shown to promote infection of epithelial cells (Menozzi *et al*., 2006) by binding to actin and inducing actin polymerization and depolymerisation which in turn prompted cytoskeletal re-arrangement, facilitating epithelial cell transcytosis (Esposito *et al*., 2011). Mueller-Oritz *et al*., (2002) corroborated the findings of Pethe *et al*. (2001) also by using a HBHA-deficient strain demonstrating that HBHA was required for extra-pulmonary dissemination, but not for adherence of *M. tuberculosis* to, and its growth in macrophages (Mueller-Ortiz *et al*., 2002). Expression of *hbhA* at various stages of *in vitro* and *in vivo M. tuberculosis* infection was assessed using RT-PCR. Expression levels of *hbhA* remained similar at the initial stage of infection, whilst increased at the early stationary phase of in an axenic culture. A549 human pneumocytes and the lungs of mice displayed increased *hbhA* expression compared to low or constant *hbhA* expression in bone marrow derived macrophages and the spleen of mice (Delogu *et al*., 2006). Since the initial studies by Pethe *et al*., 2001, little research has been conducted on adherence of HBHA to macrophages. However, a previous study showed that the C3 binding protein of *M. tuberculosis* matched the amino acid sequence of HBHA and was demonstrated to bind specifically to the cell membrane. Furthermore, J774.A1 macrophages displayed increased phagocytosis of and adherence to HBHA-coated polystyrene beads. These findings suggested that HBHA possibly uses C3 and C3 binding receptors to mediate adherence to macrophages and phagocytosis of *M. tuberculosis* (Mueller-Ortiz *et al*., 2001). However, it was subsequently shown that *M. tuberculosis* binding to C3 was not impaired in the absence of HBHA (Mueller-Ortiz *et al*., 2002). Recombinant HBHA was seen to induce apoptosis of murine macrophages by generating reactive oxygen species and reducing mitochondrial transmembrane potential (Sohn *et al*., 2011), During HBHA induced apoptosis, the endoplasmic reticulum pathway was deemed essential (Choi *et al*., 2013).

1.10.1.2. Biomarker potential of HBHA

HBHA's potential as a biomarker and diagnostic tool have been evaluated by numerous studies since Menozzi *et al*., (1996) first described that *M. tuberculosis*-infected patients produce antibodies that recognise HBHA. HBHA's lys-pro-ala-rich region of the C-terminus has been identified to be methylated

(Pethe *et al*., 2002). Methylated HBHA was more strongly recognised compared to rHBHA by antibodies of infected patients (Zanetti *et al*., 2005), thereby highlighting the importance of methylation to the antigenic functioning of HBHA. In addition, latently-infected TB individuals elicited a stronger response to methylated HBHA when compared with TB patients with pulmonary TB (Masungi *et al*., 2002) suggesting that HBHA may potentially be a suitable biomarker for latent infection. This finding was corroborated by Interferon gamma response assays (IGRA) using HBHA. HBHA IGRA exhibited the highest sensitivity and specificity for detection of latent TB when compared with similar assays using Early Secreted Antigenic Target of 6kDa protein and purified protein derivative (Hougardy *et al*., 2007). In contrast, a similar study carried out in Finland, demonstrated that BCG vaccinated and unvaccinated individuals elicited a response to HBHA, suggesting that an innate immune response may have been due to either HBHA or the possible presence of cross-reactive peptides (Savolainen *et al*., 2008). Recently, more TNF-α CD4+ T cells induced by HBHA were observed in QuantiFERON-TB gold positive diagnosed latent *M. tuberculosis*-infected individuals compared with individuals that were negative for latent infection (Smits *et al*., 2015). Greater amounts of HBHA-triggered IL-2, IFN-γ and TNF-α CD4+ T lymphocytes were present in positively diagnosed latently-infected individuals than those with active pulmonary TB. In support of these findings, individuals with pulmonary TB were shown to elicit lower amounts of HBHA-specific CD4+ T cells compared with latently infected individuals, suggesting that HBHA may be a potential diagnostic marker for latent TB infection (Hutchinson *et al*., 2015). However, HBHA has been described as a poor biomarker for pulmonary and extra-pulmonary TB differentiation (Sun *et al*., 2011).

1.10.1.3. Vaccine potential of HBHA

HBHA has previously been reported to stimulate pro-inflammatory cytokines, TNF-α and IL-6 in bone marrow derived macrophages by induction of NF-κB and MAPKs pathways (Kim *et al*., 2011), using RT-PCR and ELISA to investigate a selected number of cytokines and pathways. HBHA was also shown to induce IFN-γ, IL-2 and IL-17 co-expressing $CD4^+$ T-cells in household contacts, but not in active TB cases (Loxton *et al*., 2012). Kuvar (2016) using a HBHA-deficient strain, demonstrated that HBHA modulates host-pathogen interactions and immune response by inducing differential gene regulation in a mouse infection model (unpublished). Rampersad (2018) demonstrated HBHA was most involved with the induction of chemokines, IL-8, MIP-1 and MCP-1 (unpublished).

HBHA also demonstrated a strong immunogenic and antigenic response, thereby enabling host protection against *M. tuberculosis* infection, suggesting that HBHA could be a suitable vaccine candidate (Parra *et al*., 2004). Kohama *et al*. (2008) showed that the use of rHBHA together with cholera toxin (CT) as an

intranasally administered booster to BCG vaccine, was able to prevent dissemination of bacilli from the primary site of *M. tuberculosis* infection to other sites in the host (Kohama *et al*., 2008).

HBHA prime-booster vaccines also require aTh17 immune response, in addition to the Th1 response (Verwaerde *et al*., 2014). HBHA and Cholera Toxin fusion together with the BCG vaccine resulted in induction of both Th1 and Th17 immune responses and was effective at bacillary load reduction (Fukui *et al*., 2015). Recent studies have used a multi-antigenic approach in order to fully capitalise on the immunogenic properties of HBHA. Nanoparticle formulation of a combination of antigen85B and HBHA was used as a booster to BCG vaccination and resulted in decreased bacillary loads in lungs of *M. tuberculosis*-infected mice (Stylianou *et al*., 2014). Most recently, a novel fusion protein of MTB32C-HBHA was tested for immunogencity in the mouse model and demonstrated the ability to induce a Thl response (Teimourpour *et al*., 2017). A novel vector (*Bacillus subtilis* spores) coated in a novel fusion protein consisting of Ag85B, ACR, and HBHA used as a booster to BCG vaccination demonstrated reduction in bacterial load in lungs and elicited a superior Th1-, Th17-, and T-reg-type cytokine production (Copland *et al*., 2018). Rampersad (2018), using RNA sequencing and HBHA deficient and proficient strains, demonstrated that HBHA is involved in differential regulation of canonical pathways and upstream regulators during *M. tuberculosis* infection. Of note, the HBHA deficient strain induced increased levels of pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ), predicted activation recruitment of phagocytes and significant enrichment of role of pattern recognition receptors in recognition of bacteria and viruses compared to HBHA proficient strain (unpublished).

1.10.2. *M. tuberculosis* pili (MTP)

Bacterial pili are composed of a minor tip-associated adhesin and a major repeating subunit (Kline *et al*., 2009) and function in various roles during infection including biofilm formation, agglutination of erythrocytes, aggregation, colonization of mucosal surfaces and host tissues, cell to cell communication and twitching (Telford *et al*., 2006; Proft and Baker, 2009). In addition, bacterial pili modulate host cytokine and chemokine production thereby contributing to the host inflammatory response (Bian *et al*., 2000; Kai-Larsen *et al*., 2010).

A study by Alteri in (2005) demonstrated by transmission electron microscopy (Fig. 1.2) that *M. tuberculosis* produces structures resembling pili, and scanning electron microscopy further delineated these structures into two different pili types, *M. tuberculosis* curli pili (MTP) and type IVB pili (Alteri, 2005; Alteri *et al*., 2007).

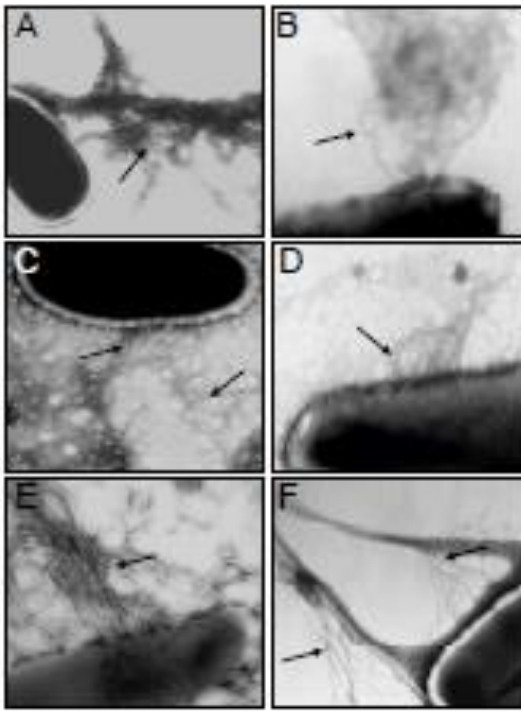

Fig. 1.2: *M. tuberculosis* pili. Electron micrographs of (A) *M. tuberculosis* H37Ra, (B) *M. tuberculosis* H37Rv, (C) *M. tuberculosis* CDC1551, (D) *M. bovis* BCG, (E) *M. smegmatis* mc2155, and (F) *M. fortuitum* visually confirming the presence of pili by mycobacteria (Alteri, 2005).

In *M. tuberculosis*, the MTP pilin subunit is encoded by *Rv3312A*. *In vitro* studies demonstrated that MTP and laminin, which is an extracellular matrix protein, bind to each other, highlighting that MTP displays adherence properties to host cellular matrix proteins and suggested that MTP possibly plays a role in host colonization (Alteri *et al*., 2007). This finding was supported by *in vitro* studies by Ramsugit and Pillay (2014 and 2016), using a MTP-deficient and -complemented strains and demonstrated that MTP is important for adhesion and invasion to macrophages and epithelial cells respectively (Ramsugit and Pillay, 2014; Ramsugit *et al*., 2016). In addition, MTP was also seen to be instrumental in the production of *in vitro* biofilms (Ramsugit *et al*., 2013). MTP was also explored as a possible biomarker and (Naidoo *et al*., 2014). Non-tuberculous mycobacteria and other respiratory bacteria demonstrated absence of *mtp* gene suggesting that the pilin subunit protein of *M. tuberculosis* is possibly a suitable biomarker for a point care test development (Naidoo *et al*., 2014). Mann *et al*., (2016) showed that MTP was not required for *M. tuberculosis* survival in the mouse model but contributes to biofilm formation and isoniazid tolerance (Mann *et al*., 2016).

Unpublished studies in our research group have also suggested that MTP is possibly a strong immunogen in mouse infection model (Nyawo, 2016) and epithelial cell model (Dlamini, 2016) respectively but further

experiments are needed to investigate the mechanism of MTP immune modulation. Nyawo (2016) demonstrated that MTP is involved in growth of *M. tuberculosis* under in vitro conditions and plays a substantial role in host-pathogen interactions by activating the innate immune and inflammatory responses, thereby impacting host immune responses critical to host defence (unpublished). Dlamini (2016) demonstrated that MTP plays a vital role in gene regulation of epithelial cells and influence host immune responses by modulating signalling pathways during infection. These studies provide evidence that MTP has the ability to modulate immune responses and therefore should be further investigated as a possible vaccine candidate (unpublished). MTP induced similar quantities of IL-1β, IL-4, IL-6, IL-8, G-CSF, IFN-γ, MCP-1 and TNF-α in the epithelial cell model. It was noted that MTP induced significantly higher concentration of TNF-α at 48 h and MTP-deficient mutant induced higher levels of IL-8 at 72 h post-infection (Ramsugit *et al*., 2016). Unpublished studies by Ramsugit (2014) and Balgobin (2018) suggests that cytokine production by macrophages was not influenced by MTP. More recently Naidoo *et al*., (2018) demonstrated MTP is detected in larger patient populations including HIV positive patients during *M. tuberculosis* infection and therefore is potentially useful in a reliable point of care test (Naidoo *et al*., 2018). MTP is not associated with host cell cytotoxicity but influence the intracellular survival of *M. tuberculosis* (Ramsugit and Pillay, in press). An unpublished study by Balgobin (2018) interrogated the whole macrophage global differential transcriptional regulation elicited by MTP using MTP deficient and proficient strains. RNA sequencing analysis revealed that MTP was involved in canonical pathway and upstream regulator enrichment. MTP also enhanced predicted activation of migration of phagocytes, inflammatory response and significant enrichment of role of pattern recognition receptors in recognition of bacteria and viruses.

1.11. Whole transcriptome analysis

Survival of pathogens in the host relies on the expression of specific gene products. These genes are regulated depending on environmental pressures (Fuchs *et al*., 2012). Understanding the pattern of up and down regulation of genes provides vital information about characteristics of a bacterium that could possibly translate into novel therapeutic target. Recent technological advances have enhanced the prospects for elucidating bacterial gene regulation. Revolutionary techniques for studying gene regulation during host-microbe interactions include microarrays, real time RT-PCR and RNA sequencing.

1.11.1. Techniques to study whole transcriptome analysis

Real time Polymerase chain reaction (PCR) offers greater sensitivity which is useful for studying gene expression of limited *M. tuberculosis* mRNA in macrophage cultures and infected tissues (Fraga *et al*., 2008). However PCR analysis is limited in number of genes that can be analysed in a single experiment. Microarray technology addressed this shortcoming (Rak *et al*., 2014). DNA microarray analysis is a high-throughput hybridization-based approach for simultaneous screening of mRNA levels of thousands of genes under varied environmental conditions (Git *et al*., 2010). Microarray and real time PCR analysis has enabled the interrogation of complex host-pathogen interaction thus elucidating further *M. tuberculosis* pathogenesis.

1.11.1.1. Microarray

Activation of macrophages with IFN-γ enhances transcriptional regulation of genes involved in inflammation and immunity (Ehrt *et al*., 2001). Transcriptional profiles in blood samples between healthy control individuals, BCG vaccinated individuals and active TB patients varied in molecular signatures (Lesho *et al*., 2011). Transcriptional biosignatures were altered during TB treatment, rapid mycobacteriocidal activity coincided with initial down-regulation of inflammatory mediators, suggesting that transcriptional biosignatures measurement is a useful during clinical trials for drug development (Cliff *et al*., 2013). An extensive microarray analysis of the macrophages infected by an extensive of range bacterial pathogens depicted commonly expressed genes by the host in response to various bacterial strains. The commonly expressed gene patterns encoded host cell receptors, signal transduction molecules and transcriptional factors. Further interrogation of *M. tuberculosis* specific response showed that *M. tuberculosis* inhibits IL-12 production during macrophage infection suggesting that this is possibly a survival mechanism (Nau *et al*., 2002). Macrophage global transcriptomic response during early infection of *M. tuberculosis* was investigated using microarrays analysis depicted induction genes encoding for ribosomal proteins, interferon-response gene *STAT1*, and cytokine and chemokine (Wang *et al*., 2003). Analysis using apoptosis pathway-specific microarray displayed differential regulation of inhibitors of apoptosis (*bcl-2* and *Rb*) and pro-apoptotic (*bad* and *bax*) between *M. tuberculosis*-infected macrophage and epithelial cells (Danelishvili *et al*., 2003). Investigation of transcriptional regulation of *M. tuberculosis* during infection of macrophages up to 7 days revealed up-regulation of IL-1β, IL-8, MIP-1α, epithelial cell-derived neutrophil-activating peptide-78, macrophage-derived chemokine, matrix metalloproteinase-7 and growth-related oncogene-β. Macrophage colony-stimulating factor-receptor and CD4 were found to be down-regulated in *M. tuberculosis* infected macrophages (Volpe *et al*., 2006). *M. tuberculosis*'s involvement in the initiation of cytosolic surveillance pathway by partial perforation of phagosomal

membrane was facilitated by the ESX-1 secretion pathway. This perforation leads to exposure of *M. tuberculosis* DNA to cytosolic receptors inducing Sting/Tbk1/Irf3 signalling culminating in IFN-β production during infection of macrophages (Manzanillo *et al*., 2012). Infection of macrophages with cell wall deficient and normal *M. tuberculosis* were observed to induce differentially expressed genes involved with cytokine-cytokine receptor interaction, nitrogen metabolism, focal adhesion and mitogen-activated protein kinase signalling pathways (Fu *et al*., 2015). Microarray technology is limited in the accuracy of transcripts present in low abundance and also only a limited selection of genes are interrogated by specific probes (Zhao *et al*., 2014).

1.11.1.2. Real time PCR

Using real time PCR analysis of intracellular and extracellular immune mediators' levels secreted by patients with infectious lung diseases, active TB patients were shown to demonstrate higher levels of mediators (SOCS, IRAK-M, IL-10 and TGF-βRII, IL-1Rn, and IDO) that counteract Th1-type and innate immunity (Almeida *et al*., 2009). Real time PCR exhibits high accuracy and sensitive and therefore is currently employed in validation of microarray and RNA sequencing analyses (Nau *et al*., 2002; Danelishvili *et al*., 2003; Volpe *et al*., 2006; Fu *et al*., 2009; Manzanillo *et al*., 2012).

1.11.1.3. RNA sequencing

RNA sequencing has demonstrated strong potential to replace microarrays as a whole transcriptome profiling. Numerous studies have displayed concordance between microarray and RNA sequencing data (Bottomly *et al*., 2011; Sîrbu *et al*., 2012; Zhang *et al*., 2012). RNA sequencing is more sensitive than microarrays for low abundance transcripts and can profile gene expression in strains for which genome sequences and gene annotations are not available (Haas *et al*., 2012). RNA sequencing was used to elucidate immune responses specific to *M. tuberculosis* compared to other bacterial pathogens. *M. tuberculosis* infection specific genes were associated with phagosome maturation, response to vitamin D, sialic acid synthesis, superoxide production and macrophage chemotaxis (Blischak *et al*., 2015). The transcriptomes of *M. bovis*-infected bovine macrophages were analysed by RNA sequencing revealing differentially expressed genes involved in transcriptional suppression of host defence and innate immune signalling (Nalpas *et al*., 2015). RNA sequencing analysis of epithelial cells infected with clinical strains dominant in KwaZulu-Natal, South Africa demonstrated strain-specific induction of immune associated pathways (Mvubu *et al*., 2016). Macrophage infection with hyper- and hypovirulent Beijing *M. tuberculosis* strains induced differential gene expression and further pathway analysis depicted activation of growth inhibitory and stress-induced Gadd45 signalling pathway by the hypervirulent strain. The hypovirulent strain was

predicted to induce upstream regulators involved in interferon activation (Leisching *et al*., 2017). Unique transcriptional analysis technology, CAGE (cap analysis gene expression), of IFN-γ and IL-4/IL-13 stimulated macrophages infected with *M. tuberculosis,* differentially expressed up-regulated genes were shown to be associated with inflammation and host immune protection response and dis-regulation of cellular function was associated with down-regulated differentially expressed genes (Roy *et al*., 2018). Gene expression studies using CAGE and RNA sequencing were comparable (Kawaji *et al*., 2014).

1.12. Significance of this work

Since both HBHA and MTP have been identified as major adhesins in *M. tuberculosis* for epithelial cells and macrophages respectively, together, HBHA and MTP represent a powerful target for therapeutic interventions. Even though HBHA was shown not to play a role in adhesion of the *M. tuberculosis* to macrophages in previous studies (Pethe *et al*., 2002), deletion of HBHA in a strain that has impacted drug resistance and overall TB burden in KwaZulu-Natal (F15/LAM4/KZN) may yield a dissimilar finding. Dual deletion of HBHA and MTP will enable assessment of the effect of HBHA and MTP together on the pathogenicity of *M. tuberculosis* during host-pathogen interactions thereby permitting the interrogation of these antigens independently and in combination on the TB pathogenesis.

1.13. Research design

1.13.1. Aim of study

To evaluate the role of HBHA and MTP on TB pathogenesis, using a double *hbhA-mtp* gene knockout and functional genomics.

1.13.2. Specific objectives

- To evaluate the adhesion, invasion and replication capacity of the wild-type and *hbhA-mtp* gene knockout mutant and complemented strains in THP-1 macrophages.
- To determine the role of the *hbhA-mtp* gene knockout on the host immune response by comparing cytokine and chemokine production by THP-1 macrophages cells relative to the wild-type.
- To evaluate host gene regulation in THP-1 macrophages infected with the *hbhA-mtp* gene knockout mutant by whole transcriptome analysis using RNA sequencing.

1.14. Thesis overview

This thesis is divided into 5 chapters. The first chapter comprises a review of the relevant literature. Chapters 2 to 4 are research chapters written in manuscript format. Chapter 5 is a synthesis of the research chapters, demonstrating how the chapters are linked, conclusion and recommendations for future work.

Chapter two:

Host cell colonization is dependent on initial adhesion and subsequent invasion of bacterial pathogens. *M. tuberculosis* possesses multiple adhesins/invasins of which Heparin-binding hemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) are two major and well characterised adhesins. This paper investigated the combined effect of HBHA and MTP on adhesion, invasion and transcript regulation elucidated using RNA sequencing during early infection in a THP-1 human macrophage model. HBHA and MTP were revealed to not act in synergy during adhesion and invasion. MTP demonstrated a more substantial role in these initial interactions with macrophages compared to HBHA. In addition, these adhesins were involved in immune recognition during early infection.

Chapter three:

Host cell signalling defines the progression of pathogenesis of *M. tuberculosis*. Cytokines are important effector molecules that activate innate and adaptive arms of immune response. HBHA was previously shown to induce the pro-inflammatory cytokines and chemokines, whilst MTP exhibited a limited role in cytokine modulation. The impact of loss of both these adhesins on transcriptional regulation using RNA sequencing and cytokine secretion using a multiplex assay were outlined in this paper. HBHA and MTP enhanced activation of NF-κB, toll-like receptor, p38 MAPK and PI3-K/AKT canonical signalling pathways. These activated patterns resulted in a predominately pro-inflammatory response during *M. tuberculosis* infection of macrophages.

Chapter four:

Transcriptional regulation during *M. tuberculosis* pathogenesis impacts host immune response and molecular signatures. HBHA and MTP individually have displayed immunogenic characteristics in macrophages and mice. HBHA and MTP combined immunogenic ability was investigated by intracellular replication assays, RNA sequencing of host global transcriptome and real time quantitative PCR of specific transcripts. The data generated in this paper proposed the engagement of alternate antigens in the absence of HBHA and MTP. Further studies to elucidate the identity of these antigens would possibly result in a more successful, novel therapeutic target combination in addition to HBHA and MTP.

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The first chapter detailed pertinent literature on TB epidemiology, general characteristics of *M. tuberculosis*, host immune response and *M. tuberculosis* adhesins HBHA and MTP. This highlighted the individual roles of HBHA and MTP in adhesion, invasion and modulation of the host immune responses. Chapter two investigated the role of dual-deletion of HBHA and MTP on adhesion, invasion and transcriptional regulation of macrophages.

CHAPTER 2: *Mycobacterium tuberculosis* heparin binding haemagglutin adhesin (HBHA) and curli pili (MTP) promote adhesion and invasion non-synergistically, and modulate cell signalling canonical pathways in macrophages.

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Running title: HBHA and MTP promote adhesion and invasion non-synergistically.

Key words: HBHA; MTP; *M. tuberculosis*; adhesion; invasion; transcriptional regulation; macrophages.

Abstract

The heparin-binding hemagglutinin adhesin (HBHA) and *Mycobacterium tuberculosis* (*M. tuberculosis*) curli pili (MTP) are two major, well characterised adhesins of *M. tuberculosis* and together represent a powerful target for therapeutic interventions. In the current study, the combined effect of HBHA and MTP on adhesion, invasion and transcription regulation was ascertained during early infection in a THP-1 human macrophage model. The relative percentage adhesion/ invasion of the mutant and complemented strains was calculated at 1 h and 2 h post-infection respectively and compared to wild-type. Differential transcriptional regulation of biological adhesion associated genes were investigated using Ingenuity Pathway Analysis (IPA) software. During adhesion, DM induced a similar decrease in percentage adhesion (33.16%) to Δ *Rv3312A* (*mtp)* (39.4%), Δ*Rv0475* (*hbhA)* (22.78%), mtp comp (24.72%) but statistically lower decrease in percentage adhesion than hbhA comp (53.85%). During invasion, DM displayed a significant decrease in percentage invasion (36.49%) compared to Δ*mtp* (61.49%) and hbhA comp (53.85%); and significantly higher decrease in percentage invasion than Δ*hbhA* (22.29%) and mtp comp (24.72%). Δ*mtp* demonstrated a 39.4% and 61.49% decrease in percentage adhesion and invasion compared to WT respectively, suggesting that MTP played a greater role in adhesion and invasion during independent knockout and complementation in the double knockout strain than HBHA. In addition, the double *hbhA-mtp* knockout mutant did not demonstrate a synergistic phenotype during adhesion and invasion, suggesting that this strain compensated for the loss of these genes possibly by employing alternate adhesins and invasins. The HBHA-MTP proficient strain induced greater transcriptional changes resulting in enhanced adhesion to phagocytes and invasion of cells. Furthermore, the HBHA-MTP proficient strain displayed the sole ability to induce activation of phagocytosis. Further investigation of canonical pathway differential regulation by HBHA-MTP proficient strain demonstrated greater induction of canonical pathways. The most differential regulated pathway was Gαq signalling canonical pathway, which is vital for migration of phagocytes. In addition, the HBHA-MTP proficient strain also enhanced activation of the acute phase response, role of pattern recognition receptors in recognition of bacteria and viruses, and production of nitric oxide and reactive oxygen species in macrophages canonical pathways. The findings suggest that in addition to their role adhesion/invasin molecules, HBHA and MTP together influence transcriptional changes that favour adhesion and invasion of macrophages. In addition, these two adhesins serve as pathogen-associated molecular patterns that enable host immune recognition during early infection of macrophages.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), was responsible for an estimated 10.4 million new cases and 1.4 million deaths in 2016 (WHO, 2017). The South-East Asian, African and Western Pacific regions contributed 45%, 25% and 17% respectively of the global TB incidence cases in 2016. South Africa is currently listed among the top 20 high burden countries for TB, TB/ Human Immunodeficiency Virus (HIV) and Multi-drug resistant TB (WHO, 2017). HIV coinfection (Bruchfeld *et al*., 2015), diagnostic delays (Dheda *et al*., 2013), lack of point of care diagnostics (Wallis *et al*., 2013), lack of effective vaccination (Mangtani *et al*., 2014) and drug resistance including multiple, extensive and total drug resistance (Calver *et al*., 2010) have contributed to this high TB burden. Biomarker discovery is imperative in the development as well as successful clinical evaluation of new diagnostic tools and vaccines (Ottenhoff *et al*., 2012). A systems biology approach, that integrates functional genomics, transcriptomics and host-pathogen interactions (Gardy *et al*., 2009) offers the opportunity to interrogate novel biomarkers that may possibly lead to targets for vaccines, point of care diagnostics and drug development.

Adhesion to host cells is a virulence characteristic possessed by most bacterial pathogens and is often facilitated by adhesins that display specificity for host target molecules (Kline *et al*., 2009). Bacterial adhesins, because of their surface location and their role in facilitating host colonisation and infection by pathogenic bacteria, may be potential targets for the design of intervention therapy. Adhesins are mostly expressed on the bacterial cell surface, and are generally the first point of contact with host cell receptors (Vidal Pessolani *et al*., 2003). Some of the known adhesins in *M. tuberculosis* include alanine- and proline rich antigenic (Apa) (Pasula *et al*., 1997), 19 kDa lipoprotein (Neyrolles *et al*., 2001), heparin binding hemagglutinin adhesin (Pethe *et al*., 2001), laminin binding protein (Pethe *et al*., 2002), Cpn60.2 (Stokes *et al*., 2004), malate synthase (Kinhikar *et al*., 2006), curli pili (Alteri *et al*., 2007), membrane protein, N-acetylmuramoyl-L-alanine amidase, and L,D-transpeptidase (Kumar *et al*., 2013).

The heparin-binding haemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) are 2 major, well characterised adhesins of this pathogen reported in literature. HBHA, encoded by *Rv0475* (*hbhA)*, is a surface-exposed protein that facilitates the interaction of the tubercle bacilli with the host by acting as an adhesin for non-phagocytic cells and plays a vital role in extrapulmonary dissemination of *M. tuberculosis* (Menozzi *et al*., 1996; Pethe *et al*., 2001). The *hbhA* gene is strongly upregulated in *M. tuberculosis* infecting type II human pneumocytes but not in bone marrow-derived macrophages (Delogu *et al*., 2006). However, the global expression of the *hbhA* gene in the THP-1 and A549 infection cell models has not yet been reported. Vaccination with HBHA (Parra *et al*., 2004), HBHA and *Mycobacterium bovis* bacillus Calmette-Guérin vaccine (Guerrero *et al*., 2010), yeast expressed recombinant HBHA and a mucosal

adjuvant cholera toxin (Kohama *et al*., 2008), and *M. smegmatis* strain expressing HBHA and human interleukin 12 (Zhao *et al*., 2012), resulted in the reduction of bacterial load in a mouse infection model suggesting protective immunity. HBHA has potential as a biomarker, as it had demonstrated an ability to distinguish between latent infection, active TB and patients under therapy by eliciting a HBHA-specific T-cell response by both recombinant and methylated *M. tuberculosis* HBHA produced in *M. smegmatis* (Zanetti *et al*., 2005; Delogu *et al*., 2011; Molicotti *et al*., 2011). However, in murine macrophage-like cell lines J774.1, MH-S and human macrophage-like cell line U937, HBHA was reported not to function as an adhesin (Pethe *et al*., 2001).

Pili are hydrophobic, proteinaceous structures that are in most instances composed of pilin or a major repeating subunit (Finlay and Falkow, 1997). Alteri *et al.*, 2007 showed for the first time that mycobacteria produce fine, aggregative, flexible pili, comprising protein subunits and of two distinct morphotypes (Type IV and curli-like pili (MTP) (Alteri *et al*., 2007). The role of MTP, encoded by the *Rv3312A* (*mtp)*, in biofilm formation and the essentiality of the *mtp* gene for piliation was recently described using functional genomics (Ramsugit *et al*., 2013). MTP was also reported to play a role in the adhesion and invasion of macrophages (Ramsugit and Pillay, 2014) and A549 epithelial cells (Ramsugit *et al*., 2016). The *mtp* gene was found to be present in the *M. tuberculosis*, but absent in non-tuberculous Mycobacteria and other respiratory bacteria, suggesting that MTP may be a suitable diagnostic marker (Naidoo *et al*., 2014).

These data collectively suggest that HBHA and MTP in combination may represent a powerful target for therapeutic interventions. Recently, a DNA vaccine based on *MTB32C* and *hbha* genes showed promise as a vaccine candidate due to its ability to stimulate an immune response in BALB/c mice when used alone or as a booster (Teimourpour *et al*., 2017). In the current study, using a previously constructed double knockout mutant strain (unpublished data) of *M. tuberculosis*, the combined effect of HBHA and MTP on adhesion, invasion and gene regulation was ascertained during early infection in a THP-1 human macrophage model. The study established that HBHA and MTP do not act in synergy during adhesion and invasion; and that MTP played a more significant role in these interactions than HBHA in macrophages. These differences are likely to be driven by the differential transcriptional regulation induced by HBHA and MTP. These adhesins in combination also enhanced host immune recognition during early infection of macrophages.

Materials and Methods

Ethical statement

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no. BE516/14).

Bacterial isolates and culture conditions

M. tuberculosis strains (Table 2.1) obtained from Medical Microbiology, University of KwaZulu-Natal, were retrieved from storage at -80°C and grown in Middlebrook 7H9 medium (Difco, Becton, Dickinson and Company, South Africa), supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase enrichment (Becton, Dickinson and Company, South Africa), 0.5% (v/v) glycerol (Merck, South Africa), and 0.05% (v/v) Tween 80 (Sigma, Capital lab supplies, South Africa), in presence of 150 µg/mL hygromycin (Roche Applied Sciences, Capital lab supplies, South Africa) for mutant strains or and 30 µg/mL kanamycin (Sigma, Capital lab supplies, South Africa) for complemented strains. Cultures were incubated with shaking (100 rpm) in a shaking incubator (NBS Shaking incubator series, ALS solutions, South Africa) at 37°C until an optical density (OD) $_{600nm}$ of 1 was reached. Optical density was measured with a spectrophotometer (WPA lightwave II, Biochrom, Labotec, South Africa) by measuring the absorbance at 600nm of a 1:5 dilution of a log-phase culture of the specific strain. Single complemented strains had been generated in the double knock-out mutant to assess the relative contribution of each adhesion. Mutants and complemented strains were confirmed by PCR amplification (Govender, unpublished). PCR to confirm the *mtp* deletion was carried out using *mtp* LL primer: 5'-ATTCGAGTACATGCGTGAT-3' and uptag primer: 5'-GATGTCTCACTGAGGTCTCT-3'. The presence of allelic exchange substrate (mutant and complement) results in an amplicon size of 700 base pairs whilst the absence if the allelic exchange substrate will result in no product. For *hbhA* deletion confirmation, PCR was carried out using the *hbhA* LL primer: 5'-GGCCGAAGTCCTTTATGT-3' and Uptag primer 5'-VGATGTCTCACTGAGGTCTCT-3'. The presence of allelic exchange substrate (mutant and complement) results in an amplicon size of 770 base pairs whilst the absence if the allelic exchange substrate will result in no product. The PCR mastermix contained the following final concentration of 1x *Taq* buffer (Roche Applied Science, Capital lab supplies, South Africa), 0.25 mM of dNTPs (Roche Applied Science, Capital lab supplies, South Africa), 1.5 µM of $MgCl_2$ (Roche Applied Science, Capital lab supplies, South Africa), 0.2 µM of each primer, 0.02 U of *Taq* Polymerase (Roche Applied Science, Capital lab supplies, South Africa) and nuclease-free water in a 25 µL reaction volume. Cycling conditions were initial denaturation of 94°C for 2 mins followed by 40 cycles of the following: denaturation at 94°C for 30 secs; annealing at 55°C (*hbhA*) or 57°C (*mtp*) for 30 secs; extension of 72°C for 1 mins and a final extension of

72°C for 10 mins. PCR products were run on a 1.5% agarose Tris-borate- Ethylenediaminetetraacetic acid gel with DNA Molecular Weight Marker IX (Roche Applied Science, Capital lab supplies, South Africa). G box (Syngene, South Africa) was used for visualization.

Table 2.1: Bacterial strains used in study. The strains used in this study were sourced from previous studies carried out in Medical Microbiology, UKZN, South Africa.

Strains	Genetic information	Origin
Wildtype (WT)	V9124 (F15/LAM4/KZN)	Tugela Ferry (KwaZulu-Natal, South Africa)
Δ*hbhA-mtp* (DM)	MTP and HBHA deficient double mutant	(Govender, *et al*, unpublished)
mtp comp (mtp comp)	MTP-overexpressing mtp-complemented strain (mtp comp) of double mutant Δ*hbhA-mtp*	(Govender, *et al*, unpublished)
hbhA comp (hbhA comp)	HBHA-overexpressing hbhA-complemented strain (hbhA comp) of double mutant Δ*hbhA-mtp*	(Govender, *et al*, unpublished)
Δ*hbhA* (ΔhbhA)	HBHA deficient mutant	(Govender, *et al*, unpublished)
Δ*mtp* (Δmtp)	MTP deficient mutant	(Ramsugit *et al*., 2013)

Infection of THP-1 differentiated macrophages

The THP-1 monocytic cell line (ATCC TIB-202) was propagated in RPMI-1640 (Lonza, Whitehead Scientific, South Africa) and supplemented with 10% fetal bovine serum (Biowest, Celtic Molecular Diagnostics, South Africa) in 5% CO_2 (Shel lab CO_2 incubator, Polychem, South Africa) at 37°C until approximately 1 x10^6/mL was reached. THP-1 cells were assessed for viability using the Trypan blue exclusion assay, counted using haemocytometer and seeded at a density of 5 x 10^5 cells/mL into either 75 cm^2 flasks (Porvair, Whitehead Scientific, South Africa) for RNA extraction experiments or 24 well plates (Porvair, Whitehead Scientific, South Africa) for adhesion and invasion experiments. Monocytes were differentiated into macrophages by the addition of 50 ng/mL of phorbol 12-myristate 13-acetate (Sigma,

Capital lab supplies, South Africa) at 37°C for 24 h. Logarithmic phase bacterial strains were centrifuged (Heraeus multifuge 3S-R, Thermofisher Scientific, South Africa) at 4000 x *g* for 10 mins and resuspended in RPMI-1640 (Lonza, Whitehead Scientific, South Africa) supplemented with 10% fetal bovine serum (Biowest, Celtic Molecular Diagnostics, South Africa). THP-1 differentiated macrophages were infected at a multiplicity of infection (MOI) of ~5:1 for 4 h at 37°C and 5% CO_2. The MOI was confirmed by plating out of 10-fold serial dilutions (10^{-1} to 10^{-6} in phosphate buffered saline (PBS) (Oxoid, Quantum Biotechnologies, South Africa) and 0.05% (v/v) Tween 80 (Sigma, Capital lab supplies, South Africa)) of inoculum on Middlebrook 7H11 (Difco, Becton, Dickinson and Company, South Africa) agar plates. Monolayers were washed with 20 mL (pH 7.3) PBS (Oxoid, Quantum Biotechnologies, South Africa) and processed as described below for Adhesion, Invasion and RNA sequencing assays.

Adhesion and Invasion assays

The adhesion and invasion assays were carried out according to Ramsugit and Pillay, 2014. For the adhesion assay, monolayers were infected for 1 h. Medium was removed and monolayers were washed with PBS three times and subsequently lysed with 0.1% Triton-X 100 (Sigma, Capital lab supplies, South Africa) by incubation at 37°C and 5% CO_2 (Shel lab CO_2 incubator, Polychem, South Africa) for 20 mins. Lysed macrophages were serially diluted and plated out on Middlebrook 7H11 medium (Difco, Becton, Dickinson and Company, South Africa) in triplicate, to assess the number of adherent bacteria. For the invasion assay, monolayers were infected for 2 h, and thereafter, treated with amikacin (300 µg/mL; Sigma, Capital lab supplies, South Africa) for 1 h at 37°C (Shel lab CO_2 incubator, Polychem, South Africa) and 5% CO_2. After removal of amikacin, monolayers were washed thrice with PBS, lysed and Colony forming units (CFU)/mL enumeration carried out as described in the adhesion assay.

RNA sequencing assay

To assess host transcriptomic changes elicited during early infection of THP-1 differentiated macrophages by V9124 (F15/LAM4/KZN) wild-type (WT) and MTP and HBHA deficient double mutant Δ*hbhA-mtp* (DM), RNA was extracted from host cells at 4 h post-infection. Monolayers were washed with 20 mL (pH 7.3) PBS (Oxoid, Quantum Biotechnologies, South Africa) and trypsinized for 10 mins. Following centrifugation (Heraeus multifuge 3S-R; Thermofisher Scientific, South Africa) at 300 x *g* for 5 mins at 4°C, the pellet was resuspended in 600 µL of RLT buffer (Qiagen, Whitehead Scientific, South Africa) supplemented with 1% (v/v) β-mercaptoethanol (Sigma, Capital lab supplies, South Africa) and stored at -80°C until further use. RNA was extracted with the RNeasy kit (Qiagen, Whitehead Scientific, South

Africa) with on-column DNA digestion using the RNase-Free DNase Set (Qiagen, Whitehead Scientific, South Africa) according to manufacturer's instructions.

RNA concentration and integrity were assessed using a Nanodrop (Thermo Scientific, South Africa) and 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma, Capital lab supplies, South Africa) gel electrophoresis (Appendix 3). RNA samples were stored in single use aliquots at -80°C until shipment on dry ice to the Agricultural Research Council (ARC) Biotechnology Platform (Onderstepoort, South Africa). After RNA quantification using the Qubit, the sequencing library was prepared using TruSeq Stranded Total RNA Library Prep Kit (Illumina, South Africa). Ribosomal RNA was removed using Ribozero (Illumina, South Africa). The Hiseq 2500 platform (Illumina, South Africa) was used to sequence 28 - 40 million, 125 base pair paired-end reads of 2 biological replicates of infected and uninfected host cells.

Bioinformatics analysis

The sequenced reads were quality controlled with the FASTQC tool (version 0.11.3; Babraham Bioinformatics, Cambridge, UK) and poor quality reads trimmed using Trimmomatic (Bolger *et al*., 2014). Reads were subsequently mapped to the Homo sapiens reference genome Hg38 (UCSC) using TopHat (2.1.0) and Bowtie2. Mapping rates to reference genome ranged from 89.4 – 93.8 %. Infected and uninfected cell differential gene expression patterns were identified using a cuffdiff package (version cufflinks-2.2.1) (Trapnell *et al*., 2012). Cuffdiff outputs were filtered for genes in common with the Gene Ontology biological adhesion dataset sourced from EntrezGeneIds using the molecular signatures database (Subramanian *et al*., 2005) with a False Discovery Rate q-value <1. Generated datasets were subsequently analysed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA). Canonical pathway enrichment analysis was further filtered for differences between WT and DM infection models of > 0.5. Heatmaps were generated using Multiple Experiment Viewer software 4.9.0.

Quantitative real-time polymerase chain reaction (qRT-PCR) validation of RNA sequencing data

In order to validate RNA sequencing data, qRT-PCR was conducted on 5 randomly selected genes using primers listed in Table 2.2. The High-Capacity cDNA Reverse Transcription kit (Roche Applied Sciences) was used to generate cDNA as per the manufacturer's instructions. cDNA was diluted to 50 ng/µL. PCR efficiences of all primer sets were determined using the dilution method and the following equation $E = 10^{(-1/slope)}$ (Pfaffl, 2001). qRT-PCR was carried out using the Ssoadvanced Universal SYBR Green Supermix (Bio-Rad, South Africa). The reaction mix included 1µL (10µM) of forward and reverse primers, 5 µL 2X Ssoadvanced Universal SYBR Green Supermix (Bio-Rad, South Africa) and 1 µL cDNA (50 ng), in a total volume of 10 µL. Amplification was performed in a CFX96 qRT-PCR Detection System (Bio-Rad, South

Africa) with cyclying conditions of 94°C for 2 min; 40 cycles of annealing and extension temperatures between 56-59ºC and 60ºC, respectively, and melting curve program (75°C to 95°C at 1-5 s increments with continuous fluorescence measurement). Gene expression data were normalised with the reference gene, GAPDH. Gene expression in infected and uninfected THP-1 differentiated macrophages was compared using the $2^{-\Delta\Delta Ct}$ relative quantitation method. The expression experiments were conducted in triplicate biological experiments and technical replicates.

Table 2.2: Primers used for the amplification of the *TGM5, TLR2, DLX3, NLRP3, CD80* and *GAPDH* genes. The listed primers were used to validate the RNA sequencing data.

Gene	Primer sequence (5'-3')	Ref
TGM5	For, AGCTGCTAGACAAGAGCCTG	
	Rev, CCACTCTGCTGACGTAGACG	(Spandidos *et al*., 2009)
TLR2	For, ATCCTCCAATCAGGCTTCTCT	
	Rev, GGACAGGTCAAGGCTTTTTACA	(Spandidos *et al*., 2009)
DLX3	For, TACCCTGCCCGAGTCTTCTG	
	Rev, TGGTGGTAGGTGTAGGGGTTC	(Spandidos *et al*., 2009)
NLRP3	For, GATCTTCGCTGCGATCAACAG	
	Rev, CGTGCATTATCTGAACCCCAC	(Spandidos *et al*., 2009)
CD80	For, GGCCCGAGTACAAGAACCG	
	Rev, TCGTATGTGCCCTCGTCAGAT	(Spandidos *et al*., 2009)
GAPDH	For, GAGTCAACGGATTTGGTCGT	
	Rev, AAATGAGCCCCAGCCTTCT	(Mvubu et al., 2016)

Statistical analysis

The rate of adhesion and invasion was expressed as a relative percentage adhesion/ invasion of the mutant and complemented strains. The percentage adhesion/invasion of each strain equals CFU/mL of adherent/ invaded bacteria divided by the CFU/mL of initial inoculum multiplied by 100. The relative percentage

adhesion/invasion equals the % adhesion/ invasion of double mutant or complemented strains divided by the % adhesion/ invasion of the wild-type multiplied by 100. All assays were carried out in triplicate biological and technical replicates. Statistical analysis was carried out using SPSS software 21.0. (IBM). One-way analysis of variance (ANOVA) was performed to compare the rates of adhesion and invasion. A Pearson correlation coefficient and paired t test were performed to compare RNA sequencing and qRT-PCR expression assays.

Results

MTP plays a greater role in adhesion to and invasion of macrophages than HBHA alone or in combination with HBHA.

To evaluate whether the simultaneous disruption of the *hbhA* and *mtp* genes resulted in a decreased interaction of *M. tuberculosis* bacilli with macrophages, the adhesion (Fig. 2.1A) and invasion (Fig. 2.1B) percentages of wild-type, DM, single mutants (Δ*mtp* and Δ*hbhA*) and complemented strains (*mtp* comp, *hbhA* comp) were compared.

The relative percentage adhesion of all mutant and complemented strains (Fig. 2.1A) was significantly reduced compared to the WT ($p = 0.00$). Adhesion rates of the DM were significantly higher than the hbhA comp ($p = 0.03$) but similar to Δ*mtp* ($p = 1.000$), Δ*hbhA* ($p = 0.32$) and mtp comp ($p = 0.79$). The difference in adhesion rates of the Δ*mtp* and hbhA comp ($p = 0.57$) and Δ*hbhA* and mtp comp ($p = 1.00$) were not statistically significant. The adhesion rate of Δ*mtp* was significantly lower than Δ*hbhA* ($p = 0.02$), whilst that of hbhA comp was significantly lower than mtp comp ($p = 0.01$).

Similarly, the relative percentage invasion of all strains (Fig. 2.1B) was significantly reduced compared to the WT ($p=0.00$). The DM demonstrated significantly less invasion compared to Δ*hbhA* and mtp comp ($p=0.00$) and significantly more invasion compared to Δ*mtp* ($p = 0.00$) and hbhA comp ($p = 0.00$). The difference in invasion rates of the Δ*mtp* and hbhA comp ($p = 0.00$) and Δ*hbhA* and mtp comp ($p = 0.01$) were statistically significant. In both these cases, the invasion rates of the complements were higher than the single mutants, whilst that of the Δ*mtp* was significantly lower compared to Δ*hbhA* ($p = 0.00$). The hbhA comp demonstrated significantly lower invasion rate compared to mtp comp ($p = 0.00$).

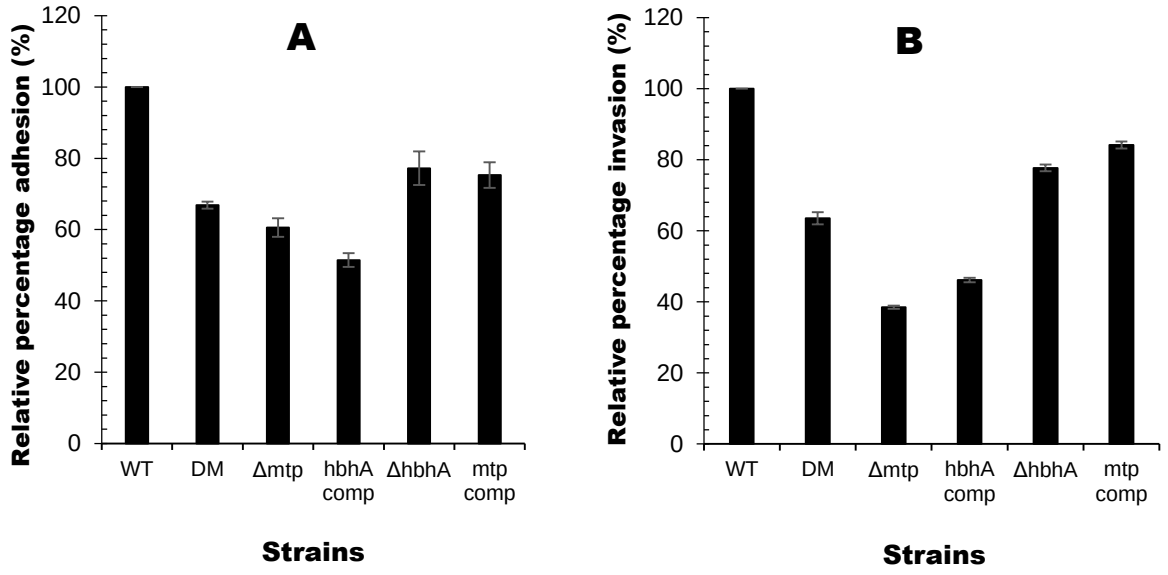


Fig. 2.1: Mean percentage adhesion (A) and invasion (B) of single mutants, DM and complemented strains relative to WT. THP-1 cells were infected with each strain (MOI = 5) for 1 h for the adhesion and 2 h for the invasion assays. Cells were lysed and serial dilutions were plated out on 7h11 agar to determine CFU/ml. Mean percentage adhesion/invasion was compared by one-way ANOVA using SPSS software 21.0. (IBM). (A). All strains demonstrated significantly less adhesion to THP-1 differentiated macrophages compared to WT strain. DM demonstrated significantly more adhesion compared to hbhA comp (*p*-value =0. 03) but similar adhesion to Δ*mtp* (*p*-value =1.00), Δ*hbhA* (*p*-value =0.32) and mtp comp (*p*-value =0.79). (B) Invasion of THP-1 differentiated macrophages was significantly lower for all strains compared to WT strain. DM demonstrated significantly less invasion compared to Δ*hbhA* (*p*-value =0.00) and mtp comp (*p*-value =0.00) and significantly more invasion compared to Δ*mtp* (*p*-value =0.00) and hbhA comp (*p*-value =0.00).

The HBHA and MTP proficient strain induced greater activation of adhesion of phagocytes, activation of phagocytosis, invasion of cells and canonical pathways during early infection of macrophages than the DM strain.

Adhesion of phagocytes, activation of phagocytosis and invasion of cells.

To further understand the role of HBHA and MTP in adhesion and invasion during early infection, the mammalian transcriptome was investigated at 4 h post infection for possible differential transcript regulation associated with these 2 adhesins. In order to assess any possible transcript regulation associated with biological adhesion, the raw RNA sequencing data with a FDR q-value < 1 was filtered using the Gene Ontology data set for biological adhesion. Transcriptome changes were subsequently assessed using biofunctions enrichment IPA analysis.

IPA enrichment analysis of biological functions of transcriptional changes revealed that the HBHA and MTP proficient WT strain induced a higher predicted activation (z-score=1.36) for adhesion of phagocytes network compared to the deficient DM strain (z-score = 0.68). Both infection models demonstrated identical biofunctions enrichment significance (*p*-value = 5.72E-16). During DM infection, Intergrin subunit X (*ITGAX*) and Transforming growth factor beta-1 (*TGFB1*) were upregulated, whilst downregulation was induced by WT infection. In contrast, S100 calcium binding protein A1 (*S10049*) was upregulated by WT infection, but downregulated by DM infection (Fig. 2.2A).

HBHA and MTP proficient WT strain induced a predicted activation (z-score= 0.64) for phagocytosis, in contrast to predicted inhibition (z-score = -0.59) by the DM strain. The biofunctions enrichment significance was identical for both infection models (*p*-value = 6.65E-22). During WT infection, Intergrin subunit beta 3 (*ITGB3*), autophagy related 5 (*ATG5*), platelet glycoprotein 4 (*CD36*), Annexin A1 (*ANXA1*), dedicator of cytokinesis 2 (*DOCK2),* C-type lectin transmembrane receptor (*CD93*) and Forkhead box P1 (*FOXP1*) were upregulated, in contrast to being downregulated during DM infection, whilst Phosphatidylinositol 4-phosphate 5-kinase type-1 alpha (*PIP5K1A*), *TGFB1* and Tyrosine-protein kinase receptor UFO (*AXL*) were upregulated during DM infection and downregulated during WT infection. Interestingly, Hematopoietic SH2 domain-containing protein (*HSH2D*) was not expressed by WT infection and was upregulated in DM infection (Fig. 2.2B).

A higher predicted activation (z-score=0.74) for invasion of cells was observed for the HBHA and MTP proficient WT compared to DM strain (z-score = 0.30). Both infection models displayed identical biofunctions enrichment significance (*p*-value = 1.06E-32). C-X-C chemokine receptor type 3 (*CXCR3*) was not expressed during WT infection, but was upregulated during DM infection, whilst Podocalyxin like (*PODXL*) was upregulated during WT infection, but not expressed during DM infection (Fig. 2.2.C). During DM infection *TGFB1*, early growth response 1 (*EGR1*), *PIP5K1A*, CD81 molecule (*CD81*), paxillin (*PXN*), catenin delta 1 (*CTNND1*), C-C motif chemokine receptor 1 (*CCR1*) and laminin subunit beta 3 (*LAMB3*) were upregulated in contrast to being downregulated during WT infection.

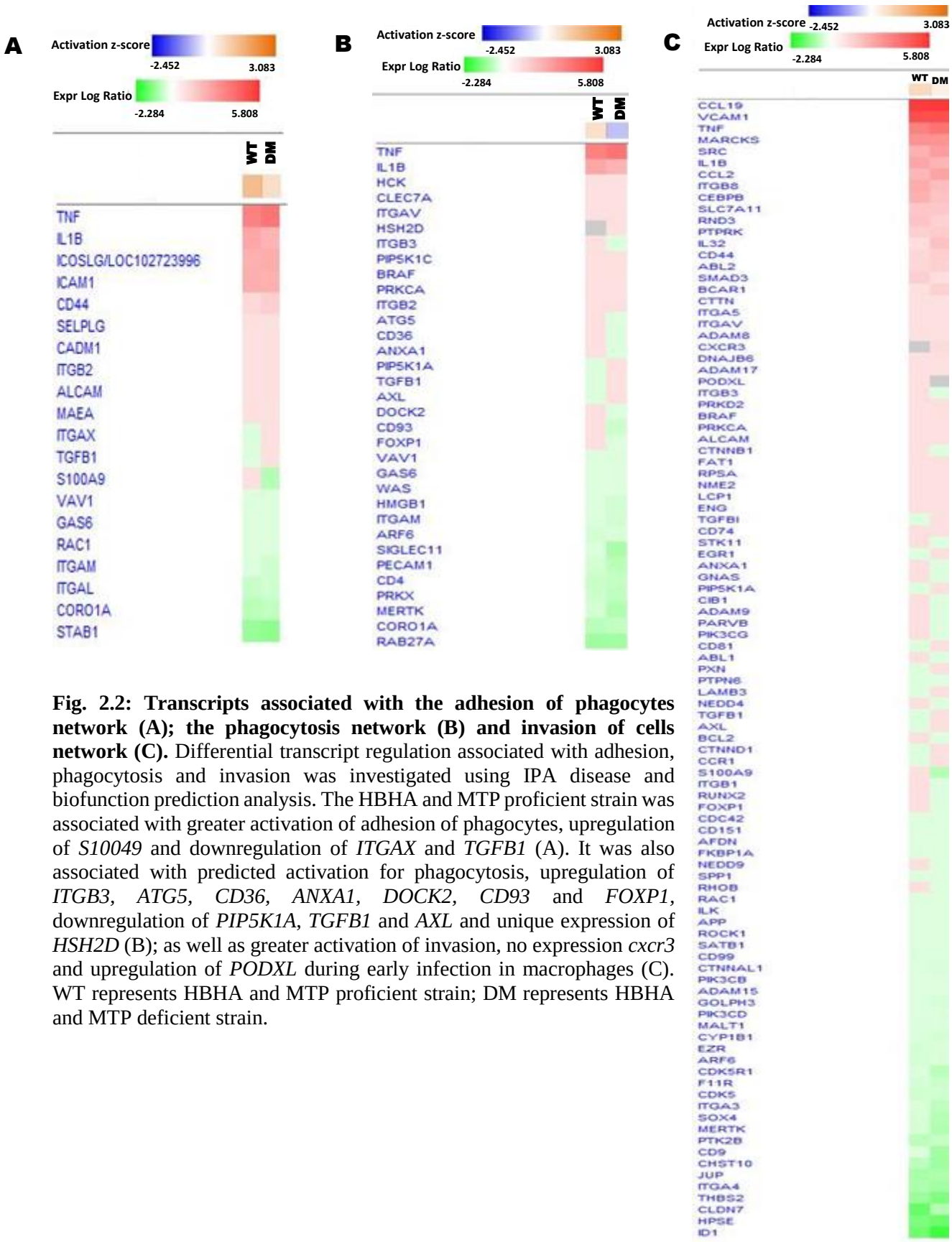


Fig. 2.2: Transcripts associated with the adhesion of phagocytes network (A); the phagocytosis network (B) and invasion of cells network (C). Differential transcript regulation associated with adhesion, phagocytosis and invasion was investigated using IPA disease and biofunction prediction analysis. The HBHA and MTP proficient strain was associated with greater activation of adhesion of phagocytes, upregulation of *S10049* and downregulation of *ITGAX* and *TGFB1* (A). It was also associated with predicted activation for phagocytosis, upregulation of *ITGB3*, *ATG5*, *CD36*, *ANXA1*, *DOCK2*, *CD93* and *FOXP1*, downregulation of *PIP5K1A*, *TGFB1* and *AXL* and unique expression of *HSH2D* (B); as well as greater activation of invasion, no expression *cxcr3* and upregulation of *PODXL* during early infection in macrophages (C). WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

Canonical pathways

To further interpret the differential transcript regulation elicited by HBHA and MTP, canonical pathway enrichment analysis was carried out using IPA software. Canonical pathways that displayed a difference in activation scores of a minimum of 0.5 between WT and DM were used for further analysis. The HBHA and MTP proficient WT strain induced overall higher predicted activation of canonical pathways compared to the DM strain lacking these adhesins (Fig. 2.3). However, similar significance for enrichment of canonical pathways was observed for host cells infected by both strains (Supplementary Table 1).

Heterotrimeric G proteins subunit Gαq (Gαq) signalling was the most differentially regulated canonical pathway in both infection models (Fig. 2.4), with a predicted activation (z-score = 0.577) by the WT compared to that of the DM (z-score = -1.732). During WT infection, guanine nucleotide binding protein, alpha stimulating complex locus (*GNAS)*, Phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit gamma (*PIK3CG*), Protein phosphatase 3 catalytic subunit beta (*PPP3CB)* and Ras homolog family member B (*RHOB*) were upregulated in contrast to being downregulated during DM infection (Fig. 2.4C).

The transcripts differentially regulated by HBHA and MTP during early infection in macrophages were interrogated during the acute phase response, role of pattern recognition receptors in recognition of bacteria and viruses, and Production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathways. The acute phase response canonical pathway (Fig. 2.5) was predicted to be activated by the HBHA and MTP proficient WT strain (z-score = 0.71), but not by the DM strain (z-score = 0) (Fig. 2.5A and B respectively). WT infection induced upregulation of *PIK3CG* in contrast to downregulation by DM infection (Fig. 2.5C). The role of pattern recognition receptors in recognition of bacteria and viruses (Fig. 2.6A and B), and Production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathways (Fig. 2.7A and B) were both more activated by the HBHA and MTP proficient WT strain than the DM strain. In the role of pattern recognition receptors in recognition of bacteria and viruses canonical pathway, during WT infection, *pik3cg* was upregulated, whilst it was downregulated by DM infection. Conversely, *TGFB1* was downregulated by WT infection and upregulated by DM infection (Fig. 2.6C). In the Production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathways, during WT infection, *RHOB* and *PIK3CG* were upregulated in contrast to being downregulated by DM infection (Fig. 2.7C).

Fig. 2.3: Activation of canonical pathways elicited by HBHA and MTP proficient and deficient strains during early infection of macrophages. IPA was used to predict the activation of canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages. Canonical pathways that displayed greater than 0.5 difference between activation z-scores elicited by WT and DM are displayed in the heatmap. Overall, HBHA and MTP were associated with greater predicted activation of canonical pathways. Gαq signalling canonical pathway displayed the highest differential regulation by the two infection models. WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

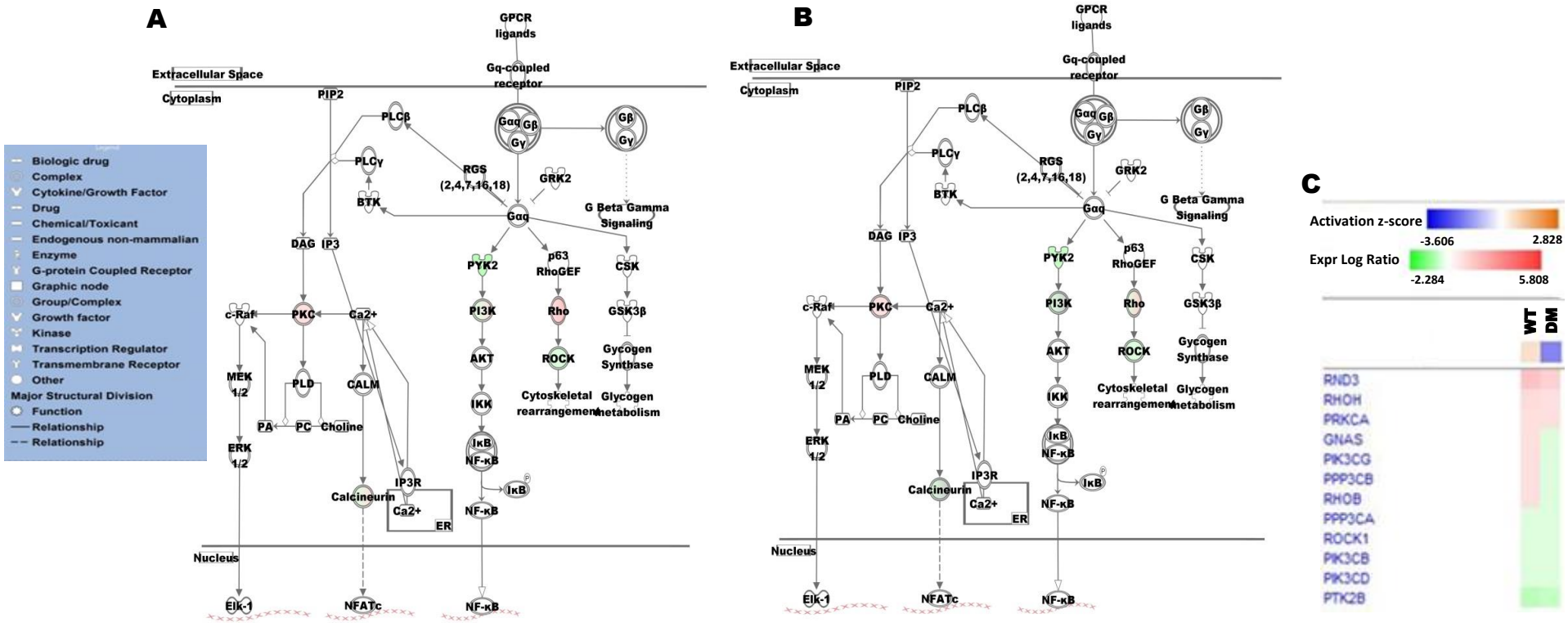
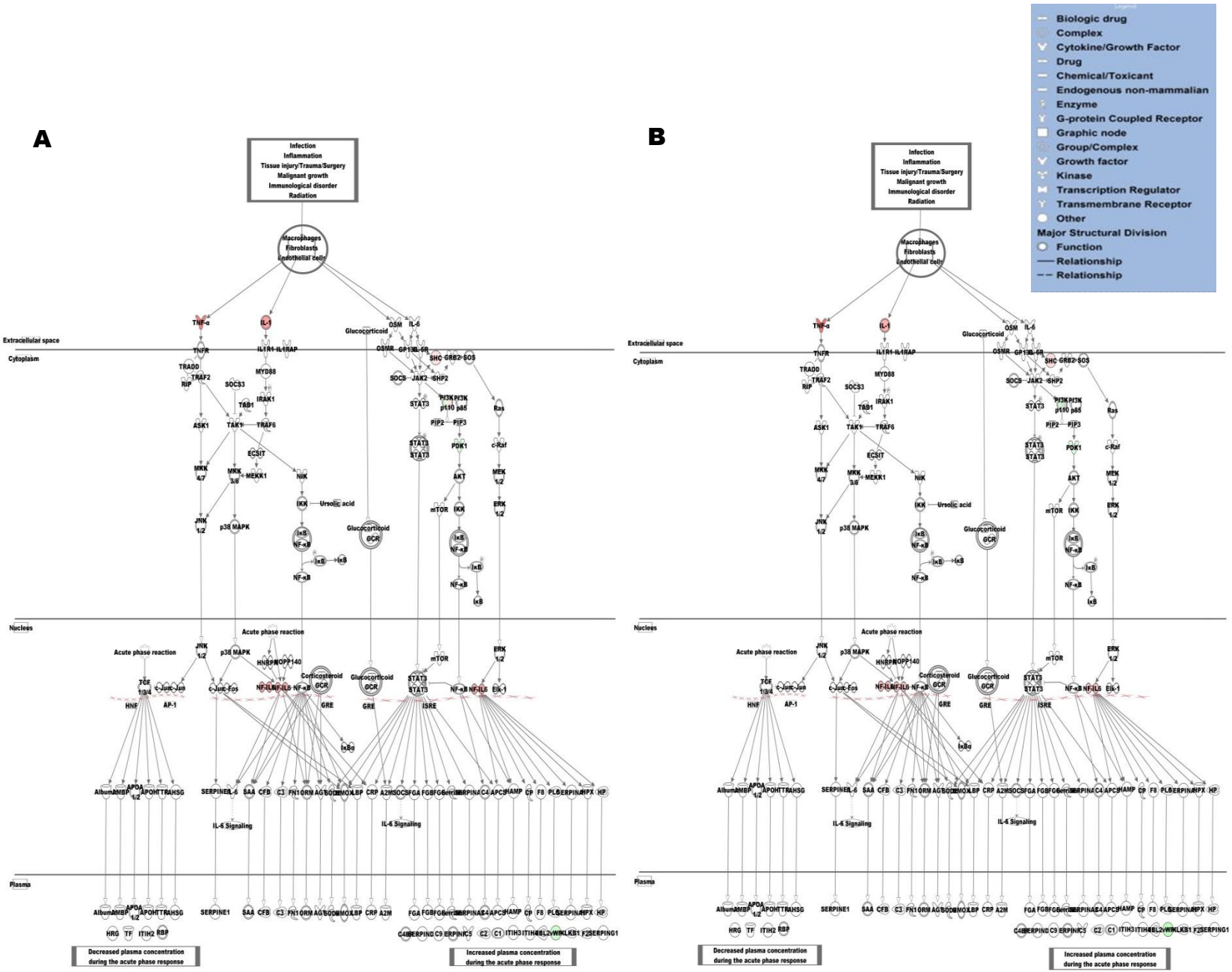


Fig. 2.4: Gαq signalling canonical pathway. Pathways enrichment elicited by HBHA and MTP proficient strain (A), HBHA and MTP deficient strain (B) and gene expression associated with pathway (C). Differential regulation of canonical pathways was investigated using IPA software canonical pathway enrichment analysis. The presence of HBHA and MTP was associated with predicted activation and whilst HBHA and MTP deficiency was associated with predicted inhibition of pathway. HBHA and MTP proficient strain upregulated the following genes *GNAS*, *PIK3CG*, *PPP3CB* and *RHOB* during early infection of macrophages. WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.


A
B
Biologic drug
Complex
Cytokine/Growth Factor
Drug
Chemical/Toxicant
Endogenous non-mammalian
Enzyme
G-protein Coupled Receptor
Graphic node
Group/Complex
Growth factor
Kinase
Transcription Regulator
Transmembrane Receptor
Other
Major Structural Division
Function
Relationship
Relationship
Infection
Inflammation
Tissue injury/Trauma/Surgery
Malignant growth
Immunological disorder
Radiation
Macrophages
Fibroblasts
Endothelial cells
Extracellular space
Cytoplasm
Nucleus
Plasma
Acute phase reaction
Ursolic acid
Glucocorticoid
Decreased plasma concentration during the acute phase response
Increased plasma concentration during the acute phase response
IL-6 Signaling

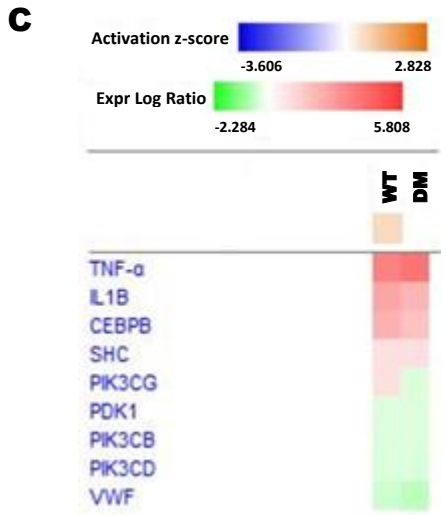
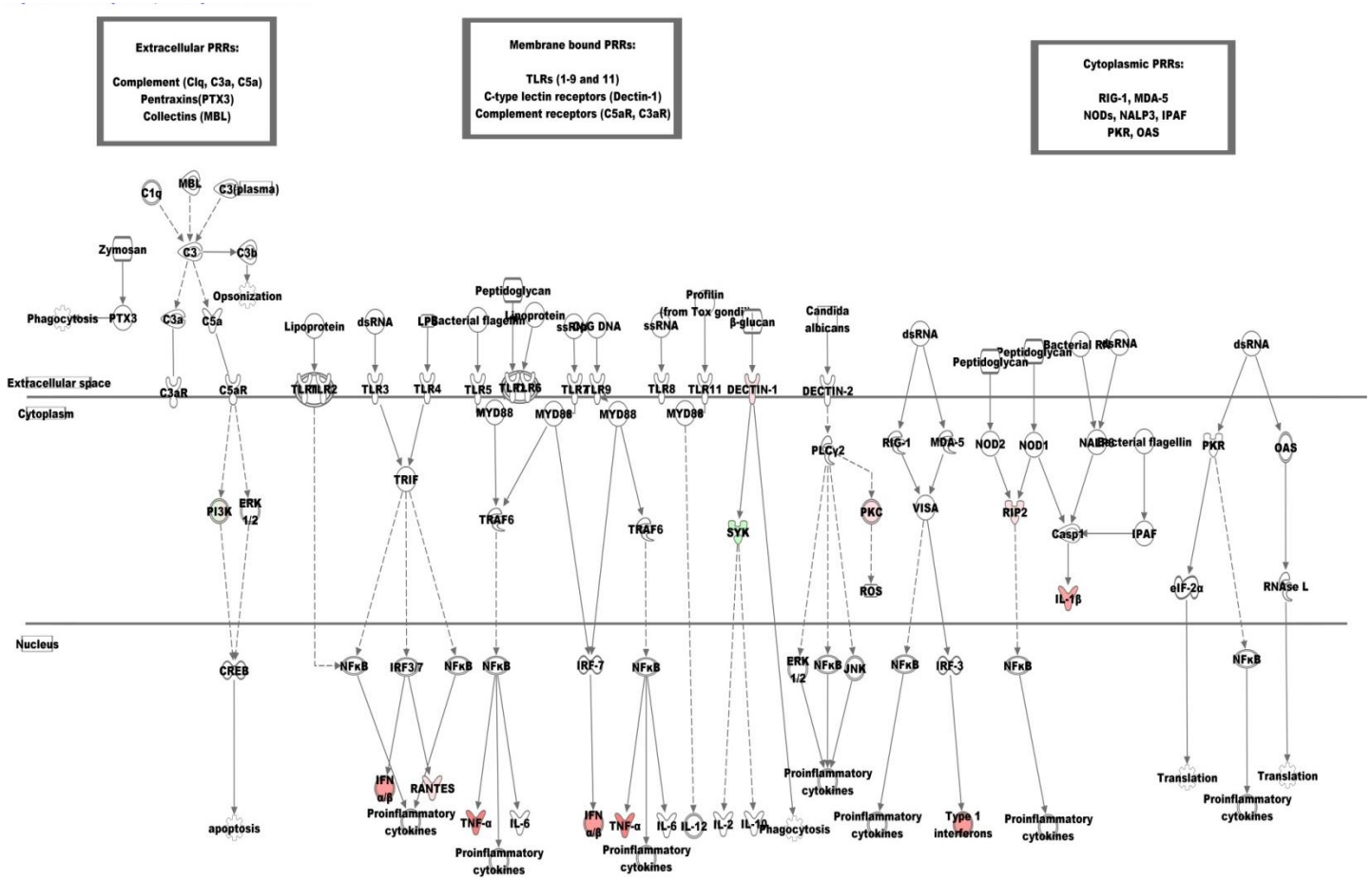


Fig. 2.5: Acute phase response canonical pathway. Pathways enrichment elicited by: HBHA and MTP proficient strain (A), HBHA and MTP deficient strain (B) and gene expression associated with pathway (C). Differential regulation of canonical pathways was investigated using IPA software canonical pathway enrichment analysis. The presence of HBHA and MTP was associated with predicted activation and whilst HBHA and MTP deficiency was associated with no predicted activation/inhibition of pathway. HBHA and MTP proficient strain upregulated the expression of *PIK3CG* during early infection of macrophages. WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

A


Extracellular PRRs:
Complement (Clq, C3a, C5a)
Pentraxins(PTX3)
Collectins (MBL)
Membrane bound PRRs:
TLRs (1-9 and 11)
C-type lectin receptors (Dectin-1)
Complement receptors (C5aR, C3aR)
Cytoplasmic PRRs:
RIG-1, MDA-5
NODs, NALP3, IPAF
PKR, OAS
C1q
MBL
C3(plasma)
Zymosan
C3
C3b
Opsonization
Phagocytosis
PTX3
C3a
C5a
Peptidoglycan
Lipoprotein
dsRNA
LPS
Bacterial flagellin
ssRNA
CpG DNA
Profilin
(from Tox gondi)
ssRNA
β-glucan
Candida albicans
dsRNA
Peptidoglycan
Bacterial PG
dsRNA
dsRNA
Extracellular space
Cytoplasm
C3aR
C5aR
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR9
TLR8
TLR11
DECTIN-1
DECTIN-2
RIG-1
MDA-5
NOD2
NOD1
NALP3
Bacterial flagellin
PKR
OAS
MYD88
MYD88
MYD88
MYD88
PLCγ2
TRIF
PI3K
ERK 1/2
TRAF6
TRAF6
SYK
PKC
VISA
RIP2
Casp1
IPAF
ROS
IL-1β
eIF-2α
RNAse L
Nucleus
CREB
NFκB
IRF3/7
NFκB
NFκB
IRF-7
NFκB
ERK 1/2
NFκB
JNK
NFκB
IRF-3
NFκB
NFκB
IFN α/β
RANTES
Proinflammatory cytokines
TNF-α
IL-6
Proinflammatory cytokines
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Proinflammatory cytokines
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Translation
Proinflammatory cytokines
apoptosis

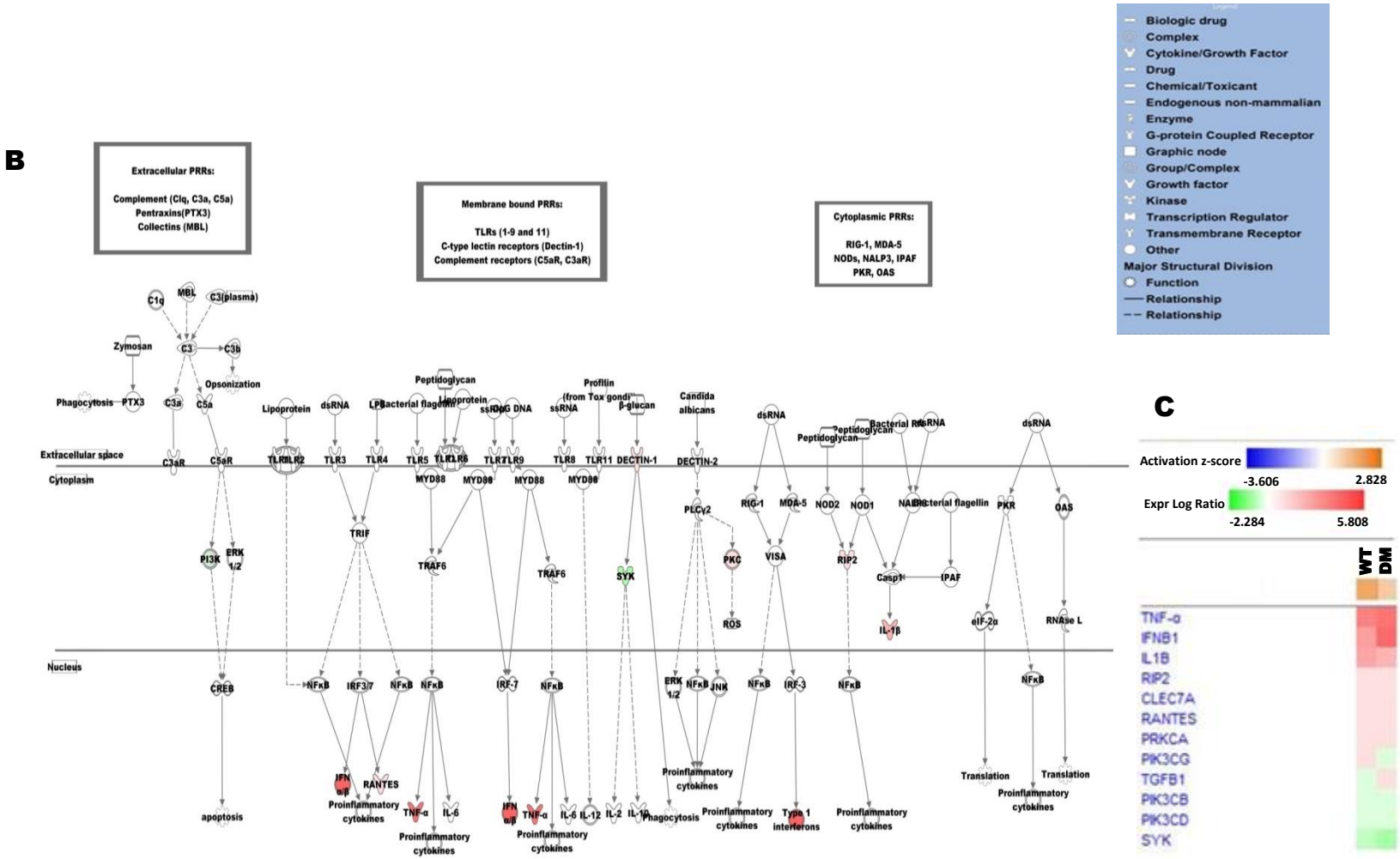
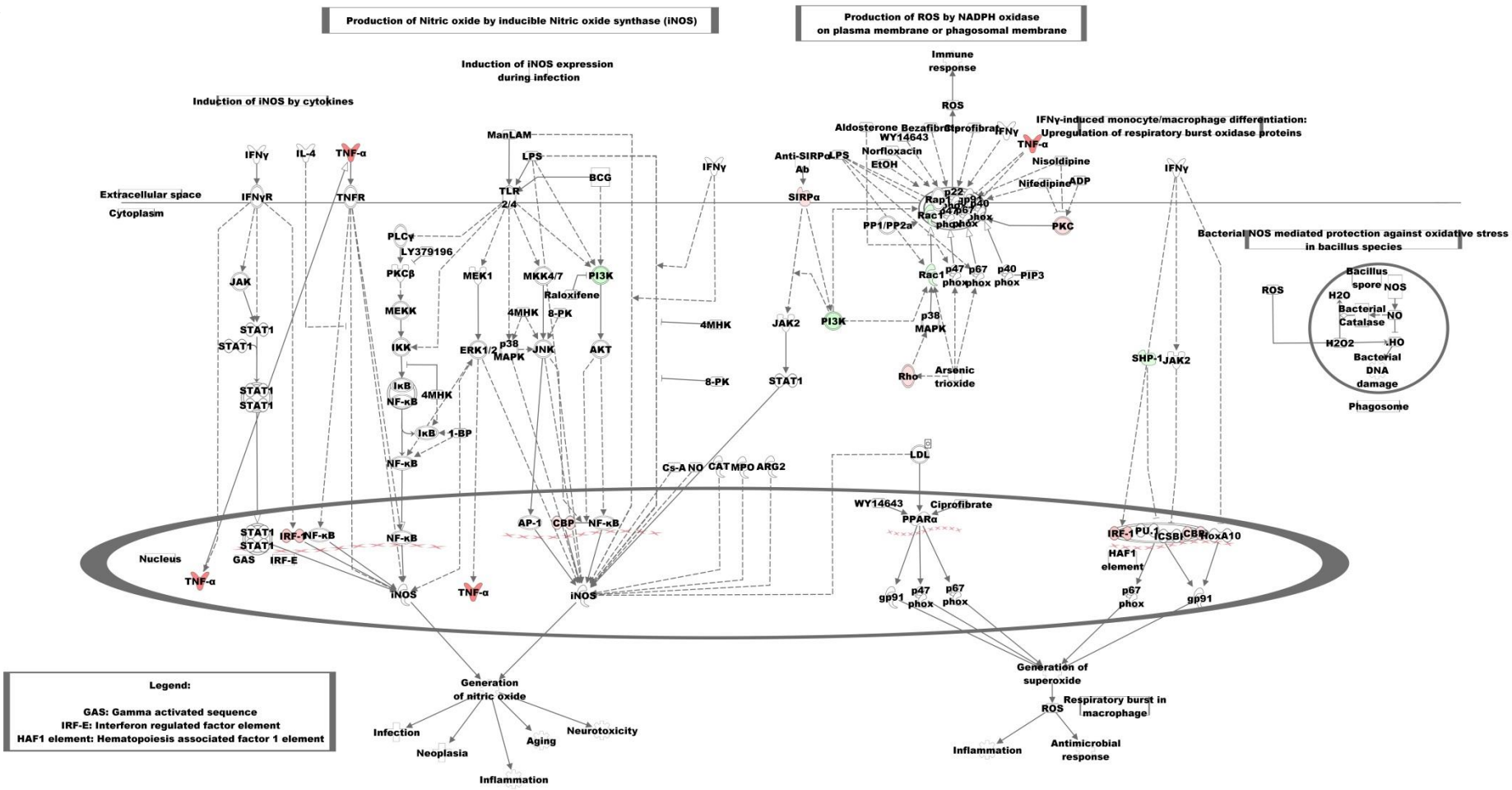


Fig. 2.6: Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses canonical pathway. Pathway enrichment elicited by: HBHA and MTP proficient strain (A), HBHA and MTP deficient strain (B) and gene expression associated with pathway (C). Differential regulation of canonical pathways was investigated using IPA software canonical pathway enrichment analysis. The HBHA and MTP proficient strain was associated with greater predicted activation, upregulated *PIK3CG* and downregulated *TGFB1* during early infection of macrophages. WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

A


Production of Nitric oxide by inducible Nitric oxide synthase (iNOS)
Production of ROS by NADPH oxidase on plasma membrane or phagosomal membrane
Induction of iNOS expression during infection
Induction of iNOS by cytokines
Immune response
ROS
IFNγ-induced monocyte/macrophage differentiation: Upregulation of respiratory burst oxidase proteins
Bacterial NOS mediated protection against oxidative stress in bacillus species
Extracellular space
Cytoplasm
IFNγ
IL-4
TNF-α
IFNγR
TNFR
ManLAM
LPS
BCG
TLR 2/4
Anti-SIRPα Ab
SIRPα
Aldosterone
Bezafibrate
WY14643
Ciprofibrate
Norfloxacin
EtOH
Nisoldipine
Nifedipine
ADP
PKC
PP1/PP2a
Rac1
p22 phox
gp91 phox
p47 phox
p67 phox
p40 phox
PIP3
PLCγ
LY379196
PKCβ
MEK1
MKK4/7
PI3K
Raloxifene
MEKK
4MHK
8-PK
IKK
ERK1/2
p38 MAPK
JNK
AKT
IκB
NF-κB
1-BP
JAK
STAT1
JAK2
Rho
Arsenic trioxide
SHP-1
Bacillus spore
H2O
NOS
Bacterial Catalase
NO
H2O2
HO
Bacterial DNA damage
Phagosome
ROS
LDL
Cs-A
NO
CAT
MPO
ARG2
PPARα
AP-1
CBP
IRF-1
PU.1
ICSBI
RhoA10
HAF1 element
IRF-E
GAS
Nucleus
TNF-α
iNOS
Generation of nitric oxide
Infection
Neoplasia
Aging
Neurotoxicity
Inflammation
Generation of superoxide
Respiratory burst in macrophage
Antimicrobial response
Legend:
GAS: Gamma activated sequence
IRF-E: Interferon regulated factor element
HAF1 element: Hematopoiesis associated factor 1 element

B

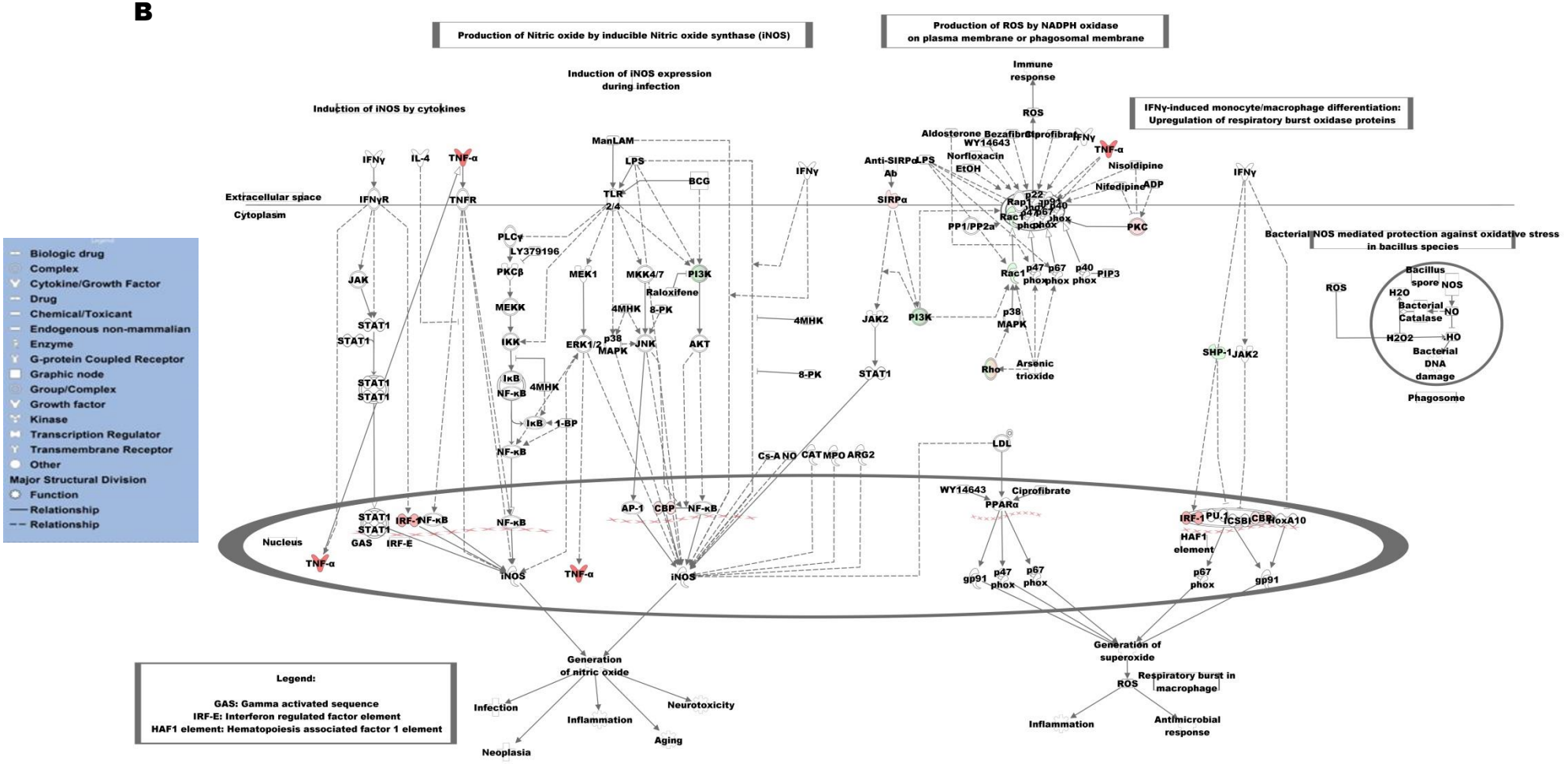

Production of Nitric oxide by inducible Nitric oxide synthase (iNOS)
Production of ROS by NADPH oxidase on plasma membrane or phagosomal membrane
Induction of iNOS expression during infection
Induction of iNOS by cytokines
IFNγ-induced monocyte/macrophage differentiation: Upregulation of respiratory burst oxidase proteins
Bacterial NOS mediated protection against oxidative stress in bacillus species
Immune response
ROS
Extracellular space
Cytoplasm
IFNγ
IL-4
TNF-α
IFNγR
TNFR
ManLAM
LPS
BCG
TLR 2/4
PLCγ
LY379196
PKCβ
MEK1
MKK4/7
PI3K
Raloxifene
MEKK
4MHK
8-PK
IKK
ERK1/2
p38 MAPK
JNK
AKT
IκB
NF-κB
1-BP
JAK
STAT1
JAK2
PI3K
SIRPα
Anti-SIRPα Ab
Aldosterone
Bezafibrate
Ciprofibrate
WY14643
Norfloxacin
EtOH
Nisoldipine
Nifedipine
ADP
PP1/PP2a
PKC
Rac1
p22 phox
p91 phox
p40 phox
p47 phox
p67 phox
PIP3
Rho
Arsenic trioxide
SHP-1
Bacillus spore
H2O
NOS
Bacterial Catalase
NO
H2O2
HO
Bacterial DNA damage
Phagosome
Nucleus
GAS
IRF-E
IRF-1
iNOS
AP-1
CBP
Cs-A
NO
CAT
MPO
ARG2
LDL
WY14643
Ciprofibrate
PPARα
PU.1
ICSBP
HoxA10
HAF1 element
gp91 phox
Generation of nitric oxide
Generation of superoxide
Respiratory burst in macrophage
Infection
Inflammation
Neurotoxicity
Aging
Neoplasia
Antimicrobial response
Legend:
GAS: Gamma activated sequence
IRF-E: Interferon regulated factor element
HAF1 element: Hematopoiesis associated factor 1 element
Biologic drug
Complex
Cytokine/Growth Factor
Drug
Chemical/Toxicant
Endogenous non-mammalian
Enzyme
G-protein Coupled Receptor
Graphic node
Group/Complex
Growth factor
Kinase
Transcription Regulator
Transmembrane Receptor
Other
Major Structural Division
Function
Relationship
Relationship

Fig. 2.7: Production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathway. Pathway enrichment elicited by: HBHA and MTP proficient strain (A), HBHA and MTP deficient strain (B) and gene expression associated with pathway (C). Differential regulation of canonical pathways was investigated using IPA software canonical pathway enrichment analysis. The HBHA and MTP proficient strain was associated with greater predicted activation, upregulated *PIK3CG* and *RHOB* during early infection of macrophages. WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

qRT-PCR expression data significantly correlate with RNA sequencing data.

The log2 (fold change) ranged from -2.34 to 2.70 via qRT-PCR and -2.90 to 4.08 via RNA sequencing (Fig. 2.8). The differences between log2 (fold change) between the two assays ranged between -1.44 to 1.39. Comparison of the qRT-PCR log2 (fold change) and RNA sequencing log2 (fold change) using a Pearson correlation coefficient of 0.97 demonstrated significant correlation between both assays (p = 0.00). In addition, a paired sample t-test showed no statistical difference between qRT-PCR and RNA sequencing (p = 0.82).

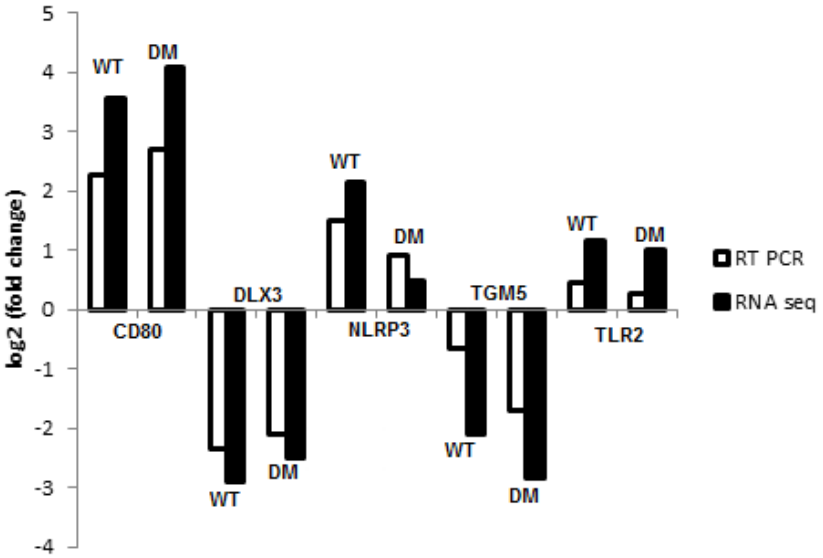


Fig. 2.8: The validation of RNA sequencing data using quantitative real time PCR for 5 genes (*cd80, dlx3, nlrp3, tgm5 and tlr2*). RNA was extracted at 4 h from THP-1 cells infected with either WT or DM respectively. Quantitative real time PCR was carried out using a CFX96 qRT-PCR Detection System (Bio-Rad) and Ssoadvanced Universal SYBR Green Supermix (Bio-Rad). The reference gene *gapdh* was utilised to normalise the gene of interest expression data. Quantitative real time gene expression of infected and uninfected THP-1 differentiated macrophages was compared using the $2^{-\Delta\Delta Ct}$ relative quantitation method. The quantitative real time PCR expression results of 3 biological experiments assayed in triplicate and the RNA sequencing expression results for 2 biological experiments are represented as log2 (fold change). Pearsons correlation coefficient showed significant correlation in the expression data between RNA sequencing and quantitative real time PCR (p-value= 0.00) as indicated by a Pearson correlation coefficient of 0 .97. Paired sample t-test showed no statistical difference between qRT-PCR and RNA sequencing (p-value= 0.82). WT represents HBHA and MTP proficient strain; DM represents HBHA and MTP deficient strain.

Discussion

Adhesins are cell surface molecules that are involved in the interactions of pathogenic bacteria and host cells. This is the precursor to invasion and colonization of host cells in an effort to establish a successful infection to ensure proliferation, survival and dissemination of *M. tuberculosis* (Smith, 2003). Despite substantial research into macrophage adhesins and invasins, translation into diagnostic and therapeutic targets is yet to yield ideal biomarkers. The individual adhesin/invasion functions of MTP and HBHA have been well documented (Pethe *et al*., 2001; Pethe *et al*., 2002; Delogu *et al*., 2006; Ramsugit and Pillay, 2014; Ramsugit *et al*., 2016). Understanding the impact of adhesins/invasins on mammalian molecular mechanisms including canonical pathways and gene regulation could possibly yield novel therapeutic biomarker targets. This is the first study, to our knowledge, that explored mammalian gene differential regulation influenced by the combination of HBHA and MTP during adhesion to and invasion of macrophages.

HBHA and MTP do not act in synergy during adhesion and invasion of macrophages.

In order to assess the impact of HBHA and MTP and decipher which adhesin plays a more significant role in adhesion to, and invasion of macrophages, single mutants and complemented strains were utilised. Δ*mtp* demonstrated a 39.4% and 61.49% decrease in percentage adhesion and invasion compared to WT respectively, suggesting that MTP plays a more significant role in invasion than adhesion. This finding correlates with results of a previous study in which Δ*mtp* displayed a 42.16% and 69.02% reduction in percentage adhesion and invasion compared to WT (Ramsugit and Pillay, 2014).

Δ*hbhA* induced a 22.78% and 22.29% decrease in percentage adhesion and invasion respectively compared to WT, suggesting that HBHA plays a minimal role in adhesion and invasion. However, a previous study demonstrated that HBHA played no significant role in adhesion and invasion of macrophages (Pethe *et al*., 2001). This may be explained by the differences in the macrophage model and strain family of mutant in the 2 studies.

During adhesion, DM induced a similar decrease in percentage adhesion (33.16%) to Δ*mtp* (39.4%), Δ*hbhA* (22.78%), mtp comp (24.72%) but statistically lower decrease in percentage adhesion than hbhA comp (53.85%). The hbhA comp which was constructed in a double mutant that lacked *mtp*, and overexpressed *hbhA*, displayed a similar decrease in percentage adhesion to Δ*mtp*. HBHA stimulation in macrophages was previously shown to induce endoplasmic reticulum stress-associated apoptosis (Choi *et al*., 2013). Therefore, we surmise that the over-expression of HBHA by hbhA comp could possibly have induced macrophage cell death at a higher rate, resulting in a higher decrease in

percentage adhesion than DM. Taken together, these findings suggest that MTP independently plays a larger role in adhesion of macrophages than HBHA.

During invasion, DM displayed a significant decrease in percentage invasion (36.49%) compared to Δ*mtp* (61.49%) and hbhA comp (53.85%); and significantly higher decrease in percentage invasion than Δ*hbhA* (22.29%) and mtp comp (24.72%). The hbhA comp and mtp comp displayed a significant decrease in percentage invasion than WT but was significantly higher compared with Δ*mtp* and *ΔhbhA* respectively. A previous study demonstrated increased expression of the *mtp* in the mtp complemented strain during infection of macrophages (Ramsugit and Pillay, 2014) and this possibly could explain the increase in invasion by the hbhA and mtp comp strains during invasion. These findings together suggests that MTP is a stronger invasin than HBHA.

Synergy occurs when the double mutant displays a greater effect than the combination of the individual mutations (Pérez-Pérez *et al*., 2009). The expected synergistic effect of HBHA and MTP on adhesion and invasion of macrophages, and postulated the reason for the lack thereof, may be due to the presence of multiple other adhesins and invasins. It is possible that *M. tuberculosis* compensated for the impairment by upregulation or use of alternate adhesins and invasins such as Apa (Pasula *et al*., 1997), 19 kDa lipoprotein (Neyrolles *et al*., 2001), laminin binding protein (Pethe *et al*., 2002), Cpn60.2 (Stokes *et al*., 2004), malate synthase (Kinhikar *et al*., 2006), membrane protein, N-acetylmuramoyl-L-alanine amidase, and L,D-transpeptidase (Kumar *et al*., 2013).

HBHA and MTP modulated transcript regulation associated with cell signalling during early infection.

In order to understand the lack of synergy in the adhesion and invasion assay, the differential regulation of specific transcripts associated with biological adhesion was analysed using IPA biological function enrichment analysis. The data elucidated that HBHA and MTP were associated with an increased predicted activation of adhesion of phagocytes by 2-fold and invasion of cells by 2.5-fold. Whilst phagocytosis was predicted to be activated by HBHA and MTP, in their absence, this process was predicted to be inhibited. Collectively, these findings provide evidence that HBHA and MTP elicit differential mammalian transcript regulation during adhesion of phagocytes and invasion, possibly accounting for the significantly lower percentage adhesion and invasion displayed by the HBHA and MTP deficient strain in the adhesion and invasion assays. In addition, since HBHA and MTP contribute to the activation of phagocytosis during infection of macrophages, this suggests recognition of these adhesins by the host immune receptors.

In the current study, HBHA and MTP demonstrated the ability to modulate transcript regulation of *GNAS, PIK3CG, PPP3CB, RHOB, S10049, ITGB3, ATG5, CD36, ANXA1, DOCK2, CD93, FOXP1*

and *PODXL*. The proteins encoded by these transcripts were associated with the following cell functions. *GNAS* is a G protein α-subunit required for the intracellular cyclic adenosine monophosphate production (Weinstein *et al*., 2004). *PIK3CG* belongs to a family of enzymes that facilitates cell growth, differentiation, proliferation, survival, intracellular signalling and motility (Brock *et al*., 2003). Knockout experiments demonstrated that the *PIK3CG* transcript is required for migration of macrophages and neutrophils dependent on chemokines, and not for phagocytic processes and bactericidal activities in neutrophils (Wymann *et al*., 2003). The *PPP3CB* transcript, which encodes for a subunit of calcineurin was demonstrated to be required for the activity of calcineurin proteins (Bueno *et al*., 2002) and prevention of chronic reactive oxygen species generation by fibroblasts (Williams and Gooch, 2014). *RHOB* was previously demonstrated to be upregulated in response to inflammatory cytokines and co-localizes with RAC1 in endosomes, thus inhibiting RAC1 activity (Marcos-Ramiro *et al*., 2016). *S10049* is associated with cell process regulation by calcium regulation (Most *et al*., 2003), microtubule assembly inhibition (Zimmer *et al*., 1998) and protein kinase C-mediated phosphorylation inhibition (Reppel *et al*., 2005). It has also been associated with production of nitric oxide by endothelial cells (Sen *et al*., 2014). *ITGB3* is a cell-surface protein associated with cell adhesion and signalling (Howard and Zwilling, 1999; Oki *et al*., 2006). *ATG5* is associated with preventing polymorphic mononuclear cell mediated immunopathology, thereby playing a role in protection against *M. tuberculosis* (Kimmey *et al*., 2015). CD36 molecule facilitates uptake of surfactant lipids and *M. tuberculosis* growth in human macrophages (Dodd *et al*., 2016). *ANXA1* demonstrated involvement in mediation of dendritic cell cross-presentation by increasing efferocytosis and antigen-presenting machinery during *M. tuberculosis* infection (Tzelepis *et al*., 2015) and is also involved in Toll-like receptor signalling activation resulting in IFN-β and CXCL10 production (Bist *et al*., 2013). *DOCK2* is required for chemotaxis of mature lymphocytes by acting as a Rac activator and has demonstrated in immature hematopoietic cells the ability to regulate CXCR4 signalling (Kikuchi *et al*., 2008). *CD93* molecule is a cell surface protein that is associated with hematopoiesis, inflammatory processes and required for phagocytosis (Nepomuceno *et al*., 1997; Norsworthy *et al*., 2004). *FOXP1* is a transcriptional factor whose downregulation has been deemed important in the processes of differentiation of monocytes and functions of macrophages *in vivo* (Shi *et al*., 2008). *PODXL* gene encoded protein has been shown to decrease cell adhesion of podocytes (Shankland, 2006) and is associated with the gene ontology biological process including cell adhesion and positive regulation of cell-cell adhesion mediated by integrin.

PIK3CB and *PIK3CD* were commonly expressed by all canonical pathways investigated in this study, and were similarly regulated by HBHA and MTP. Both these genes are catalytic subunits of phosphoinositide 3-kinase and function in cell signalling. Previous studies demonstrated that *PIK3CB*

may play an important role in cell proliferation (Bénistant *et al*., 2000) whilst *PIK3CD* is involved in B- and T-cell antigen receptor signalling (Okkenhaug *et al*., 2002).

Taken together, our transcription regulation data suggest that HBHA and MTP enable ***M. tuberculosis*** to modulate enzymatic and cellular signalling as a possible survival mechanism during early infection of macrophages. For example, previous studies have demonstrated one of *M. tuberculosis* survival strategies in macrophages involve the use of *M. tuberculosis* glycolipids to interrupt host cell signalling mediated by phosphatidylinositol 3-phosphate, which is necessary for phagolysosome biosynthesis (Roth, 2004). *M. tuberculosis* actively prevents the accumulation of phosphatidylinositol 3-phosphate on phagosomal membranes (Fratti *et al*., 2003). The prolonged survival of *M. tuberculosis* in host cells has been attributed to production of virulence factors and utilization of host molecules for the pathogen's benefit (Pieters, 2008). An example of this, is the manipulation of the calcineurin pathway during infection of macrophages. Tryptophan aspartate containing coat protein or Coronin1 is a protein that was found only in phagosomes that contained viable *M. tuberculosis*, therefore, coronin 1 is an important host factor that mediates prevention of lysosomal delivery (Ferrari *et al*., 1999). Coronin1 dependent activation of calineurin is necessary for inhibition of phagosome-lysosome fusion. *M. tuberculosis* intracellular growth in macrophages was inhibited using calcineurin blockers and activated calcineurin if required for inhibiting phagosome-lysosome fusion, thereby, promoting intracellular growth of *M. tuberculosis* (Jayachandran *et al*., 2008). In the current study, *PIK3CB, PIK3CD* and *PIK3CG*, are phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunits, involved in the synthesis of phosphatidylinositol 3,4,5-trisphosphate. The former two were unaffected by HBHA and MTP but *pik3cg* was upregulated by HBHA and MTP. *PP3CB* which encodes a subunit of calcineurin, was also upregulated by HBHA and MTP.

The absence of HBHA and MTP modulated the transcript regulation of *ITGAX, HSH2D, CXCR3, TGFB1, PIP5K1A* and *AXL*. *M. tuberculosis* has been previously show to decrease expression of integrins during infection of dendritic cells (Roberts and Robinson, 2014). *HSH2D* serves an adapter protein in tyrosine kinase signalling and plays a role in hematopoietic cell cytokine signalling and cytosketetal reorganization (Oda *et al*., 2001). *CXCR3* facilitates antigen presenting cell and T cell interaction by playing a role in T cell migration, thus enabling production of memory and effector cells (Groom and Luster, 2011). *TGFB1* is involved in the production of nitric oxide species, suppression of antigen presenting cell's costimulatory molecules and down- regulates proliferation of T cells (Bright and Sriram, 1998; Gorham *et al*., 1998). *PIP5K1A* contribute to phospholipid phosphatidylinositol-4,5bisphosphate which facilitate production of inositol-1,4,5-trisphosphate and lytic granule exocytosis. HBHA and MTP are not required for granule polarization and phosphatidylinositol3-kinase activity (Micucci *et al*., 2008). *AXL* was seen to facilitate macrophage functional compartmentalization and it was suggested that this is important for prevention of excessive inflammation (Fujimori *et al*., 2015).

The modulation of these transcripts are independent of HBHA and MTP and could be regulated by the speculated alternate adhesion. Further studies involving identification of the *M. tuberculosis* compensatory mechanisms for the loss of HBHA and MTP and their impact on macrophage transcription regulation is warranted.

The current study subsequently interrogated differential canonical pathway regulation to assess the impact of HBHA and MTP on cellular signalling canonical pathways.

HBHA and MTP activate canonical pathways during early infection of macrophages.

In the current study, HBHA and MTP present in the WT strain induced higher predicted activation scores of canonical pathways, including Gαq signalling, which was the most differentially regulated. G protein-coupled receptors and heterotrimeric G-proteins are primarily involved in signal transduction following activation of various hormones (catecholamines, angiotensin-II, prostaglandin F2-α and endothelin-1). Therefore, HBHA and MTP play an important role in regulation of physiological functions in cells (Shi and Kehrl, 2001; Johnson and Druey, 2002 ;Wettschureck and Offermanns, 2006; Yang and Xia, 2006). G protein-coupled receptors are vital for migration of phagocytes and are expressed by monocytes, macrophages and polymorphonuclear leukocytes that are important inflammatory cells (Sun and Ye, 2012).

The acute phase response canonical pathway leads to the swift inflammatory response that is initiated to protect against microbes via non-specific protection mechanisms. It is normally characterised by elevation in pro-inflammatory cytokines, fever and changes in concentration of acute phase proteins. HBHA and MTP activated this pathway, suggesting that HBHA and MTP may influence the acute phase response, possibly resulting in increase of cytokines and conditions that aid in the initial host control of *M. tuberculosis* during early infection.

Pattern-recognition receptors are vital in the initiation of the immune response. These receptors recognise pathogen-associated molecular patterns or alternately, structures of the microbe, and can be classified as either membrane bound, extracellular or cytoplasmic. The data in the present study demonstrated that HBHA and MTP enhanced activation of role of pattern recognition receptors in recognition of bacteria and viruses canonical pathway. However, HBHA and MTP are not the sole activators of this pathway as indicated by the lower activation in their absence in the DM strain. This suggests that these 2 are not the only adhesins that are utilising pattern recognition receptors during early infection. *M. tuberculosis* is known to have multiple adhesins (Govender *et al*., 2014) and this is thought to be a vital survival strategy as macrophages can serve as reservoirs. This finding also supports our speculation of an upregulation of alternate adhesins as a compensatory mechanism for HBHA and

MTP deletion and warrants further investigation. Even though activation of pattern recognition receptors in recognition of bacteria and viruses canonical pathway is beneficial to the host, *M. tuberculosis* is able to modulate macrophage activation, thus enabling survival intracellularly. *M. tuberculosis* glycolipids have previously shown an ability to evade macrophage activation. For example, cell wall mannosylated lipoarabinomannan was shown to regulate cell signalling pathways such as toll like receptor signalling (Chan *et al*., 1991) and lipoarabinomannan has been shown to inhibit the activation mitogen-activated protein kinase signalling that is the downstream product of stimuli that result in macrophage activation (Knutson *et al*., 1998).

Nitric oxide and reactive oxygen species production that is induced by nitric oxide synthase, is a vital process in activated macrophages. A known survival mechanism of intracellular *M. tuberculosis* in macrophages involves the blockage of nitric oxide and reactive oxygen species production (Cooper, 2009). Our data demonstrated that HBHA and MTP enhanced the activation of the production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathway during early infection, but are not the sole activators of this pathway as indicated by the lower activation in their absence in the DM strain. This also suggests that other molecules in addition to HBHA and MTP activate this canonical pathway. *M. tuberculosis* bacilli are able to persist in the presence of these toxic species using survival strategies such as production of catalase-peroxidase (KatG) that are able to incapacitate reactive oxygen species present in phagosomes (Li *et al*., 1998) and subunits of the *M. tuberculosis* proteasome possibly assist with the resistance of *M. tuberculosis* nitric oxide stress (Darwin *et al*., 2003).

Taken together, HBHA and MTP in addition to their role in adhesion and invasion, may function as pathogen-associated molecular patterns that enable host immune recognition during early infection of macrophages. This may lead to activation of canonical pathways necessary for effective cell signalling required for macrophage functions.

Limitations

One of the limitations of this study was that only two biological replicates were sequenced and the complemented strains were not sequenced. To address this shortcoming, three biological replicates and the complemented strains were analysed by qRT-PCR which showed good correlation with the RNA sequencing data. Another limitation of the current study was that a double complemented strain was not utilised in the adhesion/invasion assay, in order to investigate the effect of single complementation in the double mutant to ascertain the relative contribution of each adhesin. However, it is acknowledged that a double complemented strain should have been included to confirm the combined contribution of HBHA and MTP in restoring gene function. This will be addressed in future studies. In addition, the presence of alternate *M. tuberculosis* adhesins and virulence factors during macrophage infection could

not be validated in the current study, since host, and not bacterial transcriptional regulation was investigated.

Conclusion and future work

This study revealed that HBHA and MTP independently induce adhesion and invasion, but do not act in synergy in combination. MTP played a more significant role in adhesion and invasion of macrophages than HBHA. HBHA and MTP together induce transcriptional regulation that enhances adhesion and invasion of macrophages and host immune recognition during early infection of macrophages. Further research needs to be undertaken to test the hypothesis that alternate *M. tuberculosis* adhesins are expressed in the absence of HBHA and MTP. Future work will also investigate specific mechanisms that HBHA and MTP are involved in during adhesion, invasion and phagocytosis using dual complementation in addition to single complementation and functional protein studies. These studies should include assessment of mycobacterial transcriptome profiling during infection of macrophages to elucidate expression of alternate *M. tuberculosis* adhesins and further investigation of HBHA and MTP interaction with pattern recognition receptors, induction of cytokines and nitric oxide and oxygen reactive species and elucidate *M. tuberculosis* compensatory mechanisms for the loss of HBHA and MTP.

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Supplementary data

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
G alpha q Signaling	0.577350269	-1.732050808
PI3K/AKT Signaling	0.25819889	-1.807392228
EIF2 Signaling	-0.333333333	-2.333333333
Wnt/beta-catenin Signaling	1.264911064	-0.632455532
Glioma Invasiveness Signaling	1.264911064	-0.632455532
fMLP Signaling in Neutrophils	-0.632455532	-2.529822128
Melanocyte Development and Pigmentation Signaling	1.897366596	0
Regulation of eIF4 and p70S6K Signaling	0	-1.897366596
Colorectal Cancer Metastasis Signaling	1.147078669	-0.688247202
AMPK Signaling	0.904534034	-0.904534034
Small Cell Lung Cancer Signaling	0.447213595	-1.341640786
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	0.447213595	-1.341640786
Thrombin Signaling	1.154700538	-0.577350269
PPAR alpha/RXR alpha Activation	0	-1.732050808
Role of NFAT in Regulation of the Immune Response	-0.277350098	-1.941450687
PI3K Signaling in B Lymphocytes	-1.941450687	-3.605551275
CCR3 Signaling in Eosinophils	0	-1.632993162

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
Pancreatic Adenocarcinoma Signaling	0	-1.632993162
Cardiac Hypertrophy Signaling	-0.774596669	-2.323790008
Opioid Signaling Pathway	0.774596669	-0.774596669
NF-kappa B Activation by Viruses	0.25819889	-1.290994449
Glioblastoma Multiforme Signaling	0.25819889	-1.290994449
Signaling by Rho Family GTPases	-1.133893419	-2.645751311
G Beta Gamma Signaling	1.133893419	-0.377964473
RANK Signaling in Osteoclasts	-0.377964473	-1.889822365
eNOS Signaling	-0.377964473	-1.889822365
ILK Signaling	-0.365148372	-1.825741858
Type II Diabetes Mellitus Signaling	0	-1.414213562
Endothelin-1 Signaling	1	-0.333333333
Mouse Embryonic Stem Cell Pluripotency	0.333333333	-1
p70S6K Signaling	0.333333333	-1
Sphingosine-1-phosphate Signaling	0.333333333	-1
ErbB Signaling	-0.333333333	-1.666666667
NGF Signaling	-0.333333333	-1.666666667
Phospholipase C Signaling	1.091089451	-0.21821789
Ephrin Receptor Signaling	0.21821789	-1.091089451
PDGF Signaling	1.264911064	0
Role of NFAT in Cardiac Hypertrophy	0.632455532	-0.632455532
Tec Kinase Signaling	-0.40824829	-1.632993162
Actin Nucleation by ARP-WASP Complex	-0.301511345	-1.507556723

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
Adrenomedullin signaling pathway	1.154700538	0
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.732050808	0.577350269
mTOR Signaling	-0.277350098	-1.386750491
iCOS-iCOSL Signaling in T Helper Cells	-0.277350098	-1.386750491
Cell Cycle: G1/S Checkpoint Regulation	1	0
Role of Wnt/GSK-3 beta Signaling in the Pathogenesis of Influenza	0	-1
Melanoma Signaling	0	-1
Amyloid Processing	-1	-2
Leukocyte Extravasation Signaling	-0.948683298	-1.897366596
Dendritic Cell Maturation	2.828427125	1.885618083
CD28 Signaling in T Helper Cells	-1.605910137	-2.523573073
Glioma Signaling	0.447213595	-0.447213595
Ovarian Cancer Signaling	0.447213595	-0.447213595
Calcium-induced T Lymphocyte Apoptosis	0.447213595	-0.447213595
Regulation of Cellular Mechanics by Calpain Protease	0.447213595	-0.447213595
CCR5 Signaling in Macrophages	1.341640786	0.447213595
Non-Small Cell Lung Cancer Signaling	-0.447213595	-1.341640786
Osteoarthritis Pathway	0.654653671	-0.21821789
Paxillin Signaling	-1.04257207	-1.876629727
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	0.816496581	0
Thrombopoietin Signaling	0.816496581	0
GM-CSF Signaling	0.816496581	0

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
Estrogen-Dependent Breast Cancer Signaling	0.816496581	0
ErbB2-ErbB3 Signaling	0	-0.816496581
IL-9 Signaling	0	-0.816496581
Antiproliferative Role of Somatostatin Receptor 2	0	-0.816496581
Nitric Oxide Signaling in the Cardiovascular System	0	-0.816496581
Androgen Signaling	1.632993162	0.816496581
Lymphotoxin beta Receptor Signaling	-0.816496581	-1.632993162
CDK5 Signaling	-0.816496581	-1.632993162
Fc gamma RIIB Signaling in B Lymphocytes	-0.816496581	-1.632993162
Renal Cell Carcinoma Signaling	-0.816496581	-1.632993162
G alpha s Signaling	2.449489743	1.632993162
IL-8 Signaling	0.39223227	-0.39223227
ErbB4 Signaling	0.377964473	-0.377964473
EGF Signaling	0.377964473	-0.377964473
Amyotrophic Lateral Sclerosis Signaling	-0.377964473	-1.133893419
IL-2 Signaling	-0.377964473	-1.133893419
Neurotrophin/TRK Signaling	-0.377964473	-1.133893419
Chemokine Signaling	1.889822365	1.133893419
Endometrial Cancer Signaling	-1.133893419	-1.889822365
LPS-stimulated MAPK Signaling	-1.133893419	-1.889822365
Ephrin B Signaling	-1.154700538	-1.897366596
PAK Signaling	-1.147078669	-1.885618083
GDNF Family Ligand-Receptor Interactions	-1.414213562	-2.121320344

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
Acute Myeloid Leukemia Signaling	0.707106781	0
Acute Phase Response Signaling	0.707106781	0
Growth Hormone Signaling	0	-0.707106781
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	-0.707106781	-1.414213562
D-myo-inositol-5-phosphate Metabolism	-0.707106781	-1.414213562
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	-0.707106781	-1.414213562
Macropinocytosis Signaling	-0.333333333	-1
Prolactin Signaling	0.333333333	-0.333333333
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.666666667	1
3-phosphoinositide Degradation	-1	-1.666666667
Neuregulin Signaling	1.264911064	0.632455532
IL-6 Signaling	0.632455532	0
Insulin Receptor Signaling	0	-0.632455532
IL-7 Signaling Pathway	-0.301511345	-0.904534034
Renin-Angiotensin Signaling	0.301511345	-0.301511345
IL-3 Signaling	0.577350269	0
HGF Signaling	-0.577350269	-1.154700538
Cdc42 Signaling	-1.154700538	-1.732050808
Integrin Signaling	-0.420084025	-0.980196059
Rac Signaling	-1.069044968	-1.603567451
G alpha 12/13 Signaling	-1.603567451	-2.138089935
HMGB1 Signaling	0.774596669	0.25819889
PKC theta Signaling in T Lymphocytes	-1.290994449	-1.807392228

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
VEGF Signaling	-0.774596669	-1.290994449
CXCR4 Signaling	-0.242535625	-0.727606875
3-phosphoinositide Biosynthesis	-0.471404521	-0.942809042
Protein Kinase A Signaling	1.147078669	0.688247202
GP6 Signaling Pathway	-0.688247202	-1.147078669
Superpathway of Inositol Phosphate Compounds	-0.894427191	-1.341640786
B Cell Receptor Signaling	-1.705605731	-2.132007164
NF-ÎºB Signaling	0	-0.25819889
TREM1 Signaling	2.333333333	2.333333333
Role of IL-17F in Allergic Inflammatory Airway Diseases	2	2
Cholecystokinin/Gastrin-mediated Signaling	1.386750491	1.386750491
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.341640786	1.341640786
Neuroinflammation Signaling Pathway	0.962250449	0.962250449
Interferon Signaling	0.816496581	0.816496581
TGF-Î² Signaling	0.816496581	0.816496581
Aryl Hydrocarbon Receptor Signaling	0.707106781	0.707106781
JAK/Stat Signaling	0.707106781	0.707106781
Th1 Pathway	0.534522484	0.534522484
Wnt/Ca+ pathway	0.447213595	0.447213595
Retinoic acid Mediated Apoptosis Signaling	0.447213595	0.447213595
ERK/MAPK Signaling	0.40824829	0.40824829
Telomerase Signaling	0	0
FLT3 Signaling in Hematopoietic Progenitor Cells	0	0

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	DM
FGF Signaling	0	0
Agrin Interactions at Neuromuscular Junction	-0.242535625	-0.242535625
Induction of Apoptosis by HIV1	-0.447213595	-0.447213595
Huntington's Disease Signaling	-0.632455532	-0.632455532
Inhibition of Angiogenesis by TSP1	-1	-1
TNFR1 Signaling	-1	-1
IGF-1 Signaling	-1.133893419	-1.133893419
Apoptosis Signaling	-1.133893419	-1.133893419
Regulation of Actin-based Motility by Rho	-1.212678125	-1.212678125
PCP pathway	-1.341640786	-1.341640786
Fc Epsilon RI Signaling	-1.386750491	-1.386750491
PEDF Signaling	-1.414213562	-1.414213562
Actin Cytoskeleton Signaling	-1.616447718	-1.616447718
SAPK/JNK Signaling	-1.666666667	-1.666666667
PPAR Signaling	-1.889822365	-1.889822365
RhoA Signaling	-2.309401077	-2.309401077
FcÎ³ Receptor-mediated Phagocytosis in Macrophages and Monocytes	-1.876629727	-1.459600898
PTEN Signaling	0.471404521	0.942809042
Th2 Pathway	0	0.534522484
GNRH Signaling	-0.333333333	0.333333333
STAT3 Pathway	0.333333333	1
Death Receptor Signaling	-1.414213562	-0.707106781
Aldosterone Signaling in Epithelial Cells	-0.707106781	0

Table S1: Activation z-scores of Canonical Pathways elicited by WT and DM infection continued... All canonical pathways differentially regulated by HBHA and MTP during infection of THP-1 differentiated macrophages was elucidated by IPA. Table displays activation z-score for each pathway.

Canonical Pathway	Activation z-score	
	WT	**DM**
14-3-3-mediated Signaling	-0.707106781	0
Type I Diabetes Mellitus Signaling	-0.377964473	0.377964473
RhoGDI Signaling	0.39223227	1.176696811
Notch Signaling	0	0.816496581
p38 MAPK Signaling	0	0.816496581
Role of p14/p19ARF in Tumor Suppression	1	2
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	-2	-1
p53 Signaling	0.707106781	2.121320344
Remodeling of Epithelial Adherens Junctions	-0.816496581	1.632993162

Chapter 2 revealed that MTP played a more significant role in adhesion and invasion of macrophages than HBHA**.** HBHA and MTP together induce transcriptional regulation that enhances adhesion, invasion of macrophages and host immune recognition during early infection of macrophages. Chapter 3 sought to elucidate further the role of HBHA and MTP in host immune response regulation by interrogating the impact of HBHA and MTP combined deletion on cytokine profile of macrophages.

CHAPTER 3: *Mycobacterium tuberculosis* heparin-binding haemagglutinin adhesin and curli pili modulate cytokine responses through inflammasome, NF-κB, toll-like receptor, MAPK and PI3-K/AKT pathways in macrophages.

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Running title: HBHA and MTP induce a predominately pro-inflammatory response and activation of intracellular canonical pathways in macrophages.

Keywords: Cytokines; Pro-inflammatory; HBHA; MTP; *Mycobacterium tuberculosis*; macrophages; transcription.

Abstract

Macrophages are known to be critical effector cells in both the protective immune response to, and the survival of *Mycobacterium tuberculosis*. Mycobacterial antigens have previously been shown to moderate the macrophage response by inducing pro-inflammatory cytokine secretion. Heparin-binding haemagglutinin adhesin (HBHA) has previously been shown to induce pro-inflammatory and chemokine production whilst and *M. tuberculosis* curli pili (MTP) does not largely influence cytokine response elicited in epithelial cell model and THP-1 macrophages. RNA sequencing was used to explore global differential transcriptome regulation induced by HBHA and MTP in macrophages at 4 h post-infection and cytokine proteins secretion was quantified at 24, 48 and 72 h post-infection using the Bio-Plex Pro Human Cytokine Multi-Plex Panel (Bio-Rad). RNA sequencing analysis showed that the *M. tuberculosis* adhesins, HBHA and MTP, elicited differential transcriptional regulation in macrophages, and demonstrated that predicted upstream regulators were associated with cytokine production. Further investigation of canonical pathways associated with these upstream regulators and cytokine quantification revealed that HBHA and MTP activate NF-κB, toll-like receptor, p38 MAPK and PI3-K/AKT canonical signalling pathways that result in the longitudinal enhancement of a pro-inflammatory response during *M. tuberculosis* infection of macrophages. HBHA and MTP elicited greater production of IL-4 and IL-10 at 24 h; G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 48 h and G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 72 h respectively, compared to DM infection. IL-1β, IL-2, IL-6, IL-12(p70), IL-17, TNF-α, IFN-γ, colony-stimulating factors G-CSF, GM-CSF and chemokines MCP-1 and MIP-1β were produced in higher concentrations by *M. tuberculosis* infection than anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13. Therefore, HBHA and MTP activate intracellular signalling pathways that result in the longitudinal enhancement of a pro-inflammatory response during *M. tuberculosis* infection of macrophages. These findings highlight the combined immunogenic ability of HBHA and MTP to induce a protective immune response, and suggest that these antigens could possibly be a novel combination to be considered for the design of a new vaccine.

Introduction

Tuberculosis (TB) resulted in more deaths than any single infectious agent worldwide in 2016 (WHO, 2017). Most TB mortality cases could be prevented by early detection and treatment but diagnosis and intervention gaps still exist despite constant evolution in TB research and development. Drug resistance and HIV coinfection are threats to TB control, highlighting the urgent need for host- and pathogen-specific biomarkers for use in improved diagnostic and therapeutic interventions. More studies on *Mycobacterium tuberculosis* pathogenicity would enable further understanding of the host and pathogen interaction, leading to the identification of biomarkers that are essential to the development of alternate therapeutic interventions.

M. tuberculosis pathogenesis is dependent on attachment and entrance into host cell types including macrophages, to facilitate intracellular replication and survival (Kline *et al*., 2009). Cytokines play an imperative role in defining the outcome of intracellular pathogen infections (Cooper and Khader, 2008). These cytokines are involved in both the innate and adaptive arms of the immune response to mycobacterial infection. Phagocytic cells recognise *M. tuberculosis* leading to cytokine production and cell activation which are crucial for the inflammatory process (Van Crevel *et al*., 2002). Macrophages are known to be important effector cells in both the protective immune response to and the survival of *M. tuberculosis*. Alveolar macrophages, together with dendritic cells, drive the induction of the cytokine and chemokine response to the stimulus of *M. tuberculosis* phagocytosis. These cytokines/chemokines activate anti-microbial responses and recruitment of leukocytes, culminating in the formation of the granuloma (Guirado *et al*., 2013). Mycobacterial antigens have previously been shown to moderate the macrophage response by inducing pro-inflammatory cytokine secretion. The interactions between *M. tuberculosis* antigens and host immune response signalling pathways and receptors are essential for protective host immune response initiation during *M. tuberculosis* infection (Jo *et al*., 2007). Various *M. tuberculosis* cell wall components are known to be ligands for Toll-like receptors such as TLR2, thereby inducing pro-inflammatory cytokines, and activating MAPK and NF-κB signalling (Brightbill and Modlin, 1999; Jo *et al*., 2007). The MAPK signalling pathway is essential for production of immune response and anti-mycobacterial mediators like pro-inflammatory cytokine, Tumour necrosis factor alpha (TNF-α) (Jo *et al*., 2007). Pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 are up-regulated in macrophages and monocytes during early infection in patients with active pulmonary TB (Lee *et al*., 2003; Jung *et al*., 2006;). TB patients also displayed enhanced activation of p38 mitogen-activated protein kinases (p38 MAPK), extracellular signal–regulated kinases 1/2 and pro-inflammatory cytokines in neutrophils or monocytes compared to the control population (Alemán *et al*., 2004; Jung *et al*., 2006; Yang *et al*., 2008).

Heparin-binding haemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) are *M. tuberculosis* antigens that are critical for adherence to host cells. HBHA has been well studied for vaccine development (Parra *et al*., 2004; Kohama *et al*., 2008; Guerrero and Locht, 2011; Stylianou *et al*., 2014; Verwaerde *et al*., 2014; Fukui *et al*., 2015; Teimourpour *et al*., 2017). However as a biomarker (Menozzi *et al*., 1996; Masungi *et al*., 2002; Zanetti *et al*., 2005; Hougardy *et al*., 2007; Savolainen *et al*., 2008; Hutchinson *et al*., 2015; Smits *et al*., 2015), HBHA has poor ability to differentiate between pulmonary and extra-pulmonary TB (Sun *et al*., 2011a) highlighting the requirement for further biomarker discovery. Kim *et al*., 2011 determined HBHA's role in the inducing pro-inflammatory cytokines, TNF-α and IL-6 was mediated by Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K) and mitogen-activated protein kinases (MAPK) signalling pathways in bone marrow derived macrophages (Kim *et al*., 2011). HBHA was recently shown to modulate host-pathogen interactions and immune response by inducing differential gene regulation in a mouse infection model (Kuvar, 2016).

MTP induced stimulation of the host humoral immune response by producing IgG antibodies against MTP (Alteri *et al*., 2007). MTP has been identified as an adhesin for macrophages (Ramsugit and Pillay, 2014) and epithelial cells (Ramsugit *et al*., 2016). MTP also displayed a limited role in cytokine production during infection of epithelial cells (Ramsugit *et al*., 2016). The conservation of the gene sequence encoding MTP in different clinical strains of *M. tuberculosis* alluded to MTP's potential as a biomarker for TB (Naidoo *et al*., 2014). Recently, the use of a synthetic MTP peptide in a slot blot assay displayed a 97% accuracy for the detection of anti-MTP antibodies in patients' serum samples, suggesting that MTP may be useful as a biomarker in a point of care diagnostic test (Naidoo *et al*., 2018). In addition, the immunomodulatory ability of MTP in a mouse model (Nyawo, 2016) and in an epithelial cell model (Dlamini, 2016) warrant further investigation of MTP as a biomarker. MTP has not yet been evaluated as a target for vaccine development to date. *M. tuberculosis* possesses multiple adhesins that facilitate the host-pathogen interaction (Pasula *et al*., 1997; Neyrolles *et al*., 2001; Pethe *et al*., 2001; Stokes *et al*., 2004; Kinhikar *et al*., 2006; Kumar *et al*., 2013) and therefore, more than one target adhesin should be included in the design for a vaccination/therapeutic strategy to be effective.

Although numerous studies on *M. tuberculosis* antigens have enhanced the wealth of knowledge on its pathogenesis, the combined effect of HBHA and MTP has to our knowledge, not been previously explored. It was hypothesised that the loss of these 2 major adhesins would dampen the ability of *M. tuberculosis* to successfully induce the host immune responses, thereby, providing evidence that HBHA and MTP may present a previously unexplored vaccine combination. Our findings demonstrated that HBHA and MTP induce a predominately pro-inflammatory response, and influence the activation of NF-κB, toll-like receptor, p38 MAPK and PI3-K/AKT canonical signalling pathways during *M. tuberculosis* infection of macrophages.

Materials and methods

Bacterial isolates and infection of differentiated THP-1 macrophages

THP-1 monocytic cells (ATCC TIB-202) were propagated using RPMI-1640 (Lonza, Whitehead Scientific, South Africa) and 10% foetal bovine serum (Biowest, Celtic Molecular Diagnostics, South Africa) and incubated at 37°C in the presence of 5% CO_2 (Shel lab CO_2 incubator, Polychem, South Africa). Enumeration of THP-1 cells was carried out by a cell count of a dilution of actively growing cells using the trypan blue exclusion assay and a haemocytometer. Cells were resuspended in media containing phorbol 12-myristate 13-acetate (50 ng/mL) (Sigma, Capital lab supplies, South Africa) at a density of 5 x 10^5 cells/ mL. Differentiation was carried out overnight at 37°C in the presence of 5% CO_2 (Shel lab CO_2 incubator, Polychem, South Africa).

THP-1 differentiated macrophages were infected with HBHA and MTP proficient Wild-type V9124 strain (WT) and HBHA and MTP deficient double mutant Δ*Rv0475-Rv3312A* (*hbhA-mtp)* (DM) (Govender, *et al*, unpublished). A single Δ*hbhA* mutant was unmarked using resolvase-carrying phage phAE280 and DM generated by phage-based mycobacterial specialized transduction. The *mtp* gene sequence was replaced by an allelic exchange substrate containing hygromycin-resistance-*sac*B cassette. Putative double mutants were screened on 7H11 agar plates (Difco, Becton, Dickinson and Company, South Africa) containing 75 µg/mL hygromycin (Roche Applied Science, Capital lab supplies, South Africa). Confirmation PCRs for both *hbhA* and *mtp* were carried out using the following primers repectively: *hbhA* LL primer: 5'-GGCCGAAGTCCTTTATGT-3' and Uptag primer 5'-VGATGTCTCACTGAGGTCTCT-3' and *mtp* LL primer: 5'-ATTCGAGTACATGCGTGAT-3' and uptag primer: 5'-GATGTCTCACTGAGGTCTCT-3'. This generated PCR products of 770 and 700 base pairs respectively.

Bacterial strains were grown as previously described (Chapter 2, pages 63-64) were pelleted by centrifugation (Heraeus multifuge 3S-R, Thermofisher Scientific, South Africa) at 4000 x *g* for 10 mins and reconstituted in RPMI-1640 (Lonza, Whitehead Scientific, South Africa) and 10% foetal bovine serum (Biowest, Celtic Molecular Diagnostics, South Africa). THP-1 differentiated macrophages were infected at a multiplicity of infection (MOI) of ~5:1 for 4 h, following which, monolayers were washed thrice with phosphate buffered saline (PBS) (Oxoid, Quantum Biotechnologies, South Africa). Colony forming units (CFU)/mL were enumerated on the infecting inoculum to confirm the MOI and the bacillary burden at each sampling interval. Infected macrophages were lysed using 0.1% Triton X-100 (Sigma, Capital lab supplies, South Africa) for 20 mins, after which lysates were serially diluted in PBS (Oxoid, Quantum Biotechnologies, South Africa) containing 0.05% (v/v) Tween 80 (Sigma, Capital lab supplies, South Africa) and plated onto Middlebrook 7H11 medium (Difco, Becton,

Dickinson and Company, South Africa) containing 10% oleic acid-albumin-dextrose-catalase and 0.5% glycerol and incubated at 37°C (Shel lab CO_2 incubator, Polychem, South Africa) for 3 weeks.

RNA sequencing

Library preparation and RNA sequencing

Total RNA extraction at 4 h post-infection using an RNeasy kit (Qiagen, Whitehead Scientific, South Africa), library preparation using TruSeq Stranded Total RNA Library Prep Kit (Illumina, Whitehead Scientific, South Africa) and RNA sequencing on an Hiseq 2500 Ilumina platform (Whitehead Scientific, South Africa) were performed as previously described (Chapter 2, page 65-66). Sequencing reads trimmed by Trimmomatic (Bolger *et al*., 2014) were mapped to the Hg38 (UCSC) using TopHat (2.1.0) and Bowtie2. The Cuffdiff package (version cufflinks-2.2.1) was used to elucidate differential gene expression patterns (Trapnell *et al*., 2012). Significantly differentially expressed genes (SDEGs) with a p value <0.05 were used for further enrichment and functional analysis. Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA) upstream regulator and canonical pathway enrichment analysis was used to further investigate the predicted cytokine response.

Cytokine assay

Macrophage cytokine/chemokine production was quantified at 24, 48 and 72 h post-infection. Bovine serum albumin (0.5% (w/v) (Sigma, Capital lab supplies, South Africa) was added to cell culture supernatants that were filtered through a 0.2 µM filter (PALL Life Sciences, United scientific, South Africa) and stored at -80°C until further use. The Bio-Plex Pro Human Cytokine Multi-Plex Panel(Bio-Rad) was analysed in a Bio-Plex 200 System (Bio-Rad, South Africa) as per the manufacturer's instructions to determine the concentrations of the following 17 analytes: Granulocyte colony-stimulating factor (G-CSF), Granulocyte macrophage colony-stimulating factor (GM-CSF), Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, Interferon-gamma (IFN-γ), Monocyte chemotactic protein (MCP-1), Macrophage inflammatory protein (MIP-β) and Tumour necrosis factor alpha (TNF-α). Uninfected cells were used as a control to measure the cytokine production due to differentiation of the THP-1 monocytes to macrophages.

Statistical analysis

The cytokine assay data are displayed as means ± SEM of duplicate experiments performed independently in triplicate. One-way analysis of variance (ANOVA) (SPSS, IBM 22) was carried out at each time interval to compare the difference in cytokine concentrations elicited by WT and DM relative to the uninfected macrophages. IPA software predicted activation scores (z-score) are a statistical quantification of the correlation of the observed gene expression and expected relationship direction.

Results and Discussion

Successful control of *M. tuberculosis* infection requires efficient interactions between macrophages and T lymphocytes and this interaction is facilitated by cytokines. MTP has previously been shown to only minimally influence cytokine response elicited in epithelial cell model (Ramsugit *et al*., 2016) and THP-1 macrophages (unpublished data). However, *Escherichia coli* K12 curli pili (Bian et al., 2000) and *Salmonella enterica* serotype Typhimurium Fimbriae (Tükel *et al*., 2005) has been shown to induce a pro-inflammatory cytokine response in human macrophages (THP-1 cells). In contrast, HBHA was previously described to induce pro-inflammatory cytokine secretion (Kim *et al*., 2011) and chemokines (unpublished data) in macrophages, robust cytokine signatures during stimulation of $CD4^+$ T cells of household contacts (Loxton *et al*., 2012) and infants (Smith *et al*., 2012).

In this study, RNA sequencing was used to explore global differential transcriptome regulation induced by HBHA and MTP in macrophages at 4 h post-infection. This revealed that the top 5 predicted upstream regulators were associated with cytokine production (Fig. 3.1), prompting investigation of canonical pathways associated with these upstream regulators and cytokine quantification. Since the predicted transcriptional effects at 4 h was hypothesised to increase longitudinally, and post-translational secretion surmised to be limited at 4 h, the time intervals for cytokine quantification in the study were based on previous studies (Wagner *et al*., 2002; Chen *et al*., 2013; Ramsugit *et al*., 2016; Mvubu *et al*., 2018).

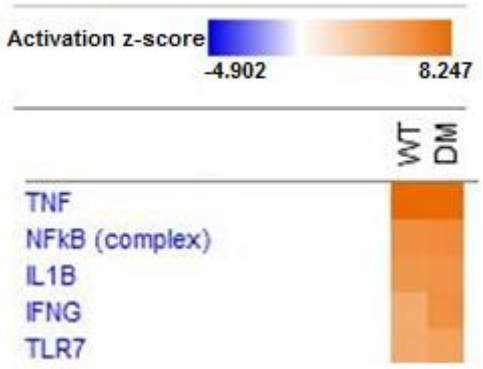


Fig. 3.1: Top 5 upstream regulators. THP-1 differentiated macrophages were infected with WT and DM. RNA extracted at 4 h post-infection was sequenced on an Ilumina platform and upstream regulator enrichment analysis was carried out using IPA. All top 5 predicted upstream regulators are associated with cytokine production. TNF-α was the most highly activated upstream regulator by both WT and DM infection at 4 h post-infection. WT represents HBHA and MTP proficient strain and DM represents HBHA and MTP deficient strain.

HBHA and MTP contribute to intracellular signalling processes through differential regulation of NF-κB signalling canonical pathway.

NF-κB is a transcriptional regulator that is essential in cell processes such as proliferation, differentiation, inflammation, immunity, and survival (Oeckinghaus and Ghosh, 2009). It's role in the inflammatory process is due to the induction of pro-inflammatory mediators central to innate immunity (Liu *et al*., 2017). HBHA induced pro-inflammatory cytokine secretion in bone marrow derived macrophages was previously shown to be dependent on NF-κB activation (Kim *et al*., 2011). In the current study, transcriptomic analysis revealed that NF-κB was among the top 5 elicited upstream regulators by both WT and DM infections (Fig. 3.1) and the activation was similar in both infections. NF-κB activation is stimulated by either canonical or non-canonical pathways, therefore, these signalling pathways are crucial for regulation of inflammation and immune responses (Beinke and Ley, 2004; Sun, 2010b).

The differential regulation of WT and DM infection on the NF-κB signalling canonical pathway was subsequently investigated (Fig. 3.2). WT infection uniquely displayed up-regulated expression of *TANK* and *MRAS* transcripts and down-regulated expression of *TNFRSF11A* transcript (Fig. 3.2.A) whilst DM infection uniquely displayed up-regulated expression NFKBIE transcript and down-regulated expression of *TGFBR2, PRKACB, MAP3K1* and *PIK3C2B* transcripts (Fig. 3.2.B). Overall, these activation differences led to WT displaying enhanced activation for the NF-κB signalling canonical pathway (z-score= 1.89) compared to DM infection (z-score= 0.66). This suggested that HBHA and MTP contribute to mediation of proliferation, differentiation, inflammation, immunity, and survival through differential regulation of the NF-κB signalling canonical pathway.

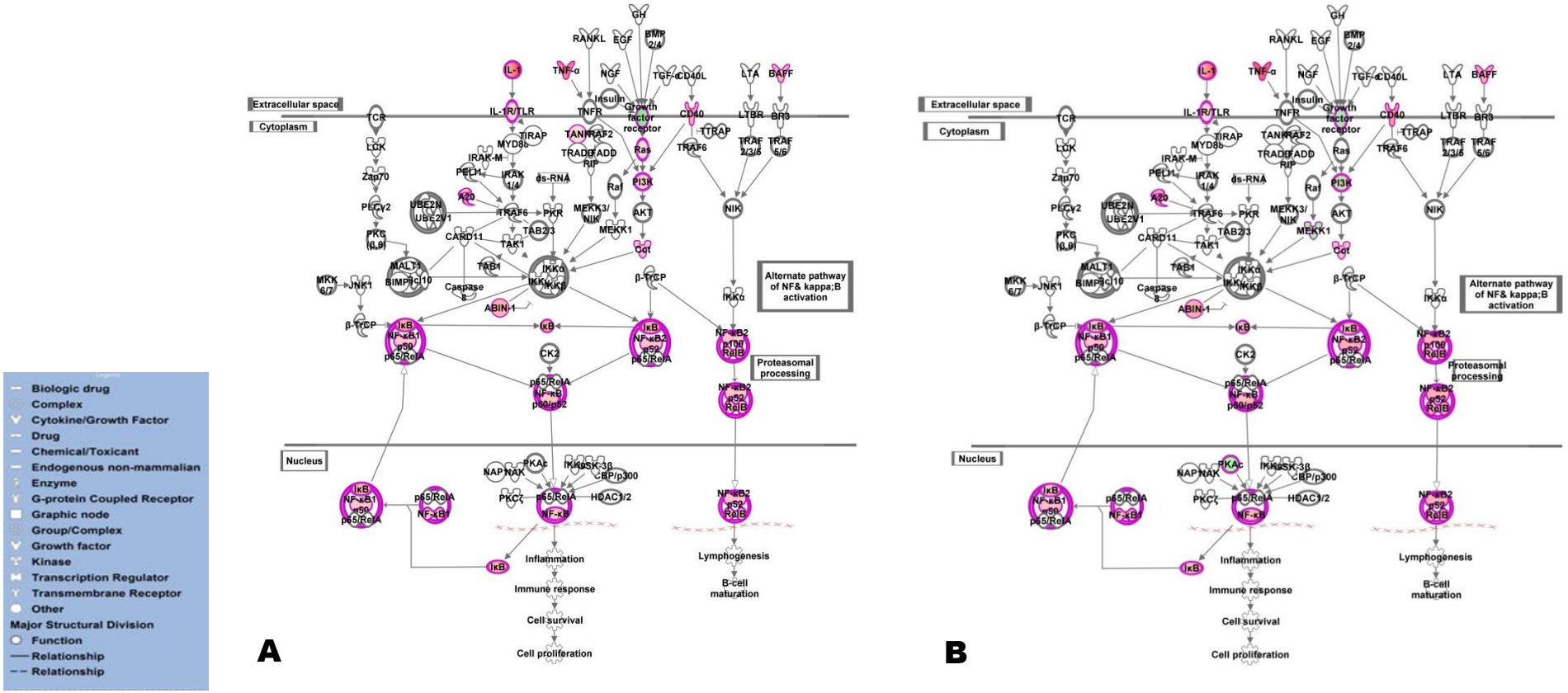


Fig. 3.2: NF-κB signalling. NF-κB signalling pathway elicited by HBHA and MTP proficient (A) and deficient strains (B). Mammalian RNA was extracted at 4 h, sequenced and analysed using tuxedo suite software and IPA canonical pathway enrichment analysis. WT infection displayed increased activation for the NF-κB signalling canonical pathway (z-score= 1.89) compared to DM infection (z-score= 0.66).

HBHA and MTP enhanced activation of Toll-like Receptor signalling canonical pathway in macrophages.

Toll-like receptors (TLR) are involved in intracellular signalling and recruitment of adapter proteins activating pro-inflammatory cytokine production and phagosome maturation (Natarajan *et al*., 2011). TLR7 was amongst the top upstream regulators elicited in this study. DM infection displayed a slightly enhanced predicted expression (z-score = 5.14) compared to WT infection (z-score = 4.68) (Fig. 3.1). TLR7 is expressed within intracellular vesicles and in macrophages, demonstrated induction of inflammatory responses by activation of NF-κB through MyD88 signalling without activation of IRF (Kawai and Akira, 2011).

In the current study, all TLRs were activated except TLR6 and TLR10, and DM infection displayed slight enhanced activation compared to WT infection for all TLRs except TLR5 which was the same in both infections (Fig. 3.3A). The TLR signalling canonical pathway was more activated by WT infection (z-score = 2.12) than DM infection (z-score = 1.89) (Fig. 3.3B and C). DM infection uniquely up-regulated *TICAM1/TRIF* and down-regulated *MAP3K1/MEKK1* transcripts. WT infection showed enhanced expression of *IL1A* transcripts compared to DM infection.

Various *M. tuberculosis* cell wall components require TLR2 activation to stimulate TNF-α in macrophages, suggestive that TLR2 facilitates pro-inflammatory cytokine production, and therefore, is essential for the protective immune response against *M. tuberculosis* infection (Underhill *et al*., 1999). *M. tuberculosis* DNA was able to induce pro-inflammatory cytokines using a TLR9 dependent pathway in macrophages (Bafica *et al*., 2005). Jung *et al*., (2011) showed evidence that methylated-recombinant HBHA expressed in *Mycobacterium smegmatis* activated TLR4-mediated MyD88 and TRIF signalling, resulting in production of pro-inflammatory cytokines by dendritic cells. *S. Typhimurium* and *E. coli* curli induced IL-1β production was dependent on TLR2 and NLRP3 in bone marrow-derived macrophages isolated from mice (Rapsinski *et al*., 2015).

In the present study, HBHA and MTP deficiency showed minimal effect on predicted expression of TLRs, however, the observed trend of slightly increased activation suggests that the absence of HBHA and MTP could possibly increase ligands for TLR4, TLR7 and TLR9 signalling. Analysis of the toll-like receptor signalling pathway that involves TLR2, revealed that HBHA and MTP together enhance the activation of this pathway and possibly could induce pro-inflammatory cytokine secretion during *M. tuberculosis* infection.

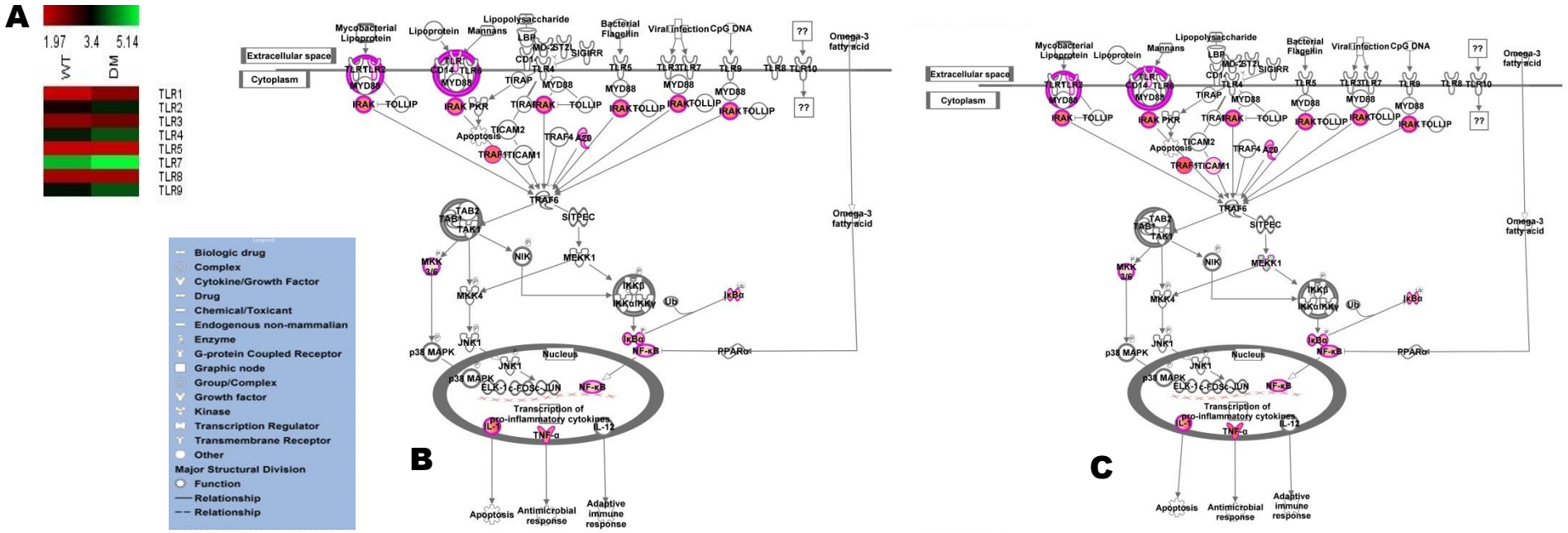


Fig. 3.3: Toll-like receptor signalling. Toll-like receptors elicited during WT and DM infection (A) and Toll-like receptor signalling pathway elicited by HBHA and MTP proficient (B) and deficient strains (C). Mammalian RNA was extracted at 4 h, sequenced and analysed using tuxedo suite software and IPA canonical pathway enrichment analysis. All TLRs were activated except TLR6 and 10 and DM infection displayed slight enhanced activation compared to WT infection for all TLRs except TLR5 which was the same in both infections (Fig 3.A). The TLR signalling canonical pathway was more activated by WT infection (z-score = 2.12) than DM infection (z-score = 1.89) (Fig. 3.B and C). WT represents HBHA and MTP proficient and DM represents HBHA and MTP deficient.

HBHA and MTP induce a predominately pro-inflammatory than anti-inflammatory response during macrophage infection.

Of the 17 analytes tested by the multiplex platform analysis in this study, Interleukin (IL)-7 was undetected and IL-8 was unmeasurable due to high concentrations that were out of range for WT, DM and uninfected samples (Data not shown). Pro-inflammatory cytokines, IL-1β, IL-2, IL-6, IL-12(p70), IL-17, TNF-α, IFN-γ, colony-stimulating factors G-CSF, GM-CSF and chemokines MCP-1 and MIP-1β (Table 3.1) were produced in higher concentrations than anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13 by *M. tuberculosis* infection (Table 3.2). Host resistance to *M. tuberculosis* infections relies on the production of pro-inflammatory cytokines, which have previously been described to be secreted by macrophages (Giacomini *et al*., 2001). The *M. tuberculosis* host immune response survival strategies are believed to include the induction of anti-inflammatory cytokines which antagonise the pro-inflammatory response initiated by *M. tuberculosis* infection (Van Crevel *et al*., 2002). Increased pro-inflammatory response results in tissue damage, in contrast to increased anti-inflammatory responses that favour the growth of *M. tuberculosis* (Van Crevel *et al*., 2002). HBHA (Kim *et al*., 2011), *E. coli* and *S. Typhimurium* curli pili (Bian *et al*., 2000; Tükel *et al*., 2005) elicits pro-inflammatory cytokine secretion from macrophage cell lines. The current study observations are in line with the above-mentioned previous studies, and suggests that HBHA and MTP together may contribute to TB immunopathology.

At 24 h and 48 h, MIP-1β displayed the highest concentration for both strains and uninfected cells whereas, at 72 h WT, DM infection and uninfected elicited the highest concentration of IL-1β and MIP-1β respectively (Table 3.1 and 3.2). WT elicited higher levels of IL-4 and IL-10 at 24 h; G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 48 h and G-CSF, GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN-γ and TNF-α at 72 h respectively, compared to DM infection. These findings are consistent with previous studies that demonstrated that monocyte derived macrophages produced IL-1, IL-6, IL-8, IL-10, IL-12(p40), IFN-γ, and TNF-α during *M. tuberculosis* infection (Giacomini *et al*., 2001; Sharma *et al*., 2003). However, this study investigated the levels of specific cytokines using ELISA (Giacomini *et al*., 2001). A separate study demonstrated that *M. tuberculosis* infected macrophages produced IL-6, IL-10 and G-CSF that played a role in the induction of Macrophage arginase 1, which is involved in the inhibition of nitric oxide production (Qualls *et al*., 2010).

Multiple studies have described mycobacterial antigens that induce a pro-inflammatory response, including the 38-kDa glycolipoprotein (Jung *et al*., 2006), 19 kDa Lipoprotein (Rao *et al*., 2005), FMN binding nitroreductase domain containing protein (Peddireddy *et al*., 2016) and macrophage-activating

protein, *Rv2882c* (Choi *et al*., 2016) and HBHA (Kim *et al*., 2011). Previous unpublished studies by our research group elucidated the macrophage cytokine profiles elicited by HBHA and MTP single mutants. These demonstrated that HBHA was mostly involved with the induction of chemokines, IL-8, MIP-1 and MCP-1, whilst MTP did not influence cytokine production. MTP induced similar quantities of IL-1β, IL-4, IL-6, IL-8, G-CSF, IFN-γ, MCP-1 and TNF-α at 24 h, 48 h and 72 h post-infection in the epithelial cell model. It was noted that MTP induced significantly higher concentration of TNF-α at 48 h and MTP-deficient mutant induced higher levels of IL-8 at 72 h post-infection (Ramsugit *et al*., 2016). This suggests that the cytokine profile demonstrated by the HBHA and MTP deficient strain may not be attributable to either HBHA or MTP individually, but rather the simultaneous deletion of HBHA and MTP.

Table 3.1: Colony stimulating factors, pro-inflammatory and chemokine concentrations (pg/mL) detected in supernatants 24, 42 and 72 h post-infection. THP-1 differentiated macrophages were infected with WT or DM for 4 h. Cytokine levels were measured at 24 h, 48 h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and data is presented as ±SEM of two independent experiments performed in triplicate.

Time-point	Strains	G-CSF (pg/mL)	GM-CSF (pg/mL)	IL-1β (pg/mL)	IL-2 (pg/mL)	IL-6 (pg/mL)	IL-12 (pg/mL)	IL-17 (pg/mL)	IFN-γ (pg/mL)	MCP-1 (pg/mL)	MIP-1β (pg/mL)	TNF-α (pg/mL)
24 h	**WT**	61.35±6.42	32.54±0.93	135.62 ± 5.93	6.32±1.14	23.04±2.86	3.61±1.14	213.86±2.12	164.81±28.64	529.97±29.32	2620.47±110.81	40.58±7.70
	DM	48.73±3.70	27.88±1.18	135.58± 8.95	3.98±1.36	16.62±3.36	2.36±1.36	206.10±4.26	156.37±12.10	469.34±41.13	2795.61±128.21	38.96±3.14
	N	5.43±0.00	16.49±1.58	11.27±0.95	1.19±0.00	2.67±0.34	0.04±0.00	98.70±5.64	67.69±6.38	218.37±62.98	840.91±72.40	8.45±1.15
48 h	**WT**	338.91±94.54	58.91±2.84	857.22±22.53	12.76±1.05	312.29±92.86	7.52±1.05	251.45±4.95	397.23±19.31	574.50±27.18	2864.90±196.64	267.56±65.36
	DM	101.90±12.14	45.55±1.53	515.13±84.61	7.24±0.90	162.14±16.00	3.64±0.90	218.73±7.38	326.82±10.40	638.39±27.26	2600.71±219.28	101.36±9.94
	N	28.25±4.77	23.41±1.30	39.84±9.07	2.43±0.00	4.96±0.22	0.04±0.00	165.56±10.37	144.96±11.81	342.05±91.33	1906.33±229.78	62.18±23.55
72 h	**WT**	405.58±90.31	116.00±2.87	17855.60±3831.74	24.56±1.18	1473.72±216.88	15.07±1.18	277.86±7.11	776.49±32.00	716.14±39.76	3202.18±209.64	495.26±98.81
	DM	118.47±12.62	74.56±8.01	834.25±278.79	14.63±1.25	340.92±113.86	2.85±1.25	212.94±9.15	506.11±60.97	630.82±21.12	3202.18±430.84	184.75±13.54
	N	34.50±4.81	24.53±1.88	60.99±18.04	1.49±0.00	4.76±0.24	0.04±0.00	172.28±8.69	149.97±14.51	294.58±101.18	2160.91±462.11	59.74±25.44

Key: WT: HBHA and MTP proficient strain; DM: HBHA and MTP deficient strain and N represents the uninfected control.

Table 3.2: Anti-inflammatory concentrations (pg/mL) detected in supernatants 24, 42 and 72 h post-infection. Supernatants collected from infected THP-1 differentiated macrophages were quantified for cytokines at 24 h, 48 h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and data is presented as ±SEM of two independent experiments performed in triplicate.

Time-point	Strains	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-10 (pg/mL)	IL-13 (pg/mL)
24 h	**WT**	7.21±0.36	12.22±1.60	3.79±0.36	6.51±1.08
	DM	6.17±0.23	6.24±2.12	2.46±0.23	6.14±1.26
	N	3.23±0.13	3.98±1.58	1.13±0.13	0.93±0.52
48 h	**WT**	9.86±0.98	15.30±1.19	10.85±0.98	8.87±1.05
	DM	8.32±1.08	6.39±1.28	7.13±1.08	6.28±0.95
	N	6.01±0.21	4.09±1.55	2.17±0.21	3.96±1.94
72 h	**WT**	16.15±1.23	17.52±1.55	18.61±1.23	12.19±1.58
	DM	7.88±1.08	6.61±1.15	4.13±1.08	7.43±1.57
	N	5.58±0.48	5.61±1.45	2.14±0.48	3.60±1.65

Key: WT: HBHA and MTP proficient strain; DM: HBHA and MTP deficient strain and N represents the uninfected control

HBHA and MTP enhance IL-1β production, inflammasome and IL-1 signalling canonical pathways during infection of macrophages.

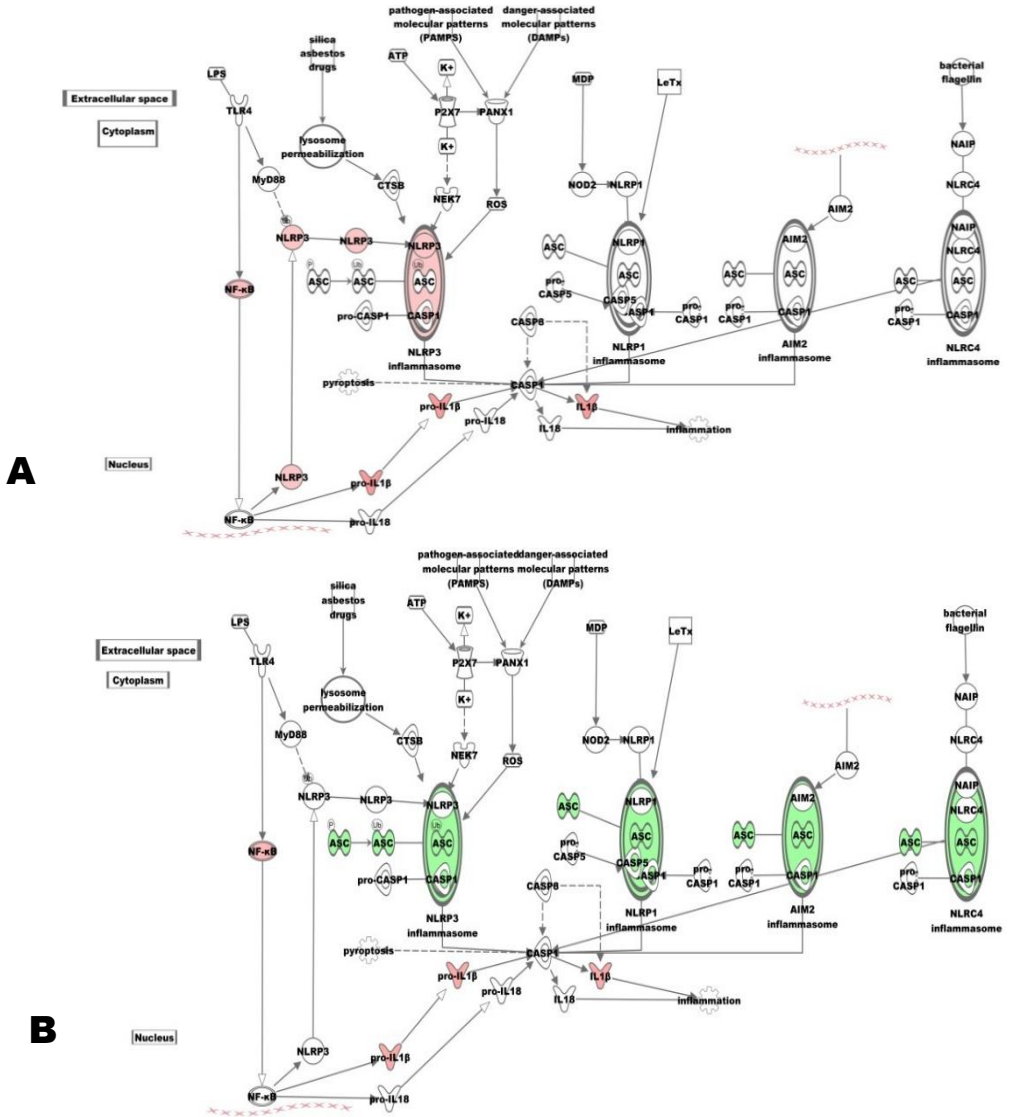
IL-1β was among the top 5 upstream regulators elicited by both infections (Fig. 3.1). Successful immune response to infection requires the production of IL-1β. The inflammasome has been identified to be involved in the production of IL-1β in macrophage and dendritic cells (Schroder and Tschopp, 2010). A recent study revealed that different *M. tuberculosis* strains produced varying levels of IL-1β, up-regulated *IL-1β* transcripts, but elicited similar levels of the immature form of IL-1β. The variation in IL-1β secretion was suggested to be not regulated by pattern-recognition receptors but possibly regulated by activation of the inflammasome and caspase-1 (Krishnan *et al*., 2013).

In the present study, differential transcript regulation of the inflammasome canonical pathway was demonstrated by WT (Fig. 3.4A) and DM (Fig. 3.4B). Nucleotide oligomerization domain (NOD)-like receptor family, pryin domain containing 3 (*NLRP3)* transcript was significantly upregulated by only WT infection, whilst apoptosis-associated speck-like protein containing a caspase recruitment domain (*ASC)* transcript was significantly downregulated by DM infection only. These regulation differences resulted a significant increase activation of pathway during WT infection (z-score = 2.236; -log (*p*-value) = 4.51) compared to DM infection (z-score = 1.342; -log (*p*-value) = 3.62). However, only a slight predicted increase of *IL-1β* transcripts were observed during WT infection compared to DM infection.

Nucleotide-binding oligomerization domain-like receptors (NLR) are receptors that facilitate the development of innate immune responses including the inflammasome (Schroder and Tschopp, 2010). NLRP3 has been identified as the sole NLR involved in induction of the inflammasome during *M. tuberculosis* infection of macrophages and dendritic cells (Carlsson *et al*., 2010; Mayer-Barber *et al*., 2010; Wong and Jacobs, 2011; Abdalla *et al*., 2012; Dorhoi *et al*., 2012). ASC functions as an adaptor during apoptotic and inflammatory signalling. ASC is integrally involved in the activation of the inflammasome by linking NLRs and caspase-1 (Sarkar *et al*., 2006). Inhibition of ASC during *Legionella* infection resulted in disruption of activation of the inflammasome (Abdelaziz *et al*., 2011). Bone marrow derived macrophages from mice deficient in ASC and caspase-1 displayed significant reduction in IL-1β production in response to *M. tuberculosis* infection (Mayer-Barber *et al*., 2010). In contrast, during in vivo infection, IL-1β concentrations were not dependent on NLRP3 or ASC and attributed to inflammasome independent IL-1β production (Mayer-Barber *et al*., 2010). Curli have been observed to contribute to production of IL-1β by caspase 1 activation as a result of activation of the NRLP3 inflammasome (Rapsinski *et al*., 2015).

The current study investigated the IL-1β cytokine concentrations elicited by WT and DM infection of macrophages. The WT generated similar concentrations of IL-1β at 24 h but enhanced at 48 ($p = 0.001$) and 72 h ($p = 0.000$) hours compared to the DM post-infection (Fig. 3.4.C). In addition, IL-1β was the most differentially elicited cytokine when both conditions were compared, suggesting that HBHA and MTP stimulate the production of IL-1β at 48 and 72 h post-infection.

The differential regulation of the IL-1 signalling canonical pathway was further interrogated in an effort to understand the implications of the increased *IL-1β* transcripts during *M. tuberculosis* infection of macrophages. The WT infection was associated with an increase in the activation (z-score = 1.89) of the IL-1 signalling canonical pathway (Fig. 3.5.A) compared to the DM infection (z-score = 0.905) (Fig. 3.5B). However, the latter infection was associated with a greater significance (-log (p-value) = 4.33) compared to WT infection of (-log (p-value) = 3.6). DM infection differentially downregulated *MEKK1 (MAP3K1)*, *PRKACB*, *GNAQ* and upregulated *NFKBIE*, whilst WT infection differentially upregulated *MRAS* (Fig. 3.5C). The pro-inflammatory cytokine IL-1 is integral in host immune response to *M. tuberculosis* infection. Mice deficient in IL-1 receptors demonstrated increased susceptibility to *M. tuberculosis* infection, as evident from the increase in mortality, bacterial burden and malformation of granulomas (Juffermans *et al*., 2000; Mayer-Barber *et al*., 2010; TeKippe *et al*., 2010). In addition, IL-1 modulated innate immune responses possibly account for the majority of MyD88-dependent host responses involved in controlling acute *M. tuberculosis* infection (Fremond *et al*., 2007). These findings collectively suggest that HBHA and MTP play a role in IL-1β production by modulation of the inflammasome canonical pathway. This results in increased IL-1 signalling, thereby activating the protective immune response to *M. tuberculosis* infection in macrophages.



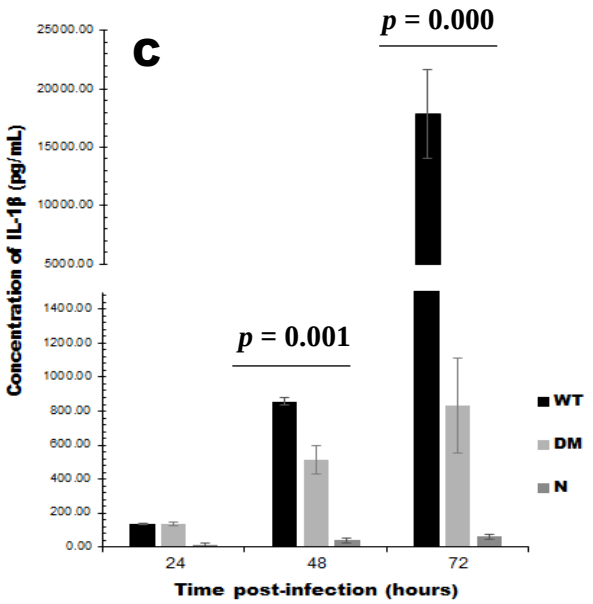


Fig. 3.4: IL-1β production in THP-1 macrophages. Inflammasome canonical pathway elicited by WT (A) and DM infection (B). Mammalian RNA was extracted at 4 h, sequenced and analysed using tuxedo suite software and IPA canonical pathway enrichment analysis. WT infection uniquely expressed *NLRP3* and deficient strain uniquely expressed *ASC*. **Concentrations of IL-1β elicited by WT and DM infection of macrophages (C).** THP-1 differentiated macrophages were infected with WT or DM for 4 h. Cytokine levels were measured at 24 h, 48 h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and data is presented as ±SEM of two independent experiments performed in triplicate. WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents uninfected control. WT infection elicited significantly more production of IL-

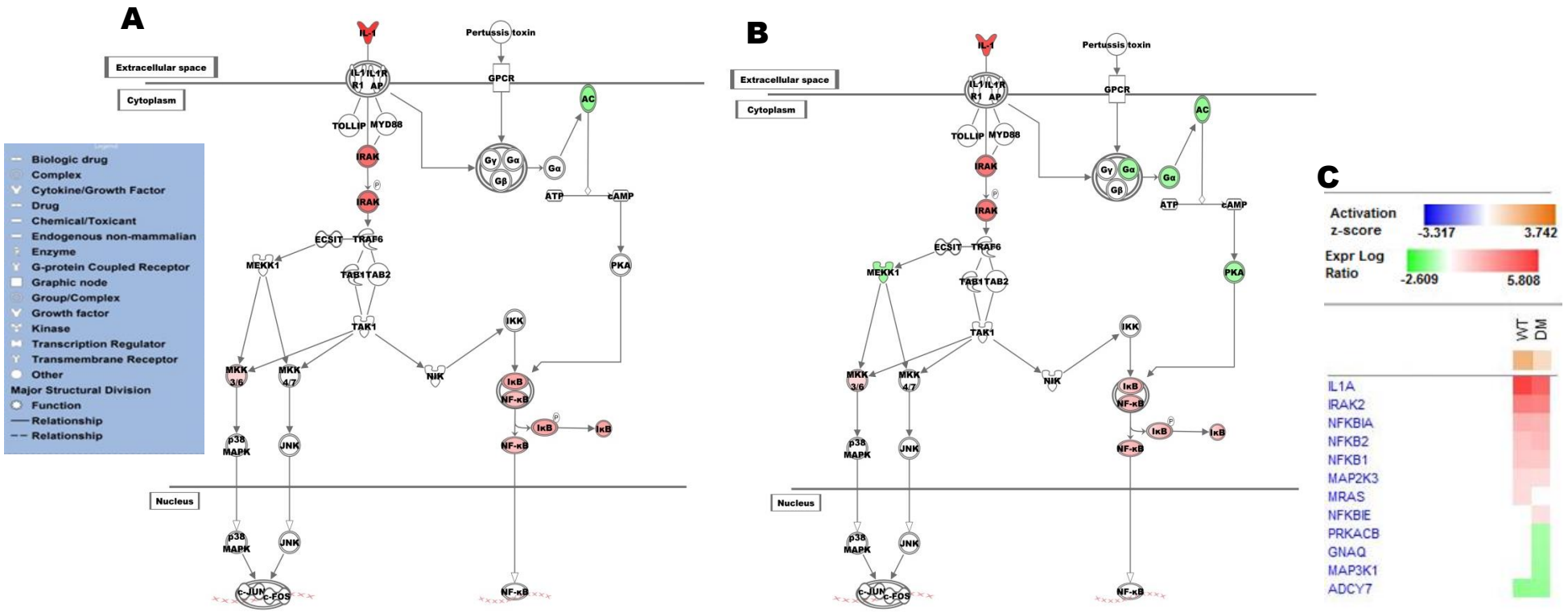


Fig. 3.5: IL-1 signalling canonical pathway elicited by HBHA and MTP proficient strain (A); HBHA and MTP deficient strain (B) infection and genes associated with pathway (C). THP-1 differentiated macrophages were infected with WT and DM. RNA was extracted at 4 h and global transcriptome regulation was investigated using RNA sequencing and IPA software canonical pathway enrichment analysis. DM infection differentially downregulated *MEKK1 (MAP3K1)*, *PRKACB*, *GNAQ* and upregulates *NFKBIE* and WT infection differentially upregulated *MRAS*. WT represents HBHA and MTP proficient strain and DM represents HBHA and MTP deficient strain.

HBHA and MTP enhance TNF-α production and differentially regulate TNF-α associated transcripts.

TNF-α has previously been shown to stimulate anti-mycobacterial activity (Sato *et al*., 2002) and also results in killing of host cells (Smith *et al*., 2002). Previously, a mycobacterial antigen (Ag85B) demonstrated enhanced induction of TNF-α in monocytes (Aung *et al*., 1996). In the current study, TNF-α was the most activated cytokine in both infections and the activation degree was similar in both absence and presence of HBHA and MTP (Fig. 3.6A). However, analysis of differential regulation of transcripts associated with TNF-α revealed that WT infection upregulated *SERPINB2, HBEGF* AND *GCLM*. DM infection upregulated *CXCL11, CD80, ICOSLG/LOC102723996, INHBA, MUC1, SERPINE1, PML, BTG2, TICAM1, DUSP5, HIVEP1, PLSCR1, NFKBIE* and downregulated *ITGA4, TGFBR2, IFNGR1* and *VCL* (Fig. 3.6A).

At 24 h, both infections generated similar concentrations of TNF-α, but WT infection generated significantly greater concentrations at 48 (p = 0.033) and 72 h (p = 0.006) post-infection (Fig. 3.6B). TNF-α is involved in host protection in the mouse model of *M. tuberculosis* infection and is required for macrophage production of reactive nitrogen during early infection (Flynn *et al*., 1995). Previously, we showed that WT infection was associated with a higher activation of the Production of Nitric Oxide and Reactive Oxygen Species in Macrophages canonical pathways than DM infection (Chapter 2, Fig. 2.7, pages 79-81). TNF-α released by human alveolar macrophages has also been implicated to facilitate intracellular bacterial growth suggesting that induction of TNF-α as a possible immune evasion mechanism (Engele *et al*., 2002).

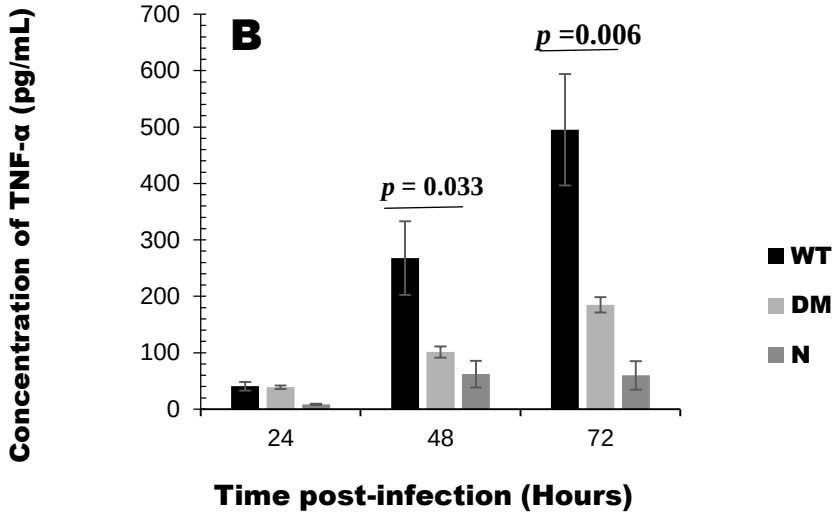


Fig. 3.6: TNF-α network and production. Genes associated with mechanistic network elicited by WT and DM infection (A).

THP-1 differentiated macrophages were infected with WT and DM. RNA was extracted at 4h and genes associated with the TNF network were investigated for differential regulation using IPA software. WT infection resulted in differential upregulation of *SERPINB2*, *HBEGF* and *GCLM*. DM infection differentially upregulated *CXCL11*, *CD80*, *ICOSLG/LOC102723996*, *INHBA*, *MUC1*, *SERPINE1*, *PML*, *BTG2*, *TICAM1*, *DUSP5*, *HIVEP1*, *PLSCR1*, *NFKBIE* and downregulated *ITGA4*, *TGFBR2*, *IFNGR1* and *VCL*.

TNF-α levels elicited by WT, DM infection and uninfected cells 24, 48 and 72 h post-infection (B). THP-1 differentiated macrophages were infected with WT or DM for 4h. Cytokine levels were measured at 24h, 48h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and presented as ±SEM of two independent experiments performed in triplicate. WT infection elicited significantly more production of TNF-α at 48h and 72 h post infection. WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents the uninfected control.

HBHA and MTP do not play a role in MIP-1β or MCP-1 regulation but enhance activation of chemokine signalling canonical pathway.

Chemokine production is necessary for inflammatory cell recruitment, and the initial activation of macrophages is vital to infection control (Guirado *et al*., 2013). In the present study, analysis of chemokine signalling canonical pathway revealed that WT infection displayed increased activation (z-score = 2.24; -log (p-value) = 2.1) compared to DM infection (z-score = 1.34; -log (p-value) = 1.38) (Fig. 3.7A and B) and predicted a higher MIP-1β production than MCP-1 by both WT and DM infection. WT infection uniquely expressed *MRAS* and the deficient strain uniquely downregulated *GNAQ*. WT and DM infection generated similar levels of chemokines, MIP-1β or MCP-1 at time intervals tested in this study (Fig. 3.7C and D). MIP-1β was generated in higher concentrations than MCP-1 during the tested time intervals and by both WT and DM infection. This finding validates the predicted levels of chemokines during the canonical pathway enrichment analysis. This suggests that HBHA and MTP do not have the ability to induce chemokine production in macrophages. Previous studies have established that *M. tuberculosis* strongly induces chemokine concentrations as part of the protective host immune response. Macrophages have previously been shown to produce CCL2, CCL3, CCL4, MCP-1, MIP-1α, MIP-1β and RANTES during *M. tuberculosis* infection (Sadek *et al*., 1998; Saukkonen *et al*., 2002).

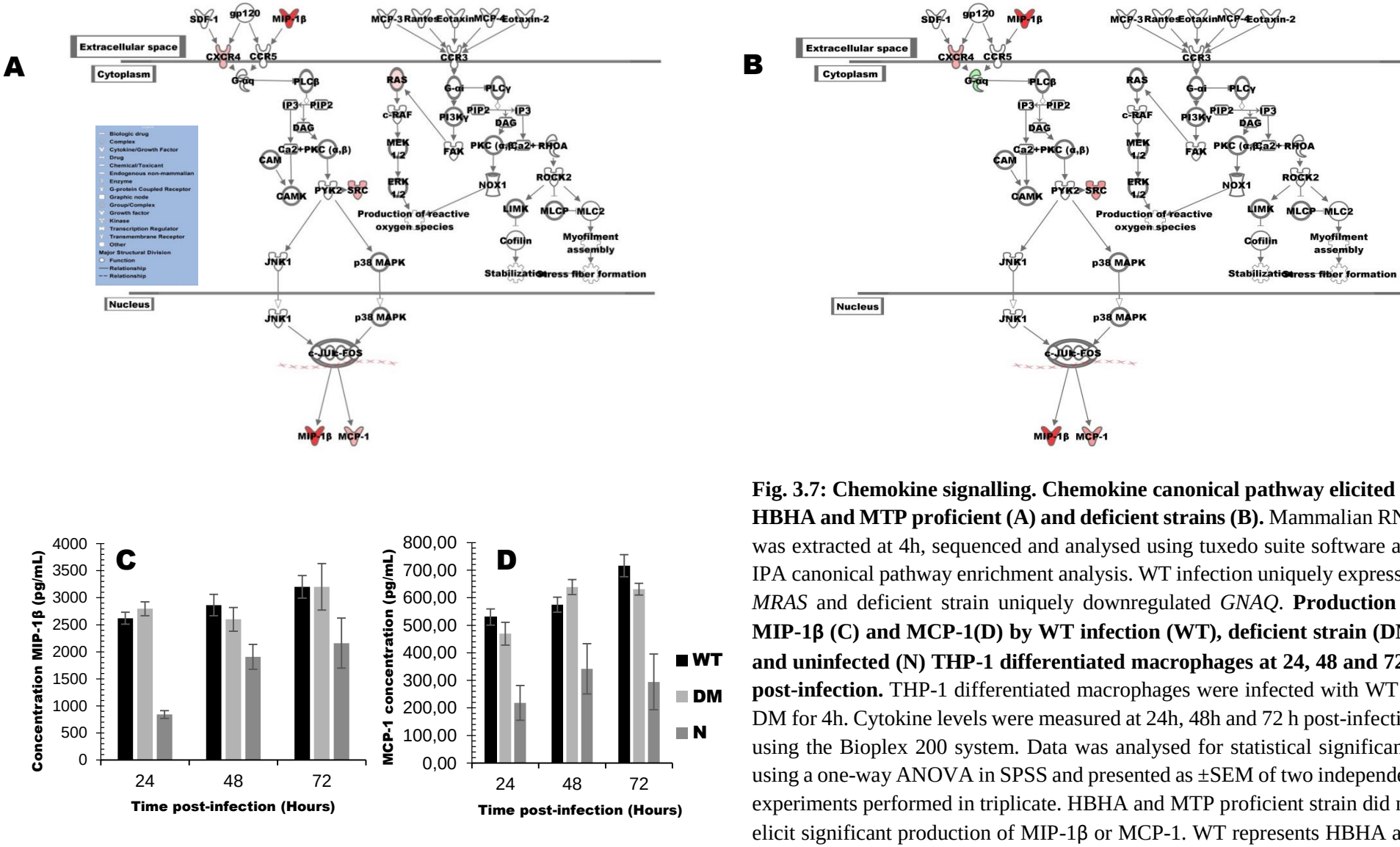


Fig. 3.7: Chemokine signalling. Chemokine canonical pathway elicited by HBHA and MTP proficient (A) and deficient strains (B). Mammalian RNA was extracted at 4h, sequenced and analysed using tuxedo suite software and IPA canonical pathway enrichment analysis. WT infection uniquely expressed *MRAS* and deficient strain uniquely downregulated *GNAQ*. **Production of MIP-1β (C) and MCP-1(D) by WT infection (WT), deficient strain (DM) and uninfected (N) THP-1 differentiated macrophages at 24, 48 and 72 h post-infection.** THP-1 differentiated macrophages were infected with WT or DM for 4h. Cytokine levels were measured at 24h, 48h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and presented as ±SEM of two independent experiments performed in triplicate. HBHA and MTP proficient strain did not elicit significant production of MIP-1β or MCP-1. WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents the uninfected control.

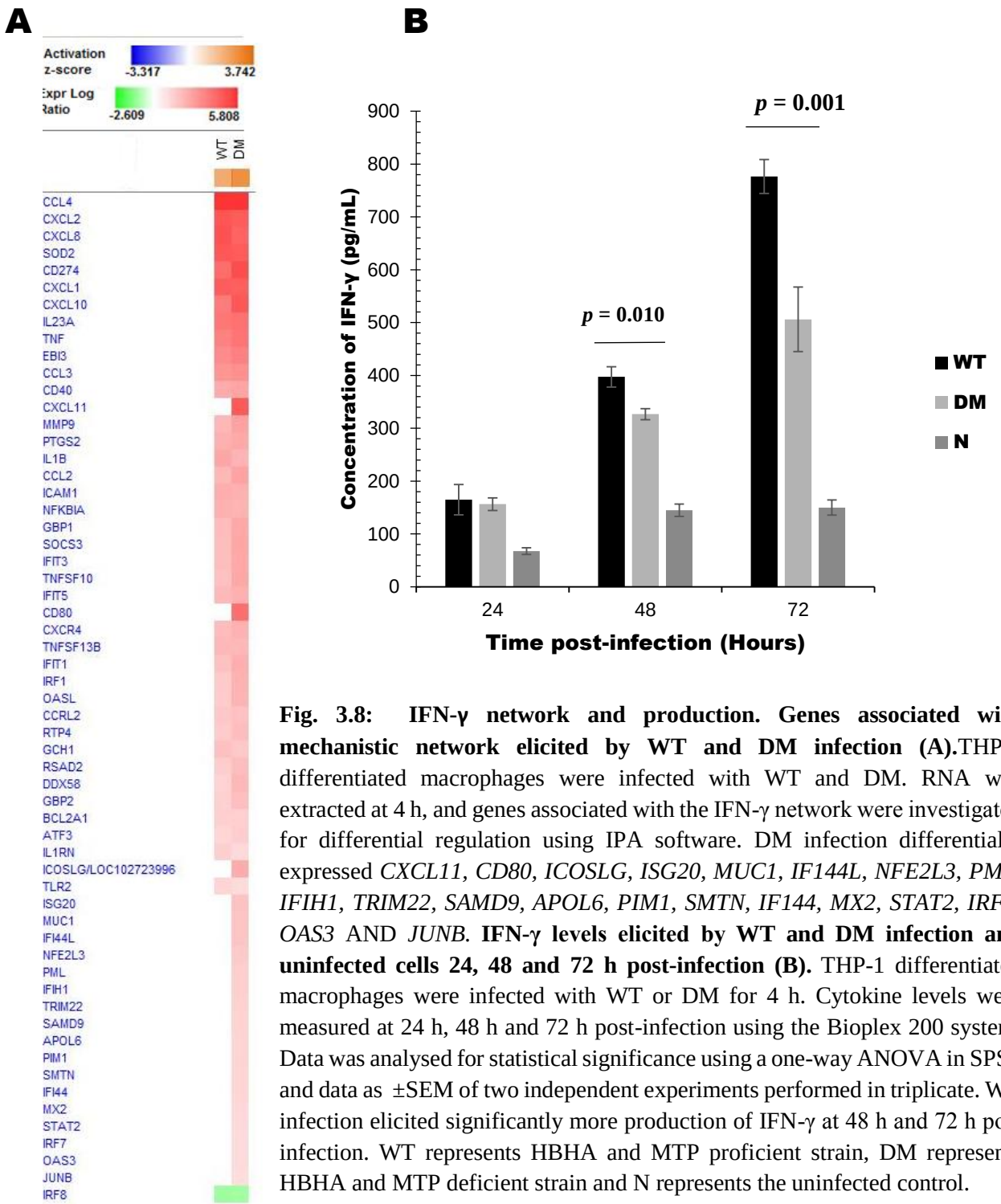


Fig. 3.8: IFN-γ network and production. Genes associated with mechanistic network elicited by WT and DM infection (A).THP-1 differentiated macrophages were infected with WT and DM. RNA was extracted at 4 h, and genes associated with the IFN-γ network were investigated for differential regulation using IPA software. DM infection differentially expressed *CXCL11, CD80, ICOSLG, ISG20, MUC1, IF144L, NFE2L3, PML, IFIH1, TRIM22, SAMD9, APOL6, PIM1, SMTN, IF144, MX2, STAT2, IRF7, OAS3* AND *JUNB.* **IFN-γ levels elicited by WT and DM infection and uninfected cells 24, 48 and 72 h post-infection (B).** THP-1 differentiated macrophages were infected with WT or DM for 4 h. Cytokine levels were measured at 24 h, 48 h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and data as ±SEM of two independent experiments performed in triplicate. WT infection elicited significantly more production of IFN-γ at 48 h and 72 h post infection. WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents the uninfected control.

HBHA and MTP enhance IFN-γ concentration and differentially regulate IFN-γ associated transcripts.

IFN-γ is required for protective host immune response against *M. tuberculosis* and has been proven to contribute to macrophage activation (Flynn *et al*., 1993). In the current study, upstream regulator analysis predicted that IFN-γ was slightly elevated in DM infection and resulted in differential regulation of the following transcripts: *CXCL11, CD80, ICOSLG, ISG20, MUC1, IF144L, NFE2L3, PML, IFIH1, TRIM22, SAMD9, APOL6, PIM1, SMTN, IF144, MX2, STAT2, IRF7, OAS3* and *JUNB* (Fig. 3.8A). Quantification of IFN-γ generated in response to HBHA and MTP in WT displayed similar concentrations at 24 h and significantly increased concentration compared to the HBHA and MTP deficient DM at 48 h ($p = 0.010$) and 72 h ($p = 0.001$) post-infection (Fig. 3.8B). The predicted elevated IFN-γ did not translate into a similar trend during quantification assays, possibly due to the interactions of induced cytokines by both infections altering the production of IFN-γ. IFN-γ produced by macrophages in response to *M. tuberculosis* infection requires augmentation by IL-12 and IL-18 (Robinson *et al*., 2010). IL-12 concentrations were elevated during WT infection compared to DM infection (Fig. 3.9C), while IL-18 was not included in the panel of analytes quantified during the current study.

Other cytokines and colony-stimulating factors elicited by HBHA and MTP.

Production of other pro-inflammatory, anti-inflammatory cytokines and colony-stimulating factors elicited by WT and DM infection respectively are displayed in Fig. 3.9. WT infection elicited significantly enhanced concentrations of IL-2, IL-12 and IL-17 at 48 h ($p = 0.003$; 0.011; 0.031 respectively) and 72 h ($p = 0.001$; 0.000; 0.000 respectively) post-infection, and IL-6 at 72 h ($p = 0.000$) post-infection respectively (Fig.3.9 A- D). IL-2 is integrally involved in granuloma formation and mediation of the cellular immune response during *M. tuberculosis* infection (Frahm *et al*., 2011; Kellar *et al*., 2011). IL-12 induces IFN-γ production (O'Neill and Greene, 1998), whilst IL-12 deficiency increased susceptibility to *M. tuberculosis* infection (Cooper *et al*., 1997; Wakeham *et al*., 1998; Wang *et al*., 1999). IL-6 displays both pro- and anti-inflammatory properties (Van Crevel *et al*., 2002) and demonstrated a detrimental role due to its involvement in inhibition of TNF-α and IL-1β (Schindler *et al*., 1990) as well as protective role in *M. tuberculosis* infection as evidenced by increased susceptibility to infection in mice that are deficient in IL-6 production (Ladel *et al*., 1997).

In addition, in a low-dose aerosol model of infection, IL-6 deficiency led to delayed IFN-γ response accompanied by a slight increase in bacterial load in the lung (Saunders *et al*., 2000). A recent study demonstrated that *M. tuberculosis* modulated the IL-6 produced by host cells in order to impede type 1 interferon signalling and thereby disease progression (Martinez *et al*., 2013).

IL-17A produced by TCR γδ T cells plays an important role in protection against *M. tuberculosis* infection by inducing granuloma formation (Yoshida *et al*., 2010). IL-17A has also been implicated in enhanced bacterial clearance of *Bacillus Calmette-Guérin* (BCG) in macrophages via a nitric oxide dependent mechanism (Ling *et al*., 2013).

Increased concentrations of the following analytes were induced by the WT compared to the DM infection: the anti- inflammatory cytokines, IL-4 and IL-10 at all-time intervals tested (p = 24 h: 0.011, 0.007; 48 h: 0.018, 0.022; 72 h: 0.000, 0.000 respectively) and IL-5 at 48 h (p = 0.001) and 72 h (p = 0.000) post-infection (Fig 9 E-H) and Colony-stimulating factors, G-CSF and GM-CSF at 48 h (p = 0.025; 0.001 respectively) and 72 h (p = 0.005; 0.000 respectively) post-infection (Fig 9 I- J).

IL-4 has previously been associated with a harmful role in intracellular *M. tuberculosis* by supressing IFN-γ production (Lucey *et al*., 1996) and activation of macrophages (Appelberg *et al*., 1992). IL-5 neutralization in SIVmac251 and *M. tuberculosis* co-infected monocytes moderately restored T cell production of TNF-α, suggesting it's possible role in modulation of TNF-α (Diedrich *et al*., 2013). IL-10 is required to maintain the balance between host inflammatory response and immunopathology. Increasing levels of IL-10 has been shown to aid mycobacterial survival (Van Crevel *et al*., 2002). IL-10 deficiency in mice has demonstrated enhanced protective immune response to *M. tuberculosis* infection (Murray and Young, 1999). In addition, IL-10 has previously demonstrated inhibition of pro-inflammatory cytokine production, and activation of antigen presenting cells and T lymphocytes (Fulton *et al*., 1998).

The function of G-CSF in *M. tuberculosis* infection is currently poorly understood, however, it has been utilized in correcting iatrogenic neutropenia caused by anti-tuberculous antibiotics without any adverse effects noted (Cormican *et al*., 2004). G-CSF has also been involved in inhibition of T cell proliferation responding to mitogen presence (Rutella *et al*., 1997). GM-CSF has been shown to be vital in lymphocyte recruitment, TH1 response expression, granuloma formation and bacterial burden control in the lung during *M. tuberculosis* infection (Gonzalez-Juarrero *et al*., 2005).

At 24 h, HBHA and MTP induced significantly greater amounts of anti-inflammatory cytokines, most likely as an immune evasion and survival strategy to control pro-inflammatory cytokine production. At 48 h and 72 h post-infection, HBHA and MTP predominately induced a pro-inflammatory cytokine profile instead of an anti-inflammatory cytokine profile, suggesting that HBHA and MTP play a role in protective immunity and immunopathology as a consequence of pro-inflammatory cytokines such as TNF-α and minimal anti-inflammatory cytokines during *M. tuberculosis* infection. These findings are consistent with previous studies that demonstrated HBHA elicited a pro-inflammatory response during infection of macrophages (TNF-α and IL-6) (Kim *et al*., 2011) and dendritic cells (IL-6, IL-12, IL-1β, TNF-α and CCR7) (Jung *et al*., 2011). In *S. typhimurium* (Tükel *et al*., 2005) and *E. coli* (Bian *et al*., 2000) infections, curli pili have been shown to induce IL-6, IL-8, and TNF-α respectively.

The inflammatory response is a host mechanism with the aim of containing *M. tuberculosis* within a granuloma, but an uncontrolled inflammatory response often leads to tissue damage and exposure of extracellular matrix, which may be utilised by pathogens as targets for binding. Both HBHA and MTP have been shown to be capable of binding to extracellular matrix. HBHA binds to heparin (Menozzi *et al*., 1996) and MTP binds to laminin (Alteri *et al*., 2007). Therefore, induction of inflammatory response could possibly be advantageous to *M. tuberculosis* proliferation.

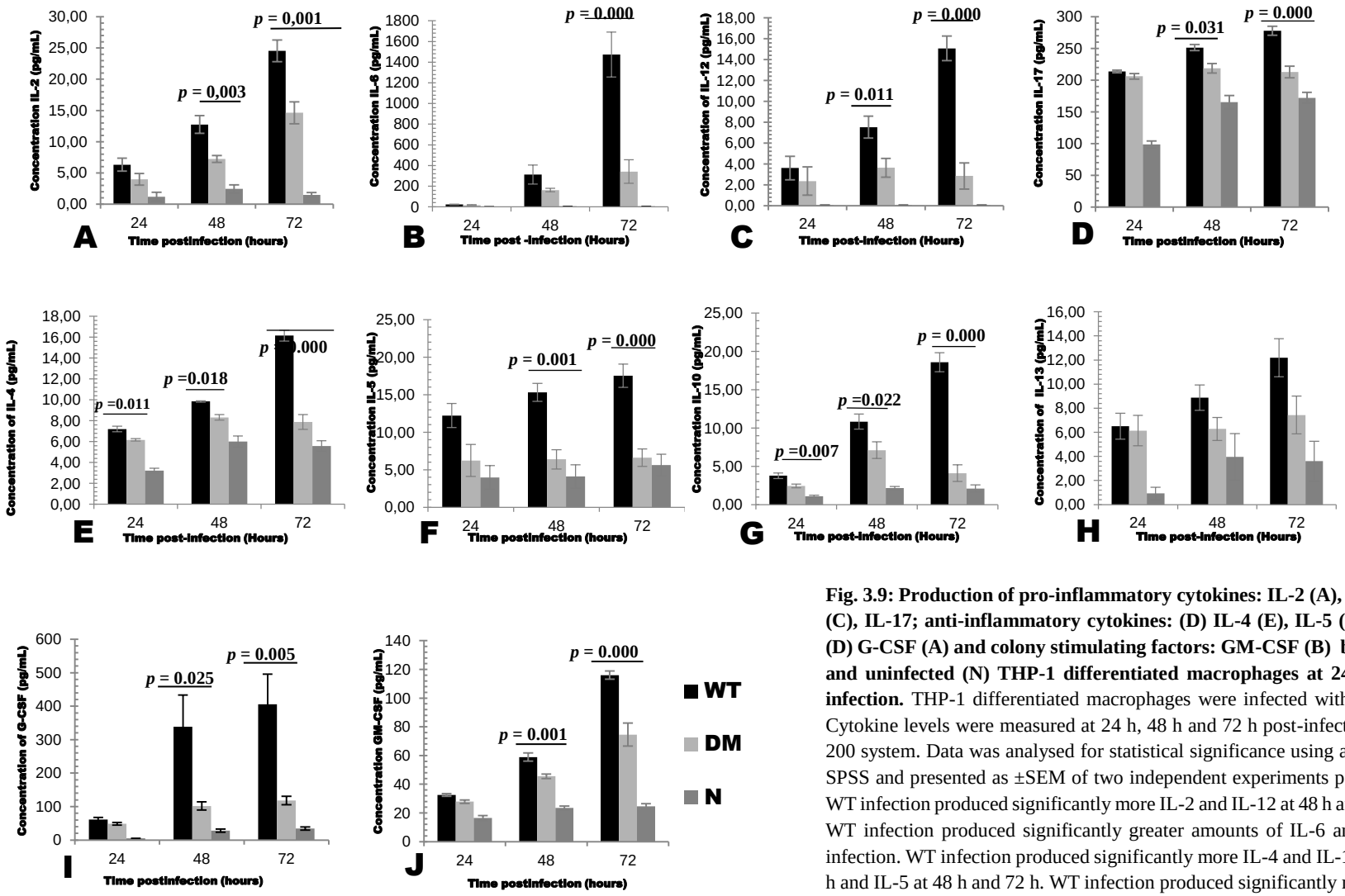


Fig. 3.9: Production of pro-inflammatory cytokines: IL-2 (A), IL-6 (B), IL-12 (p70) (C), IL-17; anti-inflammatory cytokines: (D) IL-4 (E), IL-5 (F), IL-10 (G), IL-13, (D) G-CSF (A) and colony stimulating factors: GM-CSF (B) by WT, DM infection and uninfected (N) THP-1 differentiated macrophages at 24, 48 and 72 h post-infection. THP-1 differentiated macrophages were infected with WT or DM for 4 h. Cytokine levels were measured at 24 h, 48 h and 72 h post-infection using the Bioplex 200 system. Data was analysed for statistical significance using a one-way ANOVA in SPSS and presented as ±SEM of two independent experiments performed in triplicate. WT infection produced significantly more IL-2 and IL-12 at 48 h and 72 h post-infection. WT infection produced significantly greater amounts of IL-6 and IL-17 at 72 h post infection. WT infection produced significantly more IL-4 and IL-10 at 24 h, 48 h and 72 h and IL-5 at 48 h and 72 h. WT infection produced significantly more G-CSF and GM-CSF at 48 h and 72 h post-infection. WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents the uninfected control.

HBHA and MTP enhance activation of MAPK and PI3-K signalling canonical pathways during infection of macrophages.

M. tuberculosis infection induces MAPK (Schorey and Cooper, 2003; Jo *et al*., 2007) and PI3-K signalling pathways (Maiti *et al*., 2001; Sly *et al*., 2001). The MAPK signalling pathway is implicated in pathogenesis (Yadav *et al*., 2004) and PI3-K signalling pathway is involved in anti-mycobacterial activities during infection of monocytes (Sly *et al*., 2001; Jung *et al*., 2006). In the current study, WT infection enhanced activation of both p38 MAPK signalling and PI3-K/Akt signalling canonical pathways (z-score = 1 and 2.45 respectively), compared to DM infection (z-score = 0.3 and 1.89 respectively) (Fig. 3.10). During the MAPK signalling canonical pathways, DM infection uniquely down-regulated *MEF2C* and *TGFBR2* transcripts (Fig. 3.10A) and WT infection up-regulated IL-1α compared to DM infection. During the PI3-K/AKT canonical pathway, WT infection uniquely up-regulated *MRAS* and *NFKBIE* was uniquely up-regulated and *ITGA4* transcripts were uniquely down-regulated by the DM infection in the (Fig. 3.10B). Previous studies showed that pattern recognition of *M. tuberculosis* antigens including HBHA, led to activation of MAPK and PI3-K/AKT pathways in macrophages (Schorey and Cooper, 2003; Jung *et al*., 2006; Kim *et al*., 2011). Both these signalling pathways are involved in secretion of pro-inflammatory cytokines in response to *M. tuberculosis* antigens (Kim *et al*., 2011). It is therefore possible, that HBHA and MTP enhanced activation of MAPK and PI3-K/AKT pathways resulted in enhanced secretion of pro-inflammatory cytokines quantified in the current study.

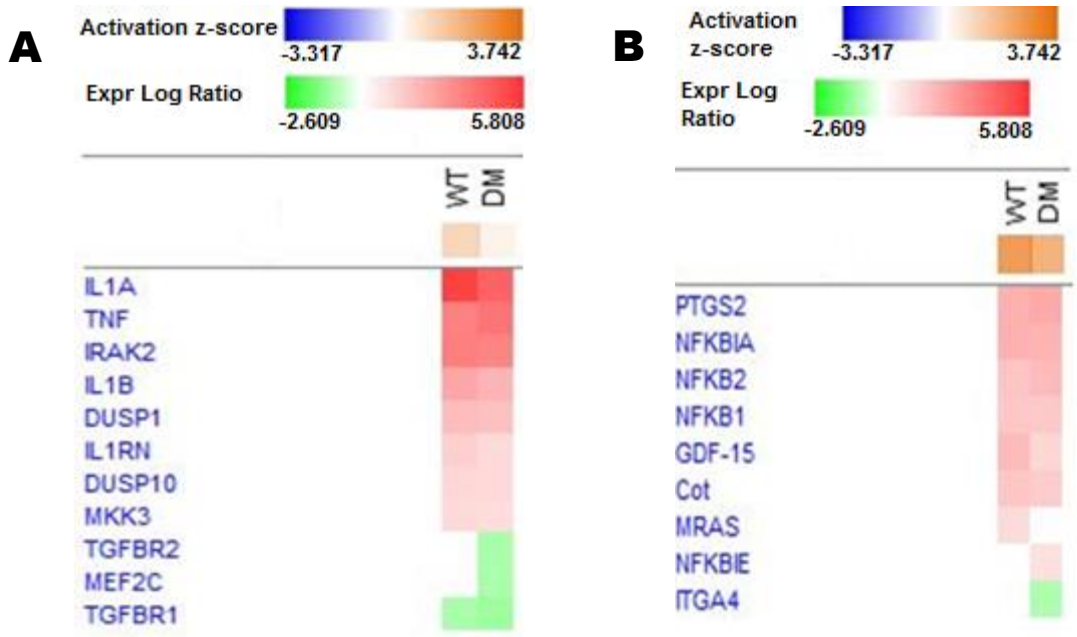


Fig. 3.10: Signalling pathways. MAPK signalling pathway elicited by HBHA and MTP proficient and deficient strains (A) and PI3-K/AKT signalling pathway elicited by HBHA and MTP proficient and deficient strains (B). Mammalian RNA was extracted at 4 h, sequenced and analysed using tuxedo suite software and IPA canonical pathway enrichment analysis. WT infection enhanced activation of both p38 MAPK signalling and PI3-K/Akt signalling canonical pathways. . WT represents HBHA and MTP proficient strain, DM represents HBHA and MTP deficient strain and N represents the uninfected control.

One of the limitations of this study was that IL-1α or IL-18 were not quantified, since both in addition to IL-1β were activated during upstream analysis of both infection models and their production has been linked to IL-1β. Both IL-1β and IL-18 production in macrophages has been demonstrated to be dependent on the inflammasome (Schroder and Tschopp, 2010). This study also did not investigate the cytokine levels induced by a HBHA-MTP complemented strain. Numerous studies have investigated the individual role of the HBHA and MTP individually on cytokine and transcriptional responses. Future studies will include a comparison of the complemented and mutant strains.

Conclusions

The data presented in this study collectively demonstrate that HBHA and MTP activate intracellular signalling pathways that result in the longitudinal enhancement of a pro-inflammatory response during *M. tuberculosis* infection of macrophages. This study provides novel information regarding *M. tuberculosis* antigens that are involved in adhesion and invasion to host cells and suggest that these intracellular signalling pathways may be important immunological processes during *M. tuberculosis* infection. HBHA and MTP combined immunogenic ability to induce a protective immune response suggests that these antigens could possibly be a novel combination for vaccine development. Further studies are required to validate the longitudinal enhancement of transcriptional activation of the intracellular signalling pathways and understand the overall impact of HBHA and MTP on *M.*

tuberculosis pathogenesis. These studies should focus on the role of HBHA and MTP in IL-1β, IL-1α and IL-18 production and signalling pathways, the effect of reintroduction of HBHA and MTP by complementation on cytokine response and functional studies assessing the role of HBHA and MTP in inflammasome, NF-κB, toll-like receptor, MAPK and PI3-K/AKT pathway regulation.

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HBHA and MTP combined ability to induce intracellular signalling pathways that result in the longitudinal enhancement of a pro-inflammatory response during *M. tuberculosis* infection of macrophages was outlined in chapter three. In chapter four, the differential transcription regulation induced by HBHA and MTP and their effect on intracellular replication in macrophages was investigated.

CHAPTER 4: *Mycobacterium tuberculosis* HBHA and MTP modulate the host immune response to facilitate intracellular replication in macrophages.

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Running title: ***M. tuberculosis*** HBHA and MTP modulate the host immune response to facilitate intracellular replication.

Keywords: ***M. tuberculosis***; HBHA; MTP; Th1; Th2; canonical pathways; transcriptional response; macrophages.

Abstract

Understanding the host and pathogen interactions via transcriptomic analysis is vital to further elucidate TB pathogenesis. This study identified the transcriptomic and molecular regulation signatures elicited by two adhesins, MTP and HBHA during early infection of macrophages in an attempt to further understand their combined influence on intracellular replication and host immune response to *M. tuberculosis*. Intracellular replication of Wild-type V9124 (WT), double mutant, Δ*hbhA-mtp* (DM), HBHA deficient mutant, Δ*hbhA* (H), MTP deficient mutant, Δ*mtp* (M), HBHA-overexpressing, *hbhA*-complemented strain of DM, (HC) and MTP-overexpressing, *mtp*-complemented strain of DM (MC) was quantified by colony forming units at 4 h, day 3 and day 6 post-infection. To understand the impact of HBHA and MTP on *M. tuberculosis* replication induced intracellular signalling, differential transcription regulation was further interrogated using RNA sequencing. The bacillary load of M was significantly less than WT at all time intervals and similar to DM whilst H was similar to WT at all time intervals. The decreased replication ability of the HBHA-MTP mutant was attributed to MTP and not HBHA, suggesting that MTP facilitates replication during infection of macrophages. A common response independent of HBHA-MTP, as well as unique responses induced by HBHA-MTP presence and deficiency were observed. The common transcriptional pattern exhibited the most enrichment for granulocyte adhesion and diapedesis canonical pathway, TNF upstream regulation and migration of cells biological function. The HBHA-MTP uniquely induced transcripts induced the most significant enrichment of Adipogenesis pathway, whilst HBHA-MTP deficiency induced the most significant enrichment of T helper cell differentiation. Unique transcripts elicited by HBHA-MTP deficiency induced less enrichment of NF-κB upstream regulator and were associated with migration of cells. The top 10 canonical pathways enriched by all transcripts were similar between both infections, but differed in molecules involved and their significance. HBHA-MTP enriched the TREM1 signalling pathway to a greater degree than HBHA-MTP deficiency in macrophages. HBHA-MTP deficiency, but not presence, enriched Th1 and Th2 Activation, Th1, Th2, Melatonin degradation, Sumoylation, Methylglyoxal degradation III, Granzyme A signalling, PCP pathways. These pathways were involved in regulation of cellular immune response, amino acid metabolism, organismal Growth and development, aldehyde Degradation and transcription. These findings suggest that HBHA and MTP, in addition to their combined role in adhesion, invasion and activating the host protective immune response, play a role in host immune response modulation as a possible survival strategy during intracellular growth in macrophages.

Introduction

Tuberculosis (TB) is currently the ninth leading cause of mortality worldwide, resulting in approximately 1.3 million deaths among HIV-negative people in 2016 (WHO, 2017). TB was the leading underlying cause of death in nearly half of South African 52 districts that combined form the 9 provinces of South Africa. Mpumalanga, Eastern Cape and KwaZulu-Natal provinces reported the highest number of deaths due to TB in 2016 (Statistics South Africa, 2016). TB diagnosis and treatment is hindered by drug resistance (Dheda *et al*., 2013) and HIV coinfection (Karim *et al*., 2010). Drug resistance is compounded by diagnostic delays due to the slow growth of *Mycobacterium tuberculosis*, lack of efficient biomarkers to develop rapid, accurate diagnostic tools (Wallis *et al*., 2013), ineffective drugs to combat drug resistance and patient non-compliance. In addition, the current available TB vaccine was developed in 1921, and provides limited efficacy against adult TB (Mangtani et al., 2014)). This highlights the imminent need for efficient biomarkers and vaccine candidates. A multi-antigen approach may address some of the shortcomings of current biomarker and vaccine discovery programmes (Triccas and Counoupas, 2016).

Interactions between *M. tuberculosis* components such as adhesins and signalling pathways are important for the protective immunity to *M. tuberculosis* infection. Cell activation and production of cytokines lead to inflammatory response and therefore, define the outcome of the disease (Van Crevel *et al*., 2002). Pathogenic bacteria produce adhesins on their cell surface that facilitate interactions with cell receptors of the host (Kline *et al*., 2009). Mycobacterial adhesins have been well studied (Govender *et al*., 2014). The heparin-binding haemagglutinin (HBHA) (Menozzi *et al*., 1996; Pethe *et al*., 2001) and *M. tuberculosis* curli pili (MTP) (Alteri *et al*., 2007; Ramsugit *et al*., 2013; Naidoo *et al*., 2014; Ramsugit and Pillay 2014; Ramsugit *et al*., 2016) have been previously described to be major mycobacterial adhesins.

HBHA has been previously investigated as a possible vaccine antigen (Parra *et al*., 2004; Kohama *et al*., 2008; Guerrero and Locht, 2011; Stylianou *et al*., 2014; Verwaerde *et al*., 2014; Fukui *et al*., 2015) resulting in a novel DNA vaccine encoding MTB32C-HBHA antigen (Teimourpour *et al*., 2017) and nanoparticles adsorbed with Ag85B-HBHA (Stylianou *et al*., 2014), that induce specific immune responses against *M. tuberculosis* after exposure to BCG vaccination. HBHA also demonstrated immunomodulatory abilities, inducing the expression of TNF-α, IL-6 and activating NF-κ B and MAPK signalling pathways in macrophages (Kim *et al*., 2011), thus, highlighting the possible role of HBHA in the pro-inflammatory response.

Understanding the host and pathogen interactions via transcriptomic analysis facilitates the identification of potential, hitherto unidentified virulence factors and novel immune response pathways

elicited by pathogens (Westermann *et al*., 2012). Therefore, global transcriptomic analysis represents a crucial link in understanding *M. tuberculosis* pathogenesis. Global transcriptomic analysis has yielded valuable results in evaluating the host immune response (Tailleux *et al*., 2008; Koo *et al*., 2012; Mvubu *et al*., 2016). Differential transcriptional regulation of macrophages by CDC1551 and HN878 determines the outcome of infection (Koo *et al*., 2012). Recently, it was shown that the Beijing strain induced lower host protective immune responses compared to the F11, F15/LAM4/KZN and F28 strains in the epithelial cell model (Mvubu *et al*., 2016). In addition, macrophages and dendritic cells demonstrate differential intracellular vescicle trafficking, phagosome acidification and oxidative stress responses (Tailleux *et al*., 2008).

Recent global transcriptomic studies in our research group have demonstrated the significant roles of MTP and HBHA in initiation of the innate immune response and inflammatory response in female Balb/C mice (Kuvar, 2016; Nyawo, 2016) as well as in epithelial cells (Dlamini, 2016) and macrophages (unpublished). These data suggest that MTP is a strong immunogen and confirms previous findings on HBHA, warranting their further development as possible vaccine and biomarker candidates.

A multiple antigen approach for vaccine design has recently gained momentum, and several combinations have been tested, including antigen85B-HBHA combination after BCG vaccination (Stylianou *et al*., 2014), MTB32C-HBHA antigen (Teimourpour *et al*., 2017), and Ag85B, ACR, and HBHA (Copland *et al*., 2018) in BCG-primed animals. Data collectively obtained thus far from the numerous studies performed independently on each adhesin, suggest that HBHA and MTP are significant modulators of the immune system, demonstrating potential as a vaccine candidates. With the multiple antigen approach in mind, the current study aimed to elucidate the combinatorial effect of HBHA and MTP on intracellular replication, host global transcriptomics and molecular signatures by RNA sequencing of macrophages infected with a double Δ*Rv0475- Rv3312A (hbhA-mtp)* knockout mutant of *M. tuberculosis*.

Methods

Ethics statement

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference no. BE516/14) approved this study.

M. tuberculosis strains

THP-1 differentiated macrophages were infected with Wild-type V9124 (WT), double mutant, Δ*hbhA-mtp* (DM), HBHA deficient mutant, Δ*hbhA* (H), MTP deficient mutant, Δ*mtp* (M), HBHA-overexpressing, *hbhA*-complemented strain of DM, (HC) and MTP-overexpressing, *mtp*-complemented

strain of DM (MC) (Govender *et al*., unpublished) (Chapter 2, page 63-64). Strains were grown in Middlebrook 7H9 medium (Difco, Becton, Dickinson and Company, South Africa), containing the following supplements: 0.5% (v/v) glycerol (Merck, South Africa), 10% (v/v) oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson), 0.05% (v/v) Tween 80 (Sigma, Capital lab supplies, South Africa), and 150 µg/mL hygromycin (Roche Applied Sciences, Capital lab supplies, South Africa) for mutant strains and 30 µg/mL kanamycin (Sigma, Capital lab supplies, South Africa) for complemented strains. For each assay, strains were grown with shaking incubator (NBS Shaking incubator series, ALS solutions, South Africa) at 37°C until an optical density (OD) $_{600nm}$ of 1 was reached. Optical density was measured with a spectrophotometer (WPA lightwave II, Biochrom, Labotec, South Africa) by measuring the absorbance at 600nm of a 1:5 dilution of a log-phase culture of the specific strain.

Intracellular replication assay

THP-1 monocytic cells (ATCC TIB-202) were differentiated into macrophages by overnight incubation with phorbol 12-myristate 13-acetate (Sigma, Capital lab supplies, South Africa) at a density of 5 x 10^5 cells/mL. Monolayers were infected with the *M. tuberculosis* strains at a multiplicity of infection of 5 to 1 for 4 h. Post-infection, monolayers were washed with Phosphate Buffered Saline (Oxoid Quantum Biotechnologies, South Africa), and fresh media was added to day 3 and 6 plates. At each time-point, macrophages were lysed in 0.1% triton-X 100 (Sigma, Capital lab supplies, South Africa) for 20 mins at 37°C and 5% CO_2. Ten-fold serial dilutions were plated out on 7H11 agar to assess the colony forming units (CFU/mL) at each time point. Three independent biological assays and 3 technical repeats were performed for each strain.

RNA sequencing

THP-1 differentiated macrophages were infected with WT and DM at a multiplicity of infection of 5 for 4 h. Total RNA was extracted using the RNeasy kit (Qiagen, Whitehead Scientific, South Africa) according to manufacturer's instructions. RNA quality and quantity was measured by Nanodrop (Thermofisher Scientific, Capital lab supplies, South Africa) and 3-(N-morpholino)propanesulfonic acid (MOPS) gel electrophoresis (Appendix 3) respectively. cDNA libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, Whitehead Scientific, South Africa). Duplicate biological replicates were sequenced using the Hiseq 2500 platform (Illumina, Whitehead Scientific, South Africa) resulting in 125 bp paired-end reads (Agricultural Research Council Biotechnology Platform, Onderstepoort, South Africa).

Mapping and transcript assembly

This was performed as previously described (Chapter 2, pages 65-66). Briefly, reads were trimmed with Trimmomatic (Bolger *et al*., 2014) and TopHat (2.1.0) and Bowtie2 mapped reads to the Hg38 reference genome (UCSC). Differential gene expression patterns were elucidated by the Cuffdiff package (version cufflinks-2.2.1) (Trapnell *et al*., 2012).

Enrichment and functional analysis

Significantly differentially expressed genes (SDEGs) with a p value <0.05 were used for further enrichment and functional analysis. Global transcriptomics and visualization of data was carried out using R version 2.4.1 (www.r-project.org), R-studio, and Bioconductor packages. Venn diagrams were created using Venny 2.1. (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Scatterplot was generated with R (version 3.2.) using the CummeRbund package (v. 2.0.). Enrichment analysis was carried out using the Molecular Signature Database (MSigDB) (http://software.broadinstitute.org/gsea/msigdb/index.jsp) to elucidate the gene ontology enriched by WT and DM infection respectively. The false discovery rate (FDR) was set at a cut-off value of $p < 0.05$. Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA) enrichment analysis was utilized to further investigate canonical pathways and upstream regulators.

Quantitative real time PCR

The influence of HBHA and MTP on gene regulation of the following genes: *CD80, DLX3, NLRP3, TGM5* and *TLR2* was investigated at 1 h, 2 h and 4 h post-infection respectively using quantitative RT-PCR (qRT-PCR). RNA quality was assessed by using MOPS gel electrophoresis and Nanodrop (Thermofisher Scientific, Capital lab supplies, South Africa) respectively, and cDNA synthesised using the High-Capacity cDNA Reverse Transcription kit (Roche Applied Sciences, Capital lab supplies, South Africa) as per the manufacturer's instructions. The qRT-PCR was run on the CFX96 qRT-PCR Detection System (Bio-Rad, South Africa) using the standard prime PCR cycling conditions and the primers in Table 4.1. The PCR reaction mix included 5 µL 2X Ssoadvanced Universal SYBR Green Supermix (Bio-Rad, South Africa), 1 µL cDNA (50 ng), 1µL (10µM) each of forward and reverse primers, in a total volume of 10 µL. *GAPDH* was used a as a reference gene to normalise data and the comparison of infected and uninfected gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantitation method. Transcript regulation experiments were conducted in duplicate biological experiments and triplicate technical replicates.

Table 4.1: Primers used for qRT-PCR of *GAPDH, DLX3, TGM5, NLRP3, TLR2* and *CD80*. The listed primers were used to investigate the gene regulation at 1 h, 2 h and 4 h post-infection.

Transcript	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Ref
GAPDH	GAGTCAACGGATTTGGTCGT	AAATGAGCCCCAGCCTTCT	**(Spandidos *et al*., 2009)**
DLX3	TACCCTGCCCGAGTCTTCTG	TGGTGGTAGGTGTAGGGGTTC	**(Spandidos *et al*., 2009)**
TGM5	AGCTGCTAGACAAGAGCCTG	CCACTCTGCTGACGTAGACG	**(Spandidos *et al*., 2009)**
NLRP3	GATCTTCGCTGCGATCAACAG	CGTGCATTATCTGAACCCCAC	**(Spandidos *et al*., 2009)**
TLR2	ATCCTCCAATCAGGCTTCTCT	GGACAGGTCAAGGCTTTTTACA	**(Spandidos *et al*., 2009)**
CD80	GGCCCGAGTACAAGAACCG	TCGTATGTGCCCTCGTCAGAT	**(Mvubu *et al*., 2016)**

Results

MTP plays a more integral role in intracellular replication than HBHA.

THP-1 differentiated macrophages were infected with WT, M, H, DM, HC and MC strains for 4 h. CFU counts were enumerated at 4 h, day 3 and day 6 post-infection (Fig. 4.1A). At 4 h, CFU/mL were significantly decreased for M (p = 0.000), DM (p = 0.018) and HC (p = 0.001), but were similar for H (p = 0.214) and MC (p = 0.753) compared to WT. On day 3, CFU/mL were significantly decreased for M (p = 0.028) and HC (p = 0.034) whilst those of H (p = 0.996), DM (p = 0.214) and MC (p = 0.885) were similar. On day 6, M (p = 0.000), DM (p = 0.000) and HC (p = 0.000) displayed significantly decreased CFU/mL compared to WT but H (p = 0.377) and MC (p = 0.073) were similar. All 6 strains increased in CFU/mL from 4 h to day 3 (p: WT= 0.000; M= 0.014; H= 0.016; MC = 0.046), however, these did not reach statistical significance for DM (p = 0.086) and HC (p-value = 0.288). The growth of all strains increased significantly from day 3 to day 6 (p: WT= 0.000; M=0.000; H= 0.002; DM = 0.030; HC = 0.007; MC = 0.004). M displayed the lowest, and WT the highest CFU/mL at all 3 time intervals. No significant differences were observed between M and HC and H and MC at all time-intervals. The bacillary load of M was significantly less than WT at all time intervals and similar to DM. H displayed a similar bacillary load to WT but higher than DM. However, at 4 h and day 3, this did not reach statistical significance (p: day 6 = 0.002). These findings suggest that MTP, but not HBHA, mediate intracellular replication of *M. tuberculosis* in macrophages.

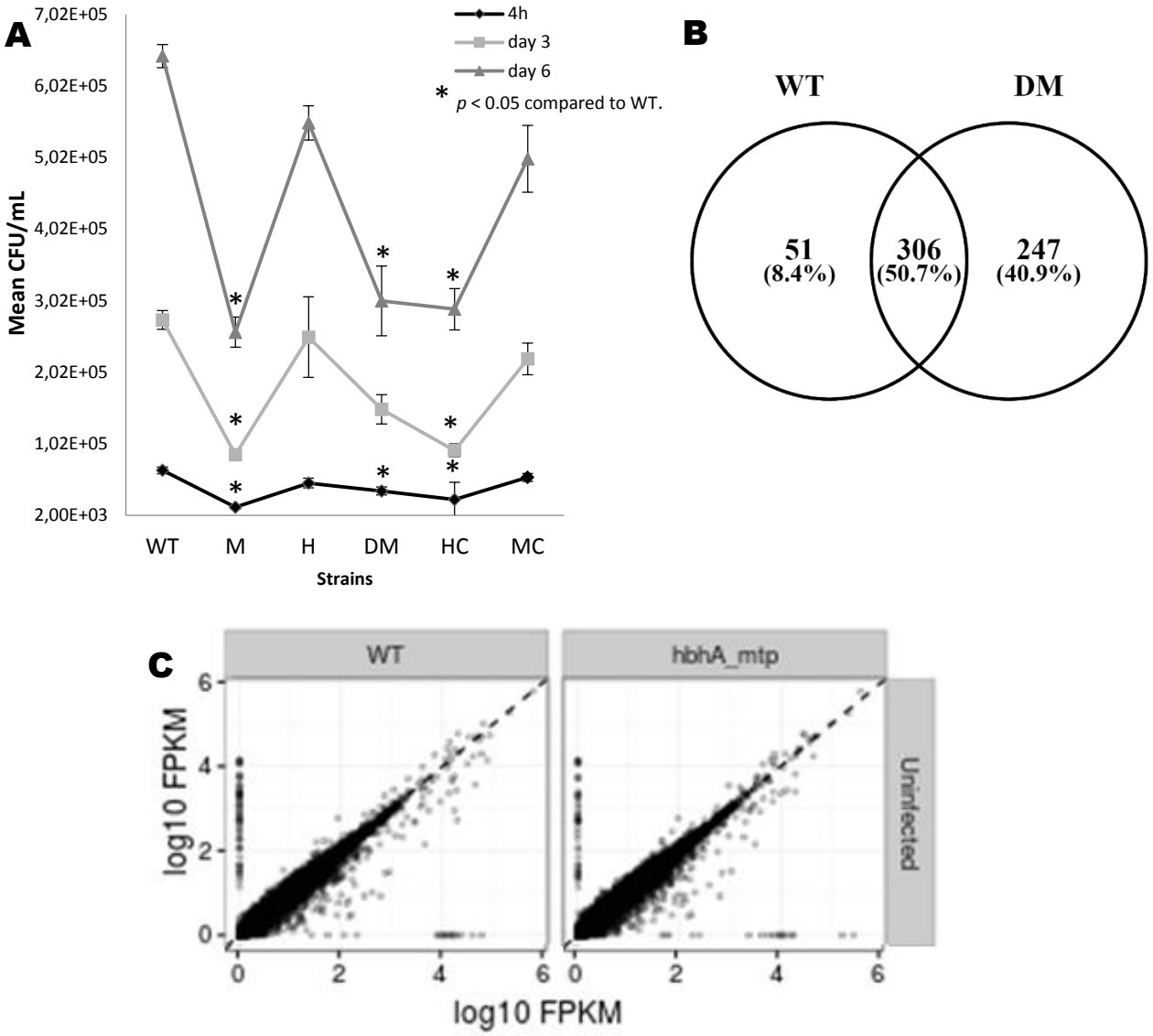

Fig. 4.1: Intracellular replication and global transcriptome of THP-1 infected macrophages. (A) Intracellular replication of WT, DM, *mtp* comp, *hbhA* comp, Δ*hbhA* and Δ*mtp* at 4 h, 3 and 6 days. THP-1 cells were infected with each strain (MOI = 5) for 4 h. At each time interval, cells were lysed and serial dilutions were plated out on 7h11 agar to determine CFU/ml. All strains except DM and HC displayed increased intracellular replication from 4 h to day 3. All strains displayed increased intracellular replication from day 3 to day 6. At 4 h, M, DM and HC displayed significantly decreases in bacillary load compared to WT, whilst H and MC displayed similar bacillary loads compared to WT. On day 3, M and HC displayed significantly decreased bacillary loads whilst H, DM and MC displayed similar bacillary loads. On day 6, M, DM and HC displayed significantly decreased bacillary loads compared to WT but H and MC displayed similar bacillary loads.
(B) Venn diagram of all genes elicited by WT and DM infection respectively. The Venn diagram was created using Venny 2.0 in order to compare the significantly differentially expressed genes. A common transcriptional pattern was observed amongst both infections. DM infection elicited a larger number of unique genes compared to WT infection. **(C) Pairwise comparison scatterplot of differentially expressed genes elicited by WT, DM and uninfected macrophages.** Differentially expressed genes elicited by both infection models as well as uninfected macrophages were analysed using R: Cummerbund. WT and DM infection elicited a differential response relative to the uninfected macrophages. DM infection elicited a greater response when compared with uninfected macrophages than WT infection. Key: WT: wild-type; DM: Δ*hbhA-mtp*; M: Δ*mtp*; H: Δ*hbhA*; HC: hbhA comp in Δ*hbhA-mtp*; MC: mtp comp in Δ*hbhA-mtp*.

Macrophage global transcriptome induced by HBHA and MTP.

To understand the impact of HBHA and MTP on intracellular replication, transcriptomic changes were investigated at 4 h post-infection of macrophages with WT and DM strains. Out of 26372 genes, 357 and 553 were significantly differentially expressed genes (SDEGs) induced by the WT and DM infection respectively (Fig. 4.1B). A total of 306 genes were common to both infections, representing 85.7 % and 55.33 % of the WT and of DM infection genes respectively. WT and DM infection induced 51 and 247 unique genes respectively (Fig. 4.1B). The scatterplot of the log10 FPKM demonstrated differences in the global distribution of genes between WT and DM infected macrophages compared with uninfected macrophages (Fig. 4.1C).

Transcript regulation patterns induced by WT and DM infection during early infection of macrophages.

WT infection elicited 291 and 66 upregulated and down regulated genes respectively, compared to 353 and 200 upregulated and down regulated genes by DM (Fig. 4.2A and B). Both strains induced up- and down-regulation of 61 % and 30.4 % commonly expressed genes respectively. The most up- and down-regulated genes elicited were *CCL4* and *TGM5* by the DM infection, and *CCL4* and *DLX3* by the WT infection, respectively.

The differentially down-regulated genes by WT, DM and complemented strains were further investigated at different time points by qRT-PCR to understand the role of HBHA and MTP in adhesion and invasion. *DLX3* was significantly less down-regulated by DM and HC at 1 h post-infection (*p*: DM =0.003; HC=0. 015) compared to WT (Fig. 4.2C). At 2 h post infection, WT and DM induced similar fold-changes, whilst HC demonstrated the least down-regulation. However, these did not reach statistical significance. At 4 h post-infection, both HC and MC displayed significantly more down-regulation (*p*: HC =0.001; MC=0. 015) than WT. *TGM5* was most down-regulated by DM at 1 h and 4 h post infection (Fig. 4.2D). However, at 2 h post-infection, HC displayed the most down-regulation, but these findings did not reach statistical significance.

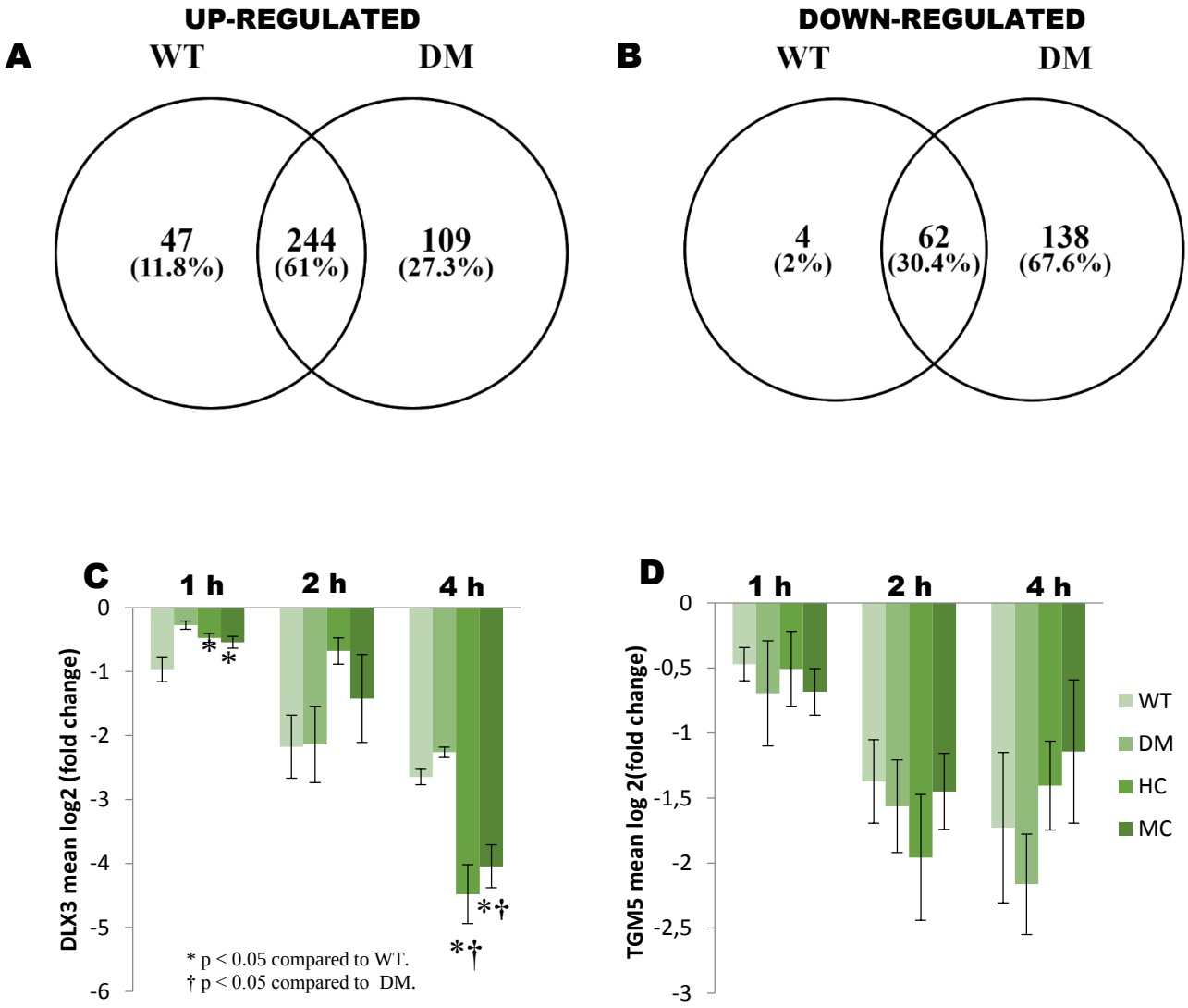

Fig. 4.2: Upregulated (A) and downregulated (B) genes elicited by WT and DM infection. Gene regulation of *DLX3* (C) and *TGM5* (D) determined by qRT-PCR at different time intervals. Gene expression elicited by both infection models were compared using a Venn diagram generated by Venny. DM infection displayed a greater number of up-regulated and down regulated genes compared to WT infection. MTP and HBHA individually might play a greater role in the downregulation of *DLX3* at 4 h, but do not play a significant role in regulation of *TGM5*.

Common transcription induced by F15/LAM4/KZN strain family during early infection.

The common transcriptional profile between WT and DM infected differentiated macrophages was conserved with slight differences between fold changes. Gene ontology analysis revealed that the commonly expressed transcripts were involved in the following top 5 biological processes such as immune system process, defence response, immune response, response to cytokine and positive regulation of response to stimulus. Comparison of the canonical pathway, upstream regulator and biological function enrichment analysis by IPA revealed similar activation for both infection models

(Fig. 4.3). The most significant canonical pathway, upstream regulator and biological function elicited was the granulocyte adhesion and diapedesis (Fig. 4.3A), TNF (Fig. 4.3B) and migration of cells (Fig. 4.3C) respectively. This possibly represents the transcriptional response elicited by the F15/LAM4/KZN strain family in macrophages.

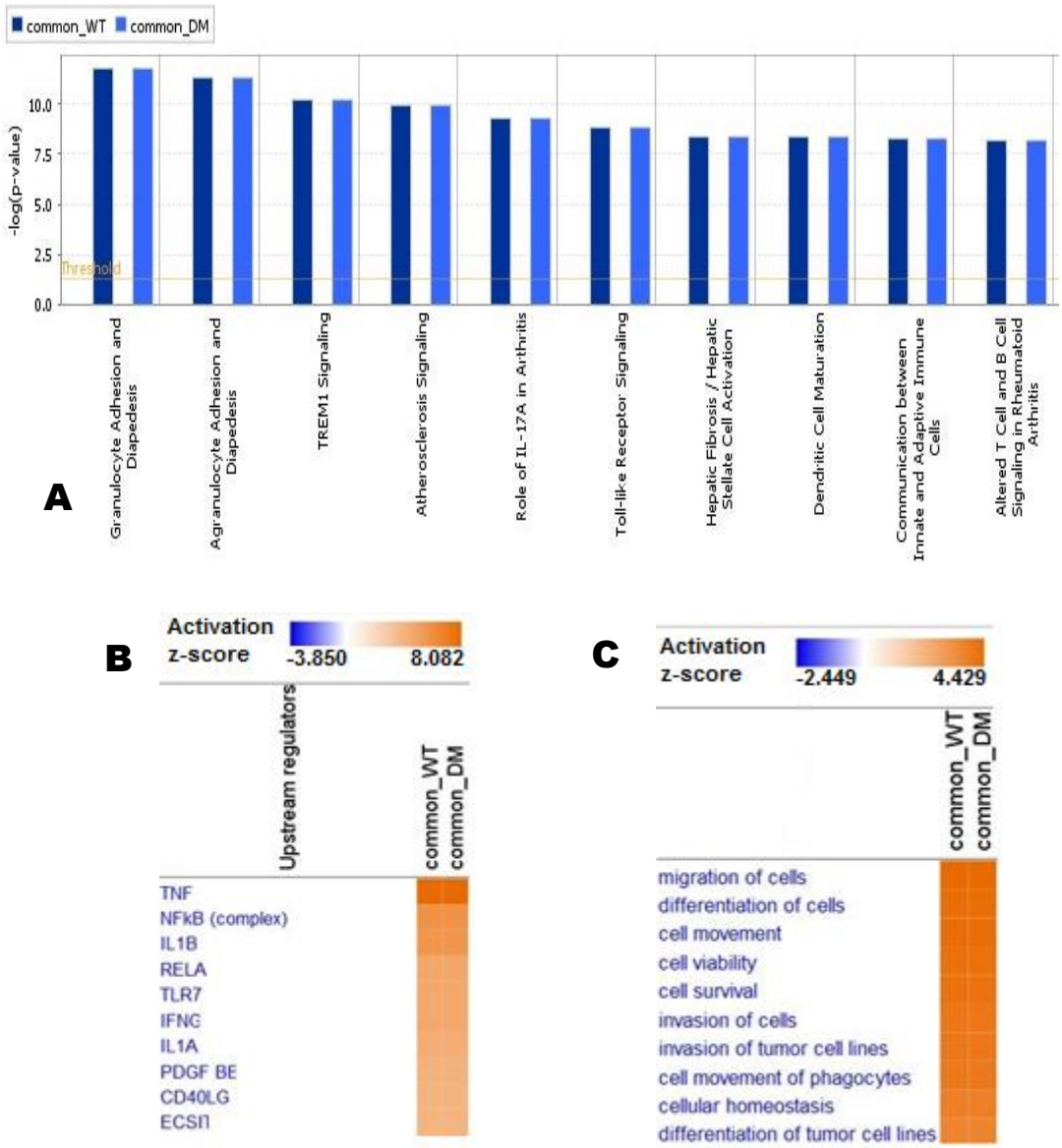

Fig. 4.3: Comparison of canonical pathway (A), upstream regulator (B) and disease and biological function activation (C) by commonly expressed transcripts of WT and DM infections. Commonly expressed transcripts that were interrogated using IPA induced identical regulation of canonical pathway, upstream regulators and biological functions. The common transcriptional pattern was associated with granulocyte adhesion and diapedesis canonical pathway, TNF upstream regulation and migration of cells biological function.

Unique transcription induced by presence or absence of HBHA and MTP during early infection. Gene ontology analysis revealed that the uniquely expressed genes during DM infection were significantly involved in the following top 5 biological processes: immune system process, response to external stimulus, response to virus, regulation of immune system process and positive regulation of immune system process. The uniquely expressed genes during WT infection were significantly involved in the following top 5 biological processes: intracellular signal transduction, regulation of response to wounding, positive regulation of multicellular organismal process, regulation of response to stress and positive regulation of response to stimulus. The functional implications of the differential unique transcriptional regulation by HBHA and MTP was explored by IPA enrichment analysis. The most significant canonical pathway enriched by unique genes in WT infection was the adipogenesis pathway (Fig. 4.4A) whilst in DM infection the T helper cell differentiation canonical pathway displayed the most significant association (Fig. 4.4B). DM infection elicited the most upstream regulators compared with WT infection (Fig. 4.4C). WT elicited unique transcripts displayed the most enrichment for NF-κB (complex), which was enriched to a greater degree compared to DM elicited unique transcripts. DM infection displayed the most enrichment for Prolactin (PRL). WT elicited unique transcripts were associated with migration of cells (Fig. 4.4D). DM elicited unique transcripts displayed decreased association with migration of cells compared to WT elicited unique transcripts and the highest association with degradation of DNA.

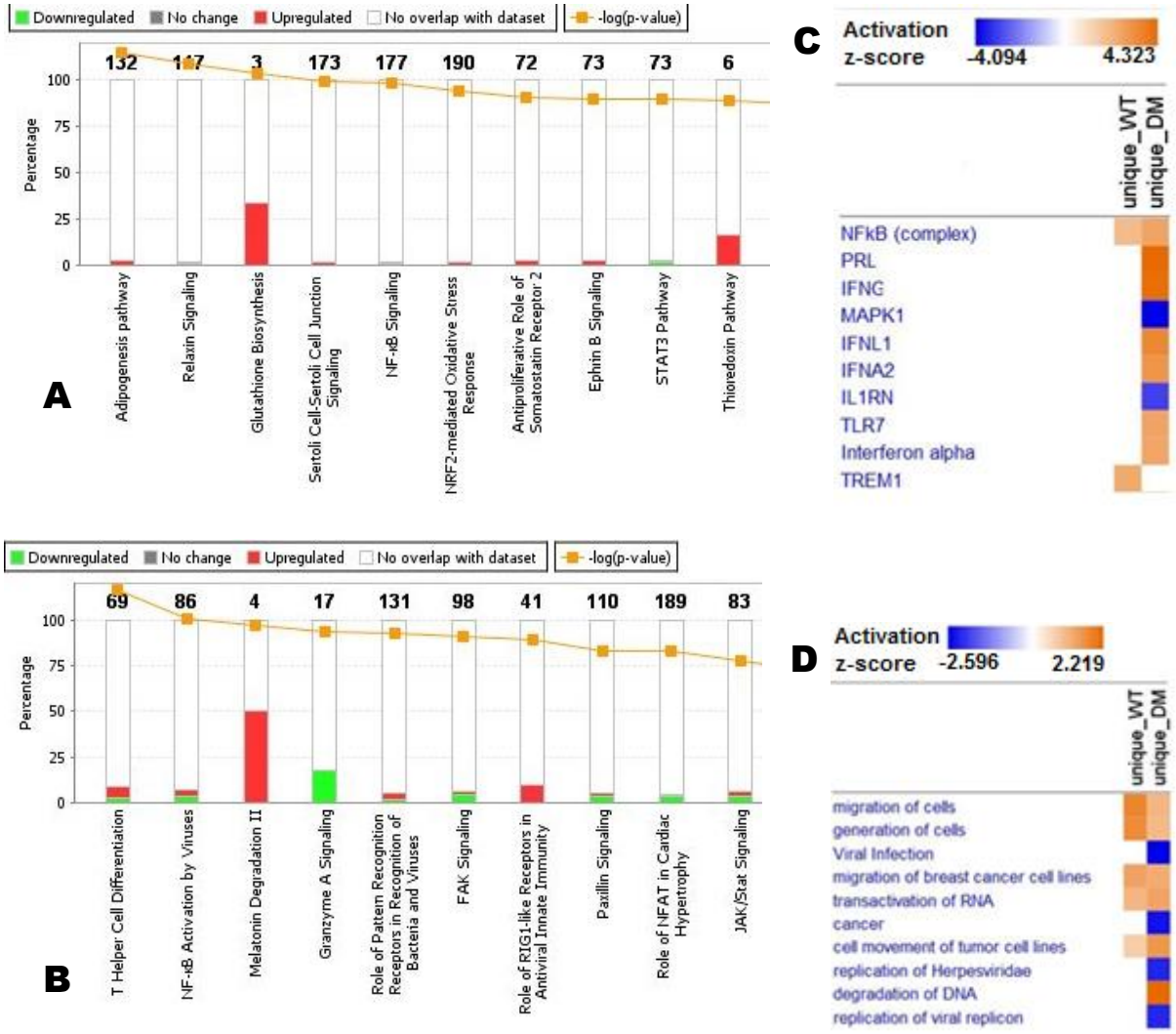

Fig. 4.4: Unique transcripts enrichment of canonical pathways elicited by WT infection (A), and DM infection (B), WT and DM infection elicited upstream regulator activation (C) and disease and biological function (D). Unique elicited transcripts were interrogated using IPA enrichment for differential regulation of canonical pathways, upstream regulators and disease and biological functions. The WT uniquely induced transcripts were associated with the most significant enrichment of Adipogenesis pathway, whilst DM infection induced the most significant enrichment of T helper cell differentiation. Unique transcripts elicited by DM infection displayed less enrichment of NF-κB upstream regulator and were associated with migration of cells.

Depletion of HBHA and MTP induced different molecules in the top 10 canonical pathways.

IPA software was used to further understand the impact of the simultaneous deletion of HBHA and MTP on canonical pathway analysis during early infection. Granulocyte Adhesion and Diapedesis, Agranulocyte Adhesion and Diapedesis, Atherosclerosis signalling, NF-κB signalling, Role of IL-17A in Arthritis, Hepatic Fibrosis / Hepatic Stellate Cell Activation, Toll-like Receptor signalling, Glucocorticoid Receptor signalling and TNFR2 signalling were enriched by both WT and DM (Fig. 4.5.A). With the exception of Glucocorticoid Receptor signalling, DM infection induced a lower

–log (p-value) which correlated to a lower significance in all the common pathways, as well as a greater number of molecules elicited than WT infection (Fig. 4.5A).

The TREM1 signalling pathway was the most differentially regulated of the top 10 canonical enriched pathways. Depletion of HBHA and MTP in the DM induced enrichment of Th1 and Th2 Activation Pathway, Th1, Th2, Melatonin degradation, Sumoylation, Methylglyoxal degradation III, Granzyme A signalling, and PCP pathways, but were not induced by the presence of these 2 adhesins in the WT infection as indicated by the absence of the dark blue bars (Fig. 4.5B).

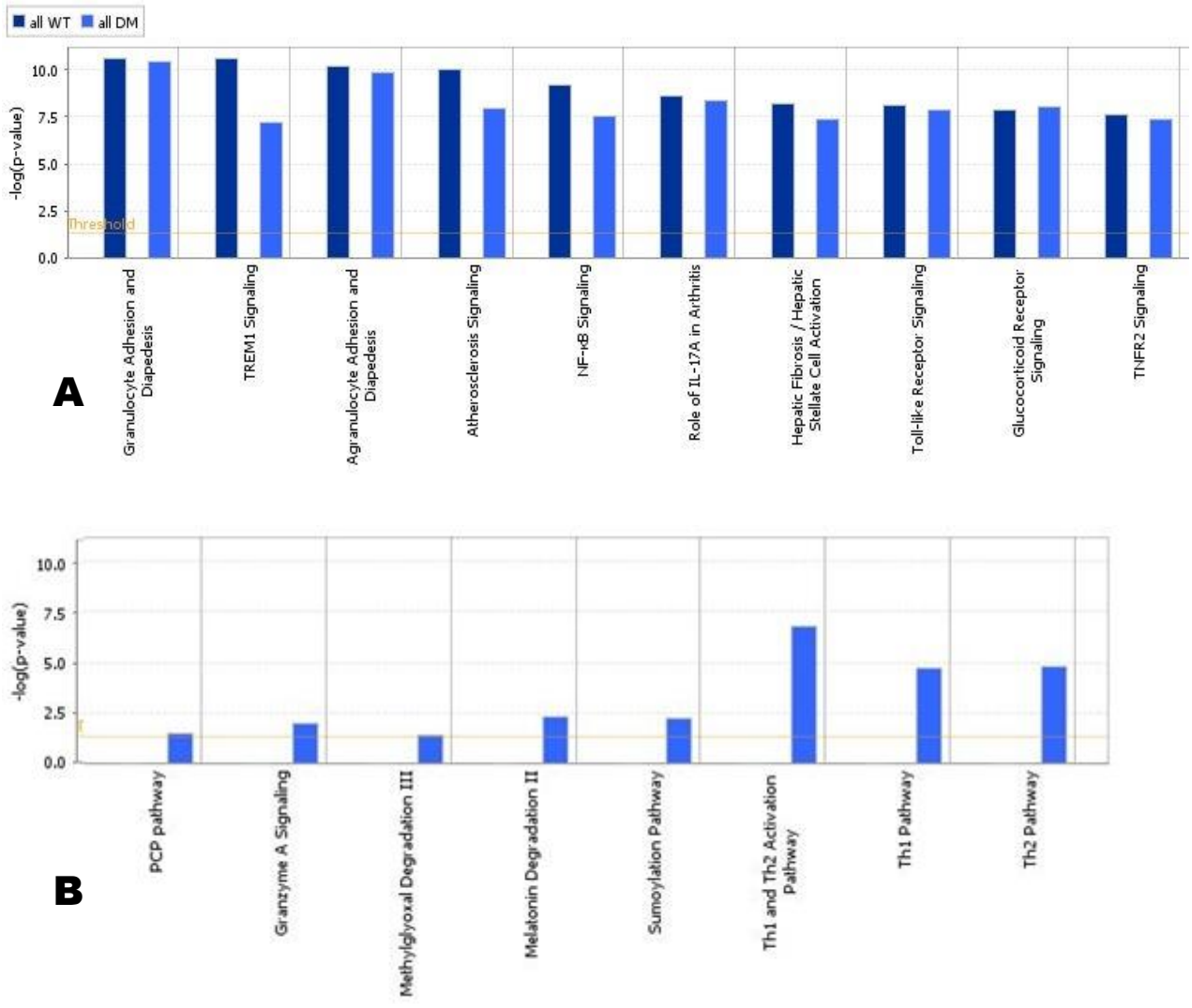

Fig. 4.5: Canonical pathways. (A) Top 10 pathways elicited by all transcripts in WT and DM infections, (B) Pathways elicited only by DM infection. Dark blue and light blue bars represent WT infection and DM infection respectively. Significance is indicated by –log (p-value). A –log (p-value) of 1.3 equates to a p-value of 0.05. The top 10 canonical pathways enriched by all transcripts were similar between both infections, but differed in molecules involved and their significance. WT infection enriched the TREM1 signalling pathway to a greater degree than DM infection in macrophages. DM infection, but not WT, enriched Th1 and Th2 Activation, Th1, Th2, Melatonin degradation, Sumoylation, Methylglyoxal degradation III, Granzyme A signalling, PCP pathways.

HBHA and MTP enhance TREM1 signalling in infected macrophages.

Triggering receptor expressed on myeloid cells 1 (TREM-1), 12 kDa transmembrane protein (DAP12)-associated receptor that is involved in regulation of innate and adaptive immune responses (Colonna and Facchetti, 2003), was the most differentially enriched canonical pathway in the current study. The presence of HBHA and MTP resulted in WT infected macrophages displaying a higher –log (p-value) (10.6) for TREM1 signalling than DM infection (-log (p-value) = 7.2) (Fig. 4.5A). Both strains elicited a similar number of molecules, n= 14 and 13) respectively (Fig. 4.6A) as well as activation z-scores of 3.742 and 3.606 respectively. DM infection resulted in increased levels of *IL-8*, *TNF-α*, *CCL3*, *CD40*, *MCP-1*, *STAT5A* and *NFB1*, whilst WT infection resulted in increased levels of *IL-1β* and *CD54*. NLR family pyrin domain containing 3 (*NLRP3*) was the only SDEG elicited by WT infection and not DM (Fig. 4.6A). qRT-PCR analysis at different time points demonstrated that *NLRP3* was similarly regulated by WT, DM, HC and MC at 1 h post-infection (Fig. 4.6B). At 2 h post-infection, WT displayed the highest up-regulation and the least up-regulation was displayed by HC but this did not reach statistical significance. At 4 h post-infection, WT produced significantly more NLRP3 compared to DM (p = 0.037) and HC (p = 0.038). Toll like receptor 2 (*TLR2*) was the least upregulated SDEGs by both infections (Fig. 4.6A). qRT-PCR analysis at different time points demonstrate that at 1 h and 2 h post-infection, *TLR2* was least up-regulated by DM infection, however, this did not reach statistical significance. At 1 h post-infection, TLR2 was most up-regulated by DM infection and at 2 h post-infection was most up-regulated by WT and MC infection, however, this also did not reach statistical significance. At 4 h, WT (p = HC: 0.000; MC: 0.000) and DM (p = HC: 0.000; MC: 0.000) infection elicited similar levels of TLR2 whilst HC and MC elicited significantly less TLR2 than WT and DM infection (Fig. 4.6C).

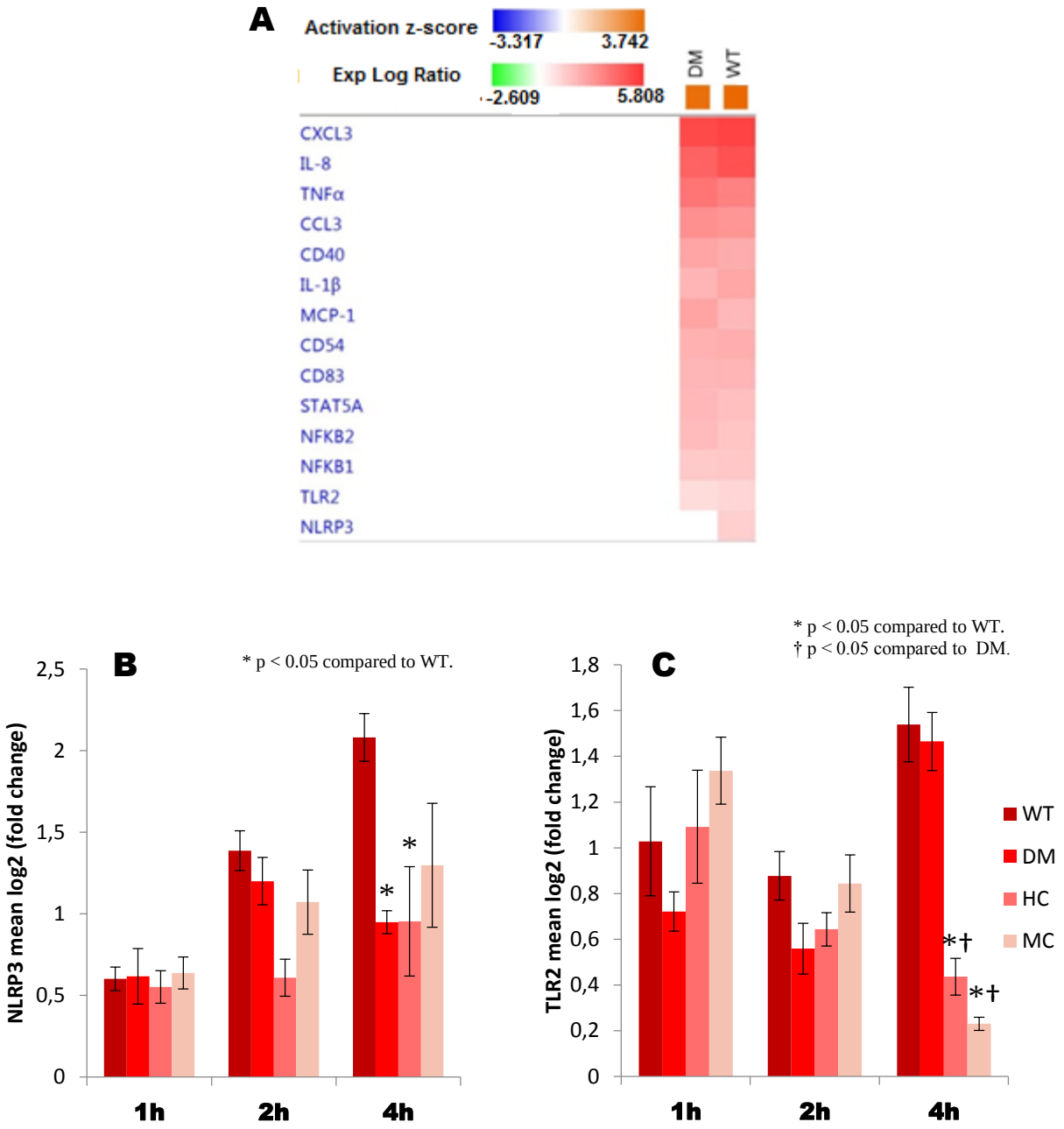

Fig. 4.6: TREM1 signalling. (A) Heatmap of transcripts enriched in the pathway by WT and DM infection respectively. Gene expression by qRT-PCR of *NLRP3* (B) and *TLR2* (C). Colour intensity corresponds to the degree of regulation: red indicating activation, green indicating repression, grey represents genes that were not differentially expressed and white represents genes in the pathway not represented in the dataset, relative to the uninfected control. All DM and all WT represents DM and WT infection respectively. WT infection more significantly enriched TREM1 signalling pathway than DM infection. Investigation of *NLRP3* and *TLR2* transcription regulation demonstrated that MTP possibly modulates *NLRP3* regulation. Both HC and MC displayed decreased levels of transcription of *TLR2*, alluding to a possible compensatory mechanism during double deletion of HBHA and MTP as individually HBHA and MTP impair expression but double deletion restores *TLR2* expression to WT levels.

Lack of HBHA and MTP enriches unique pathways in macrophages.

***Th1 and Th2 Activation, Th1, Th2, Melatonin degradation, Sumoylation, Methylglyoxal degradation III, Granzyme A signalling, PCP pathways*.**

The most significant unique canonical pathway elicited by DM infection was the Th1 and Th2 Activation pathway (-log (*p*-value) = 6.76), whilst the least significant was Methylglyoxal degradation III (-log (p-value) = 1.4). The activation patterns of Th1 and Th2 Activation, Melatonin degradation, Methylglyoxal degradation III, Granzyme A signalling and PCP pathways could not be predicted possibly due to insufficient elicited molecules to accurately predict outcome of these enriched pathways. Th1, Th2 and Sumoylation pathways were activated with z-scores of 0.905, 1.387, and 0.378 respectively. In the Th1 canonical pathway, DM infection uniquely upregulated *CD80, LICOS, STAT4* and *IL-10R* and down-regulated *IFNGR1* and *PIK3C2B* (Fig. 4.7A). In the Th2 canonical pathway, DM infection uniquely upregulated *CD80*, *LICOS*, *STAT4* and down-regulated *TGFBR1* and *PIK3C2B* (Fig. 4.7B). qRT-PCR showed that *CD80* was least upregulated by DM, whilst WT and HC induced similar levels at 1 h post-infection (Fig. 4.7C). At 2 h post-infection, MC induced the highest levels of *CD80*, whilst WT, DM, HC induced similar levels. However, both these findings did not reach statistical significance. At 4 h, DM induced significantly greater expression of *CD80* (p = 0. 003) compared to WT, whilst both HC and MC displayed significantly less than up-regulation (p = HC: 0. 003; MC: 0.001) compared to DM. This suggests that the dual deletion of MTP and HBHA at 4 h influences *CD80* transcription regulation.

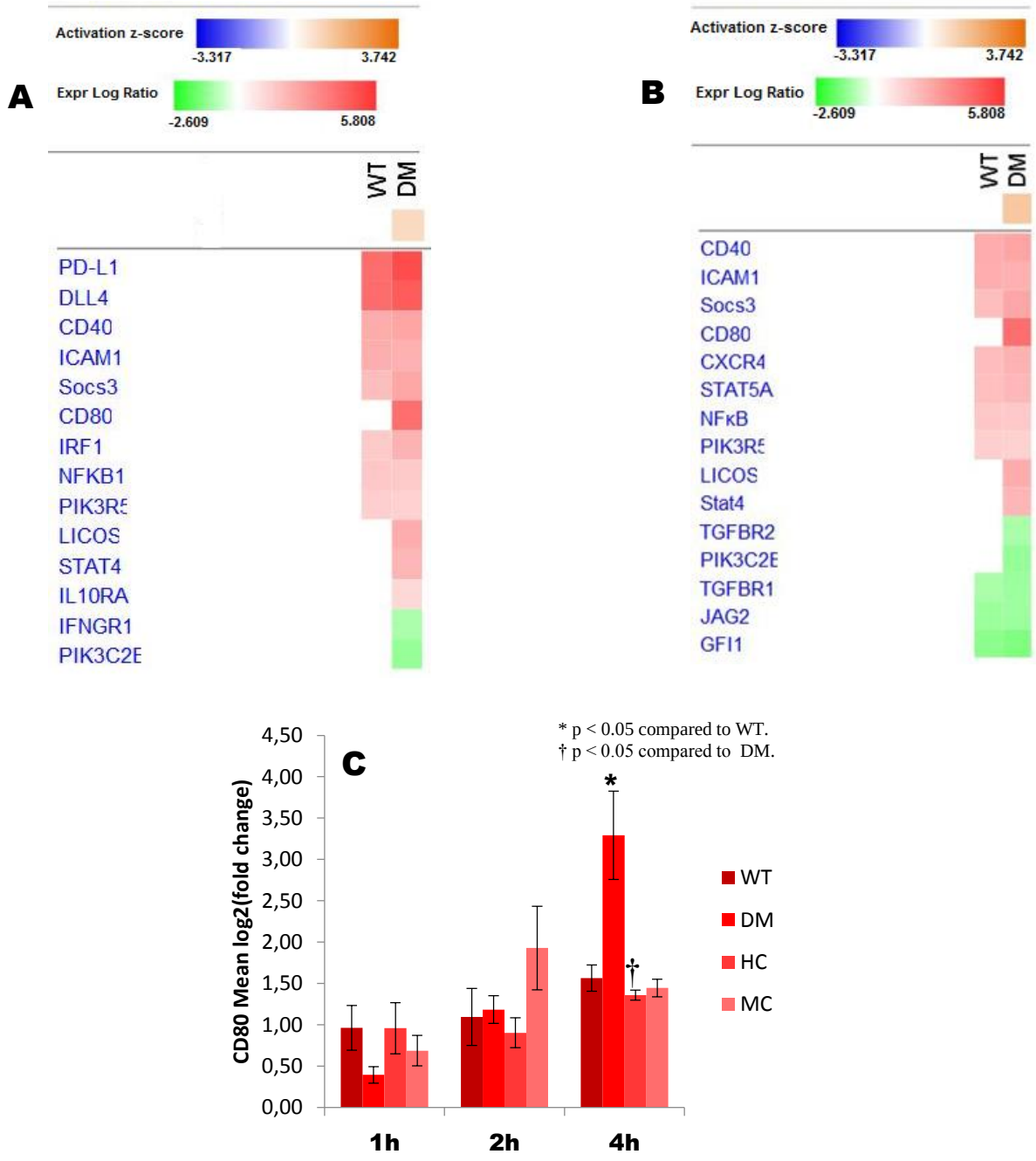

Fig. 4.7: Th1 and Th2 canonical pathway. Heatmap of genes enriched in the (A) Th1 pathway and (B) Th2 pathway by WT and DM infection respectively. (C) Gene expression of *CD80* elicited by WT, DM, HC and MC infection at 1 h, 2 h and 4 h post infection as determined by qRT-PCR. Colour intensity corresponds to the degree of regulation: red indicating activation, green indicating repression, grey represents genes that were not differentially expressed and white represents genes in the pathway not represented in the dataset, relative to the uninfected control. All DM and all WT represents DM and WT infection respectively. DM infection uniquely activated the Th1 and Th2 canonical pathways. At 4 h, DM induced significantly greater expression of *CD80*, whilst both HC and MC displayed significantly less than up-regulation. This suggests that the dual deletion of MTP and HBHA at 4 h influences *CD80* transcription regulation.

Discussion

TB is still the leading cause of death worldwide from a single infectious agent (WHO, 2017), and ongoing, sustained research efforts in TB pathogenesis are essential to elucidate valuable diagnostic and therapeutic biomarkers, and vaccine candidates to adequately control the disease. Early interactions of *M. tuberculosis* and macrophages are facilitated by adhesins and define the outcome of infection (O'Garra *et al*., 2013). This makes adhesins attractive therapeutic targets (Kline *et al*., 2009). RNA sequencing studies have revolutionised pathogenesis research, and facilitated the study of the host immune response to infection (Haas *et al*., 2012). This study identified the transcriptomic and molecular regulation signatures elicited by two adhesins, MTP and HBHA during early infection of macrophages in an attempt to further understand their combined influence on intracellular replication and host immune response to *M. tuberculosis* and thereby evaluating the potential of the combined use of these antigens as a vaccine/biomarker candidate.

MTP, but not HBHA, mediates intracellular replication during infection of macrophages.

None of the mutant strains used in this study achieved complete attenuation of growth in macrophages. It is well-known that *M. tuberculosis* possesses multiple adhesin/invasin molecules and possibly compensate for the loss of HBHA and MTP during macrophage infection by use of, or upregulation of alternate adhesins/invasins. Various *M. tuberculosis* antigens have been previously implicated as adhesins/invasins during infection of macrophages such as 19-kDa antigen (Diaz-Silvestre *et al*., 2005), PstS-1 (38-kDa antigen) (Esparza *et al*., 2015), Ag85A (Armitige *et al*., 2000), Cpn60.2 molecular chaperone (Hickey *et al*., 2009), DnaK molecular chaperone (Hickey *et al*., 2010). In addition, Glutamine synthetase A1 (Tullius *et al*. 2003), *M. tuberculosis* lipoprotein LprE (Padhi *et al*., 2017), *M. tuberculosis* PE_PGRS41 (Deng *et al*., 2017) and Protein tyrosine kinase (Wong *et al*., 2018) have been implicated in intracellular growth of *M. tuberculosis* in macrophages.

Simultaneous deletion of HBHA and MTP resulted in CFU/mL similar in statistical significance to both HBHA and MTP single deletion at 4 h and day 3, but similar to MTP and significantly decreased compared to HBHA single deletion at day 6. These data collectively suggest that MTP, but not HBHA, mediates intracellular replication during infection of macrophages. These results obtained for HBHA were similar to earlier findings using a MT103 *hbhA* mutant in a J774.1 macrophage-like cell line (Pethe *et al*., 2001). These findings are comparable to a recent study (Ramsugit and Pillay, in press) where MTP was shown to impact intracellular survival macrophages but was shown to not influence intracellular survival in epithelial cells.

Macrophages display transcriptional response that is unaffected by HBHA and MTP.

A recent study by Roy *et al*., (2018) demonstrated a more robust transcriptional response at 4 h than 12, 24 or 48 h post-infection. With this in mind, we elucidated the effect of combined HBHA and MTP at

4 h post-infection. On comparison of global transcriptome alterations in the infection models, HBHA and MTP played a limited role in regulation of commonly expressed transcripts. This could possibly represent a common macrophage transcriptional signature elicited by the F15/LAM4/KZN strain family, all *M. tuberculosis* strains or other pathogens. Macrophages have previously demonstrated a common response, to activation by different pathogens, including genes related to receptors, transcriptional factors and signal transduction resulting in immune response activation (Nau *et al*., 2002). Distinct differences in the responses were reported by intracellular and extracellular pathogens (Chaussabel *et al*., 2003). Previous studies have shown induction of genes encoding various ribosomal proteins, cytokines, chemokines, signal transducer and activator of transcription 1 (STAT1) (Wang *et al*., 2003), and cell migration and homing functions (Ragno *et al*., 2001). In the current study, the most commonly expressed genes were associated with the immune system response. Whilst STAT5A was among the commonly expressed genes by both infections, and STAT2 and STAT4 were expressed by DM infection alone, STAT1 was not expressed in this study. Signal transducer and activator of transcription (STAT) proteins control various intracellular signalling events that are vital for growth, homeostasis and regulation (Aaronson and Horvath, 2002).

Deletion of HBHA and MTP resulted in the expression of more unique genes, suggesting stimulation of a more robust transcriptome, possibly due to the DM strain compensating for the loss of these genes by potential upregulation of alternate adhesins during infection of macrophages.

CCL4/macrophage inflammatory protein-1β (*MIP-1β*), the most upregulated gene, was not affected by the deletion of HBHA and MTP. The membrane fraction, but not the cytosolic fraction of *M. tuberculosis*, was demonstrated to induce *MIP-1β* production. *MIP-1β* has previously been described to decrease the intracellular growth of *M. tuberculosis* (Saukkonen *et al*., 2002). It is therefore, evident that the regulation of *MIP-1β* is independent of HBHA and MTP presence or absence.

The most downregulated gene, *TGM5*, induced by the deletion of HBHA and MTP, belongs to the transglutaminase enzyme family. Previous studies have demonstrated that tissue transglutaminase increased cell survival by inhibiting apoptosis (Oliverio *et al*., 1999). HBHA and MTP does not play a significant role in regulation of *TGM5* regulation as demonstrated by lack of a significant trend in induced expression by mutant and complements in the current study.

The presence of HBHA and MTP resulted in the most downregulated gene being the distal-less homeobox 3 (*DLX3*). The significant down-regulation demonstrated by the complements suggest that MTP and HBHA individually might play greater role in the downregulation of *DLX3* than in combination. While *DLX3* demonstrated the ability to regulate osteogenic differentiation and cell viability (Viale-Bouroncle *et al*., 2012) and played an important role in terminal differentiation of keratinocytes (Park and Morasso, 1999), its role in *M. tuberculosis* infection if any, is unknown.

HBHA and MTP influence cellular immune response, cellular signalling and pattern recognition receptors.

The higher number of SDEGs induced by the mutant suggests that the simultaneous absence of both *hbhA* and *mtp* genes resulted in greater host transcriptional changes than the WT strain. However, the WT strain induced a more significant response for all canonical pathways, with the exception of the Glucocorticoid Receptor signalling. This suggests that HBHA and MTP do play a role in cellular immune response, cellular signalling and pattern recognition receptors but their absence results in impaired, albeit incomplete attenuation of these functions. The absence of HBHA and MTP enriched various cellular immune response, cellular growth and metabolite degradation pathways in contrast to the WT infection.

HBHA and MTP enriched TREM1 signalling.

This pathway was significantly enriched to a greater extent by the WT than DM infection. TREM1 associates with DAP12 (Transmembrane adapter molecule) to mediate activation of neutrophils, as well as monocytes, and could possibly play a vital role in the inflammatory process. TREM1 is involved in calcium mobilization and phosphorylation of molecules such as extracellular signal-related kinase 1, extracellular signal-related kinase 2 and phospholipaseC-γ (Bouchon *et al*., 2000). Previous studies have shown TREM1 activation did not induce upregulation of antimicrobial pathways targeting *M. tuberculosis*, but resulted in a production of pro-inflammatory cytokines. In addition, TREM1 activation showed only slight improvement in phagocytosis and resulted in the enhanced T cell proliferation and IFN-γ production (Bleharski *et al*., 2003). In the current study, upstream regulator analysis predicted slight differences in pro-inflammatory cytokines induced by WT and DM infection, possibly due to alternate methods of pro-inflammatory cytokine induction such as toll-like pathway signalling.

Nucleotide binding domain, leucine rich repeat-containing family proteins (NLRs), nucleotide oligomerization domain (NOD)-like receptors, are required in the formation of a multi-protein complex called an inflammasome. *NLRP3* was shown to be vital for IL-1β secretion from human and mouse macrophages infected with *M. tuberculosis* (TeKippe *et al*., 2010). The inflammasome consists of caspase-1, the apoptotic speck protein containing a caspase recruitment domain (ASC) adaptor and a NLRP3 scaffold (Kanneganti *et al*., 2006). *Mycobacterium* muramyl dipeptide and 19-kDa lipoprotein have demonstrated synergized induction of cytokines dependent on both TLR/ nucleotide-binding oligomerization domain 2 (NOD2) signalling (Ferwerda *et al*., 2005). In addition, ESAT-6 positively regulated the *NLRP3* inflammasome leading to necrosis in human macrophages (Mishra *et al*., 2010; Welin *et al*., 2011). In contrast, nitric oxide supresses IL-1β processing that is *NLRP3* inflammasome dependent (Mishra *et al*., 2013) and the leucine rich repeat Fli-I interacting protein 2 (LRRFIP2) inhibits the NLRP3 inflammasome activation (Jin *et al*., 2013). *NLRP3*, a molecule involved in the TREM signalling pathway (Fig. 4.3C) was not significantly expressed during DM infection. These findings

support the current observation of lowered IL-1β expression by the mutant in response to the lack of significant NLRP3 expression compared to the WT.

The decreased expression demonstrated by the hbhA complemented strain suggest that MTP may contribute to a greater extent than HBHA to NLRP3 gene regulation. WT and DM infection both demonstrated similar expression of *TLR2* suggesting that HBHA and MTP do not play a role in gene regulation of *TLR2*, however both complementary strains were impaired in their ability to induce expression of *TLR2*. This suggests that the possible alternate adhesins induced by the deletion HBHA and MTP may be TLR2 ligands such as *M. tuberculosis* PE_PGRS33 (Basu *et al*., 2007), 19-kDa antigen (Lopez *et al*., 2003), heat shock protein 70 (Bulut *et al*., 2005), Early Secreted Antigen ESAT-6 (Chatterjee *et al*., 2011), LprG (Gehring *et al*., 2004) and PPE18 (Nair *et al*., 2009). Further studies are required to elucidate the mechanisms of HBHA and MTP in transcript regulation of *NLRP3* and to explore the *M. tuberculosis* regulation of *TLR2*.

Pathways enriched only in absence of HBHA and MTP.

Th1 and Th2 canonical pathways

The absence of HBHA and MTP enriched Th1 and Th2 Activation, as well as Th1 and Th2 Pathways. These pathways are important in cellular immune response mounted against *M. tuberculosis* (Schluger and Rom, 1998). Naïve T-cell differentiation results from antigenic stimulation of a T cell receptor and CD4 with an antigen-MHC II complex. T cells may be differentiated into five major types of T helper (Th) cells, Th1, Th2, Th17, Tfh, and inducible T regulatory (iTreg) cells. T helper cells play an important part in the adaptive immune system response. Th1 cell differentiation is dependent on the presence of IL-2, IL-12, tumor necrosis factor (TNFα)-, and interferon gamma (IFN-γ), whereas Th2 cell differentiation relies on IL-2, IL-4, IL-7 and thymic stromal lymphopoietin (TSLP) (Zhu and Paul, 2011). Th1 helper cells target intracellular bacteria, viruses, and protozoa by interacting with CD8+ NK / CTL cells and macrophages, leading to killing of infected host cells. Th2 helper cells target extracellular parasites by interacting with B-cells, eosinophils, and mast cells, producing humoral antibodies as well as granulocytic responses (Kaiko *et al*., 2008; Wan, 2015).

In the current study, the Th2 pathway was more activated than the Th1 pathway in the absence of HBHA and MTP. Successful immunity to *M. tuberculosis* requires a strong Th1 response. Higher amounts of Th2 markers were reported in patients than community controls, while these were lower in household contacts compared to the latter (Lienhardt *et al*., 2002). This implies that a stronger Th2 activity is associated with a greater risk of infection than household and community contacts. In addition, higher Th1 activity and lower Th2 responses were also associated with clinical healing (Lienhardt *et al*., 2002). This suggests that in the absence of HBHA and MTP, the stronger Th2 response would possibly exacerbate disease progression. This further supports the notion that HBHA and MTP may be beneficial to the host protective immune response.

In the current study, the deletion of MTP and HBHA significantly increased *CD80* transcription at 4 h post-infection. This could be attributable to the induction of alternate *M. tuberculosis* adhesins/invasins such as AG85B or ESAT-6 (Xu *et al*., 2010) and/or cell wall–associated antigen PPE60 (Su *et al*., 2018). CD80 present on antigen-presenting cells interact with CD28 on T cells resulting in optimal activation T-cells and prevent apoptosis of activated T-cells during *M. tuberculosis* infection (Rajavelu and Das, 2008). The complemented strains and HBHA-MTP proficient WT strain displayed similar expression of *CD80*. This suggests that HBHA and MTP together modulate the regulation of *CD80* to prevent activation of T-cells as a possible immune evasion strategy by *M. tuberculosis*. Future studies are required to elucidate the mechanism of regulation of CD80 employed by HBHA and MTP.

Melatonin Degradation pathway, Granzyme A signalling, PCP pathway, Methylglyoxal Degradation III pathway and Sumoylation pathways.

The unique pathways elicited by DM infection are involved in Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry, Auditory and Vestibular System Development and Function, Embryonic Development, Organ Development, DNA Replication, Recombination, and Repair, Antimicrobial Response, Cell Death and Survival, Endocrine System Development and Function, Energy Production, Lipid Metabolism, Gene Expression; Cellular Development, Cellular Growth and Proliferation.

Indoleamine melatonin, a hormone secreted by the pineal gland of vertebrates, displays immunomodulatory, anti-inflammatory and antioxidant properties. Even though melatonin is ubiquitously present in all cells, mitochondria possess the highest intracellular concentration of melatonin (Slominski *et al*., 2005; Fischer *et al*., 2006; Hardeland 2008). Melatonin has been previously shown to increase the microbicidal activity of isoniazid against *M. tuberculosis* (Wiid *et al*., 1999). Thus, degradation of melatonin is potentially beneficial to the survival of *M. tuberculosis*, and in the current study, may represent a survival strategy of *M. tuberculosis* lacking HBHA and MTP.

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells utilize the granule exocytosis pathway to eradicate infected cells using perforin and granzymes. Granzyme A and granzyme B induce cell death and are delivered to cells via perforin. The tryptase granzyme A stimulates cell death through a caspase-independent mechanism resulting in apoptosis and causes single-stranded DNA nicking, which is distinctive from normal apoptosis (Andersson *et al*., 2007; Lucken-Ardjomande and Martinou 2008; Lieberman 2011). This pathway forms part of cellular immune response mounted by the host cells, suggesting that the presence of HBHA and MTP may hinder the antimicrobial response of host cells to *M. tuberculosis* infection.

The PCP pathway, in conjunction with the Wnt signalling pathway components, transduces extracellular polarity cues, induces intracellular cytoskeleton rearrangements, impacts cell behaviour and induces cell migration (Bejsovec, 2005; Gordon and Nusse, 2006; Inoki *et al*., 2006). This could possibly play a role in the cellular mediated immune response, and the lack of the enrichment of this pathway in the presence of HBHA and MTP, could possibly result in an impaired cellular immune response.

Methyl-glyoxal is produced in small amounts during glycolysis and is highly toxic. In the Methylglyoxal Degradation III pathway, methylglyoxal is reduced to acetol by the action of various enzymes possessing L-glyceraldehyde 3-phosphate reductase activity (Vander Jagts *et al*., 1992; Rachman *et al*., 2006). The survival of host cells is essential for the proliferation of *M. tuberculosis*. It must be noted that this pathway displayed the lowest significance of all the uniquely enriched pathways by DM infection.

The Sumoylation pathway involves post-translational modification capable of changing stability, gene regulation, subcellular localization, and protein-protein interactions. This plays an important role in cell processes such as cell cycle control, oncogenesis, apoptosis, nucleocytoplasmic trafficking and response to virus infection (Rodriguez *et al*., 2001; Comerford *et al*., 2003; Gareau and Lima, 2010). The activation of this pathway could be beneficial to the host immune response because cell processes such as apoptosis would aid in host protection. Together, the enrichment of these pathways only in the absence of HBHA and MTP provides further evidence of the HBHA and MTP are involved in immunomodulation during *M. tuberculosis* infection of macrophages as a possible immune evasion strategy as HBHA and MTP presence prevents enrichment of these pathways.

Limitations

In the current study, the use of single complementation rather than double complementation in the double mutant may skew the interpretation of the study findings. However, the rationale for this choice was to assess the independent effect of MTP and HBHA deletion on replication and transcriptional regulation. Only 3 time-points and a few selected genes were assessed by qRT-PCR. These time-points were chosen due to the characterisation of HBHA and MTP as adhesins/invasins and their involvement in initial cellular signalling.

Conclusion and recommendations for future studies

Overall, the combined, global transcriptomic and canonical pathways analysis revealed that the HBHA and MTP deficiency led to advanced immune activation and decreased intracellular growth. This advanced immune activation would potentially be detrimental to *M. tuberculosis* establishing a successful infection and would suggest that HBHA and MTP play a role in host immune response

modulation as a protective measure during initial infection. These findings contribute to the increasing evidence demonstrating the potential of HBHA and MTP in combination as vaccine candidates. Future work should involve analysis of bacterial transcriptomics during infection of macrophages with the HBHA-MTP double knockout strain and double complemented strain in an attempt to elucidate possible *M. tuberculosis* compensatory mechanisms associated with intracellular replication and host immune activation. In addition, further studies are required to elucidate the mechanisms of HBHA and MTP individual and combined role in transcript regulation of *NLRP3*, *TLR2* and *CD80*.

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CHAPTER 5: Synthesis

In 2016, *M. tuberculosis* the causative agent of Tuberculosis (TB), was the single infectious agent responsible for the highest mortality rates globally (WHO, 2017). The TB burden is currently not reducing rapidly enough to support the targets of the End TB strategy of 80% reduction in incidence by 2030 (WHO, 2017). This is attributed to comorbidities such as Human Immunodeficiency Virus (Karim *et al*., 2010) and diabetes (Dooley and Chaisson, 2009). In addition, this is further exacerbated by drug resistance (Gandhi *et al*., 2006; Moodley *et al*., 2011), lack of point of care diagnostics and lengthy treatment regimens. Efforts to achieve the End TB strategy targets need to be multifaceted, focusing on TB prevention and care cascade (Naidoo *et al*., 2017). New rapid TB diagnostic tools and effective treatment alternatives are dependent on innovative research elucidating pathogenicity of *M. tuberculosis* to identify novel biomarkers for this purpose (WHO, 2017). Adhesins have the potential to address this current requirement due to their easy accessibility and immunogenic characteristics (Stones and Krachler, 2015). Despite much research elucidating the influence of heparin-binding haemagglutinin adhesin (HBHA) and *M. tuberculosis* curli pili (MTP) in independent studies, to date the influence of dual-deletion of these adhesins has not been explored. Therefore, in this study, the effect of *mtp* deletion in a previously generated *hbhA* deficient strain was assessed by comparing the adhesion, invasion, replication, immunogenic capacity and global transcriptomics of macrophages infected with HBHA-MTP proficient and -deficient strains. The findings elucidated the role of the combined adhesins on the pathogenicity of *M. tuberculosis*, and provided insight into their eligibility as biomarkers for drug and vaccine design.

HBHA and MTP influence adhesion and invasion of *M. tuberculosis* to macrophages but do not display synergy during dual deletion.

Bacterial colonization of mammalian host cells is an integral step in pathogenesis (Kline *et al*., 2009). *M. tuberculosis* displays multiple molecules involved in adhesion and invasion of phagocytic and non-phagocytic cells (Adhesins/Invasins) (Govender *et al*., 2014). HBHA mediates adhesion and invasion to non-phagocytic cells (Pethe *et al*., 2001) whilst MTP mediates adhesion and invasion to phagocytic (Ramsugit and Pillay, 2014) and non-phagocytic cells (Ramsugit *et al*., 2016). To test the hypothesis of HBHA and MTP synergistic adhesion to and invasion of macrophages, percentage relative adhesion and invasion rates were calculated and compared between single mutants (Δ*Rv3312A (mtp)* and Δ*Rv0475* (*hbhA*)), single complements (mtp comp and hbhA comp), double mutant (HBHA and MTP deficient strain) and wild-type (HBHA and MTP proficient strain) strains. Deletion of *mtp* in a *hbhA* deficient strain significantly impaired the adhesion and invasion ability of the HBHA and MTP deficient strain in macrophages. However, the MTP single mutant independently demonstrated the most significant loss in adhesion and invasion capacity compared to all strains, suggesting that deletion of *mtp* independently plays a more significant role in adhesion and invasion of macrophages. The reason

for the lack of synergy may be due to the presence of multiple adhesins and invasins. Additionally, the loss of HBHA and MTP possibly resulted in *M. tuberculosis* employing or upregulating alternate adhesins and invasins (Chapter 1).

HBHA and MTP modulate transcript regulation associated with cell signalling during early infection.

Pathogenic bacteria employ adhesion to modulate host cell signalling as a survival and immune evasion strategy, thereby supporting proliferation and pathogenesis (Stones and Krachler, 2015). Macrophages are a well-known reservoir of growing and surviving *M. tuberculosis* bacilli (Van Crevel *et al*., 2002; Guirado *et al*., 2013). In order to understand the mechanisms underlying the combined HBHA and MTP involvement in adhesion and invasion, host transcripts obtained by RNA sequencing, were interrogated at 4 h post infection of macrophages by HBHA and MTP deficient and proficient strains. Expressed transcripts were screened for association with biological adhesion ontology dataset and Ingenuity Pathway Analysis (IPA) software was used to interpret the impact of differential transcriptional regulation.

In addition to their role in adhesion and invasion, HBHA and MTP were observed to induce differential mammalian transcript regulation associated with adhesion of phagocytes, phagocytosis and invasion of cells (Chapter 1). This suggests that HBHA and MTP mediate adhesion and invasion by manipulating the macrophage transcriptional profile to mediate attachment to and internalization of macrophages which is advantageous to *M. tuberculosis* survival.

The HBHA and MTP proficient strain also displayed enhanced activation of the following canonical pathways: Gαq signalling, the acute phase response, role of pattern recognition receptors in recognition of bacteria and viruses, production of nitric oxide and reactive oxygen species in macrophages (Chapter 1). These findings together suggest that the role of HBHA and MTP is not confined to adhesin/invasin functionality during early infection, but these antigens also play a role in shaping the host immune response and intracellular signalling. This alluded that HBHA and MTP may play a significant role as pathogen-associated molecular patterns that are recognised by the host immune response.

HBHA and MTP contribute to intracellular signalling processes through differential regulation of NF-κB signalling canonical pathway, Toll-like Receptor signalling, MAPK and PI3-K signalling canonical pathways.

Mycobacterial HBHA has previously been shown to induce pro-inflammatory cytokines via PI3-K and MAPK crosstalk (Kim *et al*., 2011) and to induce chemokine production (unpublished data from our research group) in macrophages. In Gram negative bacteria, curli pili of *Escherichia coli* K12 (Bian *et al*., 2000) and *Salmonella enterica* serotype Typhimurium Fimbriae (Tükel *et al*., 2005) induce pro-inflammatory cytokines production in macrophages. However, *M. tuberculosis* curli pili (MTP) has

previously been shown to only minimally influence cytokine response in epithelial cells (Ramsugit *et al*., 2016) and macrophages (unpublished data from our research group). The significantly expressed transcripts induced by WT and DM were interrogated further by IPA to assess the influence of HBHA and MTP on host molecular signatures. The present study demonstrated that the HBHA and MTP proficient strain contributed to increased activation of the induced NF-κB signalling canonical pathway, Toll-like Receptor signalling, MAPK and PI3-K signalling canonical pathways (Chapter 2). Therefore, it was speculated that the HBHA and MTP proficient strain would induce a strong pro-inflammatory cytokine response.

HBHA and MTP induces a predominantly pro-inflammatory than anti-inflammatory response during macrophage infection.

Phagocytic cells produce pro-inflammatory cytokines in response to *M. tuberculosis* antigens (Jo *et al*., 2007). These, in conjunction with chemokines, drive the protective host immune response by initiation of cell activation during *M. tuberculosis* infection (Van Crevel *et al*., 2002). Recent unpublished studies elucidated the role of HBHA and MTP independently on cytokine secretion using single mutants. HBHA was described to enhance secretion of chemokines, IL-8, MIP-1 and MCP-1 and MTP did not significantly enhance secretion of cytokine during infection of macrophages. To assess if HBHA and MTP simultaneous deletion impairs macrophage cytokine secretion, protein levels of cytokines/chemokines elicited by HBHA and MTP proficient and deficient strains were quantified using a Bioplex multiplex array at 24 h, 48 h and 72 h post-infection. HBHA and MTP proficient strain produced a strong pro-inflammatory cytokine response supporting the findings of transcript profiling during early infection. Imbalance of the pro-inflammatory response results in tissue damage and an imbalance of the anti-inflammatory response favours the growth of *M. tuberculosis* (Van Crevel *et al*., 2002). Both HBHA (Kim *et al*., 2011) and curli pili (Bian *et al*., 2000; Tükel *et al*., 2005) have previously been demonstrated to induce pro-inflammatory cytokines, suggesting that HBHA and MTP possibly influence TB immunopathology. HBHA and MTP's combined immunogenic ability to induce a protective immune response suggests that these antigens could be considered a novel combination for vaccine development.

HBHA and MTP together influence intracellular replication of *Mycobacterium tuberculosis* to macrophages, but in a non-synergistic manner.

In order to assess the impact of the pro-inflammatory response and differential transcription regulation elicited by HBHA and MTP on the growth of *M. tuberculosis* in macrophages, the intracellular bacillary loads were quantified at 4 h, day 3 and day 6. Deletion of *mtp* in a *hbhA* deficient strain significantly decreased the replication capacity of *M. tuberculosis* in macrophages. However, the most significant decrease was evident in macrophages infected with the independent *mtp* deletion mutant (Chapter 3). The lack of a synergistic loss in replication capacity may again be ascribed to the upregulation of

alternate *M. tuberculosis* antigens and growth factors. This hypothesis can be tested in future studies on *M. tuberculosis* RNA extracted from infected macrophages. These findings provide more evidence suggesting that MTP may be involved in adhesion, invasion and replication in macrophages to a greater extent than HBHA.

HBHA and MTP deletion induces robust global transcriptome changes in macrophages during early infection.

Transcriptional regulation plays a critical role in pathogenesis (Westermann *et al*., 2012). Host global transcriptome analysis has identified various *M. tuberculosis* strain specific responses in previous studies (Tailleux *et al*., 2008; Koo *et al*., 2012; Mvubu *et al.*, 2016). Unpublished studies have demonstrated that HBHA deficient strain enhanced predicted levels of pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ), predicted activation recruitment of phagocytes and significant enrichment of role of pattern recognition receptors in recognition of bacteria and viruses canonical pathway, thereby, suggesting that the aforementioned activation is attributed to alternate molecules. Similar analysis of MTP, demonstrated that MTP influenced canonical pathway and upstream regulator enrichment.

Comparison of whole transcriptomes of macrophages infected with HBHA and MTP-proficient and deficient strains demonstrated a common transcriptional profile induced by F15/LAM4/KZN strain family during early infection of macrophages (Chapter 3). The common transcriptional pattern revealed the most enrichment for granulocyte adhesion and diapedesis canonical pathway, TNF upstream regulation and migration of cells biological function. Deletion of HBHA and MTP resulted in the expression of more unique genes and canonical pathways compared to the WT. This suggests stimulation of a more robust transcriptome, possibly due to compensation for the loss of these genes by the HBHA and MTP deficient strain by potential upregulation of alternate adhesins during infection of macrophages. The HBHA-MTP uniquely elicited transcripts induced the most significant enrichment of Adipogenesis pathway whilst HBHA-MTP deficiency induced the most significant enrichment of T helper cell differentiation. Unique transcripts elicited by HBHA-MTP deficiency induced less enrichment of NF-κB upstream regulator and were associated with migration of cells.

In addition to the validation of RNA sequencing data, the role of HBHA and MTP in gene regulation of selected genes was also investigated using real time PCR (RT-PCR). Comparison of the RT-PCR and RNA sequencing data confirmed significant correlation between both assays. *TGM5* was regulated independently of HBHA and MTP presence or absence, whilst their presence resulted in the most downregulated gene being the distal-less homeobox 3 (*DLX3*). The data also demonstrated that MTP may contribute to a greater extent than HBHA to *NLRP3* transcript regulation. In addition, the loss of both HBHA and MTP at 4 h most likely induced *TLR2* expression in compensation, since both complementary strains were impaired in their ability to induce expression of *TLR2*. *M. tuberculosis*

possibly compensates for loss of HBHA and MTP by upregulation of TLR2 ligands. *CD80* transcript regulation was evaluated in the current study, which demonstrated that the dual deletion of MTP and HBHA increased *CD80* transcription at 4 h post-infection. This could be attributable to the induction of alternate *M. tuberculosis* adhesins/invasins such as AG85B or ESAT-6 (Xu *et al*., 2010) and/or cell wall–associated antigen PPE60 (Su *et al*., 2018). These findings together suggest that in the absence of HBHA and MTP the presence of multiple, alternate antigens stimulate the intracellular signalling and transcriptional regulation *in vitro*. Further investigation into the identity of these antigens would possibly result in a more successful, novel therapeutic target combination in addition to HBHA and MTP.

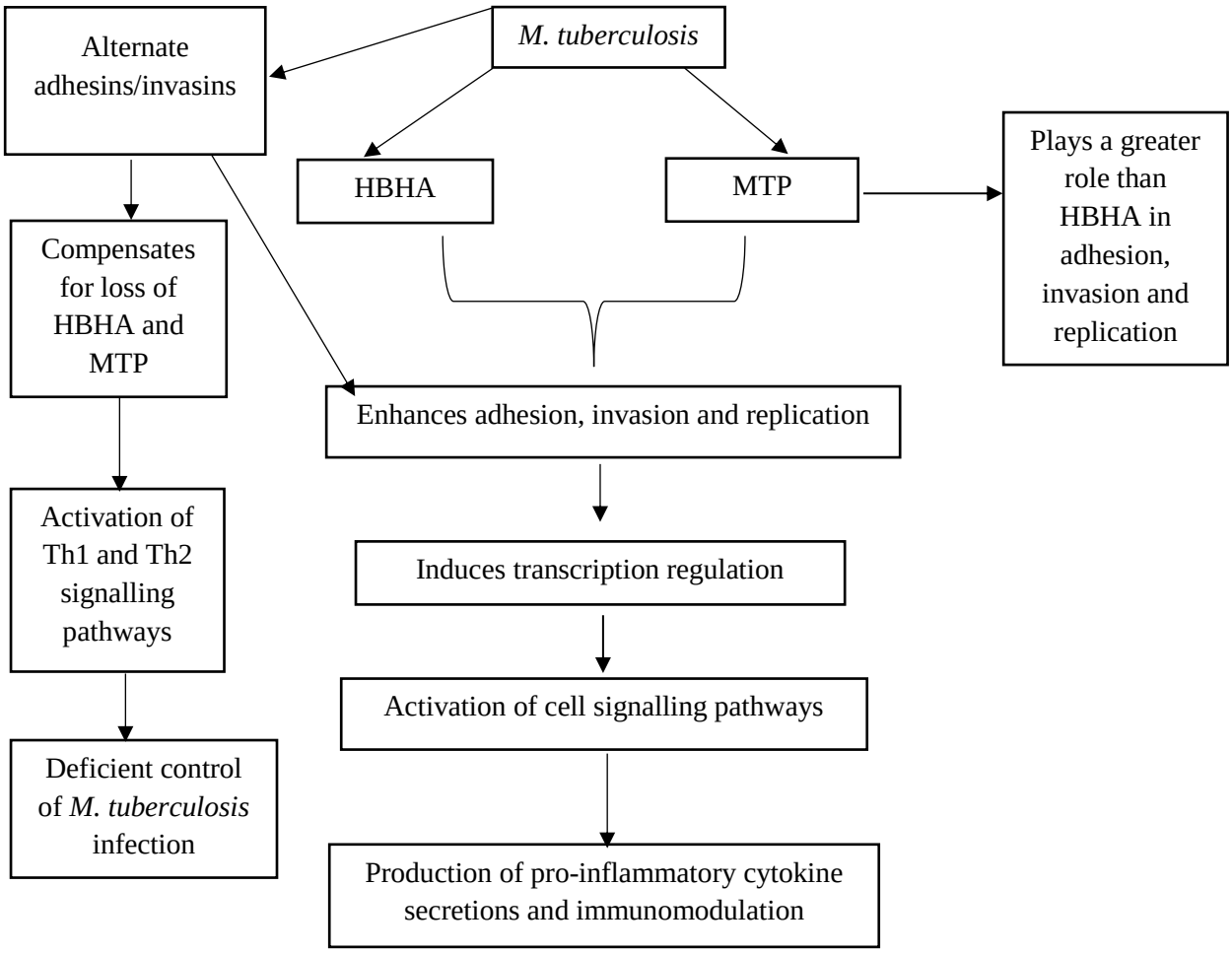

Figure 5.1: Graphical summary of discussion. The impact of double knockout of HBHA and MTP on the *M. tuberculosis* and macrophage interaction is graphically represented above.

Conclusion

This study demonstrated that the deletion of *mtp* in a previously generated *hbhA* single mutant resulted in the impairment of the ability of resultant HBHA and MTP deficient strain to adhere to, invade and replicate in macrophages. The single MTP deficient strain demonstrated greater impairment in ability to adhere to, invade and replicate in macrophages, suggesting that it is a more effective adhesin and invasin than HBHA in this host cell. HBHA and MTP in addition to their role in adhesion, invasion and replication, together play a specific role in the host immune response by inducing various canonical pathways and a strong pro-inflammatory response that is necessary for adequate protective host immune responses during *M. tuberculosis* infection. HBHA and MTP dual-deletion induces a robust differential global transcriptome regulation, suggesting the influence of other adhesins on the host immune response. These findings collectively suggest that the combination of HBHA and MTP represent a novel, powerful immunogenic target as a vaccine candidate. The inclusion of other adhesins would enhance this potential even further.

Limitations

The following limitations may impact on the interpretations of the study findings:

1. RNA sequencing and cytokine protein quantification assays were investigated for a limited selection of time-points. The time-points were chosen based on previous studies by our research group, as well as previous published studies in an attempt to select the most relevant intervals for the current study.
2. RNA sequencing and cytokine protein quantification assays did not include the complemented or single mutant strains. However, RT-PCR was carried out with selected genes, including the complemented strains to investigate some of the gene regulation findings and data from previous studies with single HBHA and MTP mutants have been discussed where applicable.
3. A double complemented strain was not included in the study design. Previous studies in our research group and by other researchers have extensively investigated the role of single mutants, and therefore, this study aimed to elucidate the effect of deletion of *mtp* in *hbhA* mutant strain. With this in mind, it was deemed more valuable in the current study to focus on the independent effect of genes deleted using single complementation to firstly establish the effect of deletion of HBHA and MTP. Future studies will address this limitation.

Recommendations for future work

The current findings should be further explored in future studies including a double complement and functional protein analysis such as RNA sequencing of *M. tuberculosis* transcriptome from infected macrophages to ascertain if *M. tuberculosis* compensates by the use of alternate adhesins during infection of macrophages. Selected canonical pathways should be further investigated using phenotypic assays to elucidate the actual mechanism in which HBHA and MTP independently, and in combination,

activated these pathways. These should include the inflammasome pathway, NF-κB signalling canonical pathway, Toll-like Receptor signalling, MAPK and PI3-K signalling that are important in the signalling of pro-inflammatory cytokines. The role of MTP in the inflammasome pathway regulation and with particular emphasis on NLRP3 regulation should also be further explored. In addition, fusion proteins of MTP and HBHA could be produced and tested in *in vitro* and *in vivo* models of infection to ascertain the protective potential of these antigens. Finally, alternate antigens should be explored, as in combination with HBHA and MTP may evoke a more powerful immunogenic response.

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APPENDICES

Appendix 1: BREC approval

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI

RESEARCH OFFICE
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Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

16 May 2018

Ms Suventha Moodley (207502021)
113 Robin Street
Kearwastan
4092
suventha@gmail.com

Dear Ms Moodley

PROTOCOL: The role of heparin binding haemagglutinin adhesion and curli pili on the pathogenicity of *Mycobacterium tuberculosis*:
Degree Purposes (PhD) **BREC REF: BE516/14**

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	12 May 2018
Expiration of Ethical Approval:	11 May 2019

I wish to advise you that your application for Recertification received on 30 April 2018 in relation to the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The approval will be ratified by a full Committee at a meeting to be held on 12 June 2018.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc: pillayc@ukzn.ac.za

Appendix 2: Media Recipes

Middlebrook 7H9 broth (1L)

- 900 mL distilled water
- 4.71g Middlebrook 7H9 powder (Difco, Becton, Dickinson and Company, South Africa)
- 100 mL oleic acid-albumin-dextrose-catalase (Becton, Dickinson and Company, South Africa)
- 10 mL of 50% (w/v) glycerol
- 2.5 mL 20% Tween-80 (Sigma, Capital lab supplies, South Africa

1. Dissolved 4.71g of Middlebrook 7H9 powder (Difco, Becton, Dickinson and Company, South Africa in 900 mL of distilled water
2. Autoclaved at 121ºC for 15 mins and cooled.
3. Added 10 mL of 50% (w/v) glycerol, 2.5 mL of 20% Tween-80 (Sigma, Capital lab supplies, South Africa) and 100 mL of oleic acid-albumin-dextrose-catalase (Becton, Dickinson and Company, South Africa).
4. Stored at 4°C until further use.

Middlebrook 7H11 Agar (1L)

- 21 g Middlebrook 7H11 powder (Difco, Becton, Dickinson and Company, South Africa)
- 900 mL distilled water
- 100 mL oleic acid-albumin-dextrose-catalase (Becton, Dickinson and Company, South Africa)
- 10 mL of 50% (w/v) glycerol (Merck, South Africa)

1. Dissolved 21 g of Middlebrook 7H11 powder (Difco, Becton, Dickinson and Company, South Africa) in 900 mL of distilled water.
2. Autoclaved at 121ºC for 15 mins and cooled.
3. Added 10mL of 50% (w/v) glycerol (Merck, South Africa) and 100ml of oleic acid-albumin-dextrose-catalase (Becton, Dickinson and Company, South Africa) were added after
4. Dispensed approximately 12.5 mL was aliquoted into 65mm petri dishes, and
5. Stored at 4°C until further use.

50% (v/v) Glycerol (100 ml)

- 50 mL glycerol (Merck, South Africa)
- 50 mL autoclaved distilled water

1. Dissolved 50 ml of glycerol (Merck, South Africa) in approximately 50 ml of autoclaved distilled water
2. Filtrate sterilised through a 0.22µm membrane.
3. Stored at 4°C until further use.

20% (v/v) Tween-80

- 20 mL Tween-80 (Sigma, Capital lab supplies, South Africa)
- 80 mL autoclaved distilled water

1. Dissolved 20 mL Tween-80 (Sigma, Capital lab supplies, South Africa) in 80 mL autoclaved distilled water.
2. Filtrate sterilised through a 0.22µm membrane.
3. Stored at 4°C until further use.

Phosphate buffered saline (PBS)

- 10 PBS tablets (Oxoid, Quantum Biotechnologies, South Africa)
- 1000 mL distilled water

1. Dissolved 10 PBS tablets in 1000mL autoclaved distilled water.
2. Autoclaved at 121°C for 15 mins.
3. Added 2.5 ml 20% Tween 80 for serial dilutions (omitted for PBS used in wash steps).
4. Stored at 4°C until further use.

Diethylpyrocarbonate (DEPC-treated) water (1L)

- 1 mL 0.1% DEPC (Sigma, Capital lab supplies, South Africa)
- 1 L autoclaved distilled water

1. Added 1 ml of 0.1% DEPC (Sigma, Capital lab supplies, South Africa) to 1 L of autoclaved distilled water.
2. Left at room temperature for overnight.
3. Autoclaved at 121°C for 15 mins.
4. Stored at room temperature until further use.

MOPS Buffer (1L)

- 41.9 g MOPs (Sigma, Capital lab supplies, South Africa)
- 8.2 g Sodium acetate.$3H_2O$ (Sigma, Capital lab supplies, South Africa)
- 3.72g Ethylenediaminetetraacetic acid (EDTA) (Sigma, Capital lab supplies, South Africa)

1. Dissolved MOPs in 1000 mL of DEPC-treated water.
2. Autoclaved at 121°C for 15 mins.
3. Stored at room temperature in darkroom until further use.

Appendix 3: MOPS gel electrophoresis

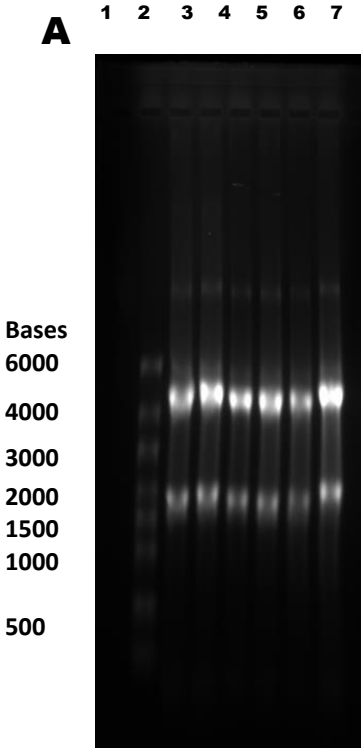
Gel preparation:

1. Added 0.5 g of agarose (Sigma, Capital lab supplies, South Africa) into 36 ml DEPC-treated water.
2. Heated until agarose (Sigma, Capital lab supplies, South Africa) dissolved and cooled till 60°C
3. Added 5 mL 10 x MOPS buffer (Sigma, Capital lab supplies, South Africa) and 9 mL 37% formaldehyde (Sigma, Capital lab supplies, South Africa).
4. Poured gel into casting tray and allowed to set for 30 mins.

RNA sample preparation and Electrophoresis:

1. Added 2.5 µl of RNA to 2.5 µl of RNase-free water (1:1 dilution).
2. Incubated samples at 65°C for 10 mins and cooled on ice for 2mins.
3. Added 5 µl of RNA gel loading dye (Thermofisher Scientific, South Africa).
4. Loaded and ran at 65 V until dye has migrated ¾ of gel.
5. Visualised in the G box. Expose for 80 ms.

A

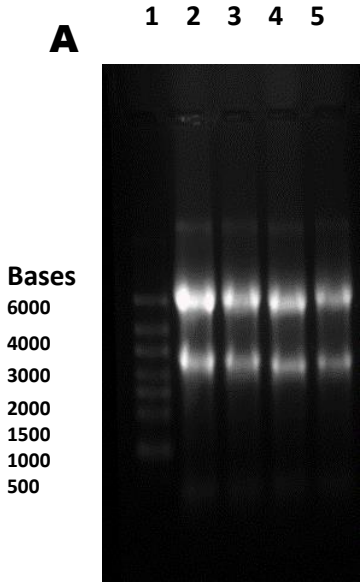


B

Lanes on gel	Sample ID	Conc.	Dilution	260/280	260/230
1	Riboruler	-		-	-
2	WT1 4 h	1745 ng/ µL	1:1	2.11	2.27
3	DM1 4 h	2075.2 ng/ µL	1:1	2.1	2.23
4	WT2 4 h	1295.7 ng/ µL	1:1	2.13	1.79
5	DM2 4 h	1428.3 ng/ µL	1:1	2.1	2.29
6	WT3 4 h	1167.5 ng/ µL	1:1	2.12	2.29
7	DM3 4 h	2042.2 ng/ µL	1:1	2.13	2.06

Fig. 1: RNA quality control of samples sent for sequencing. MOPS gel electrophoresis (A) and sample loading information and nanodrop readings (B).

A

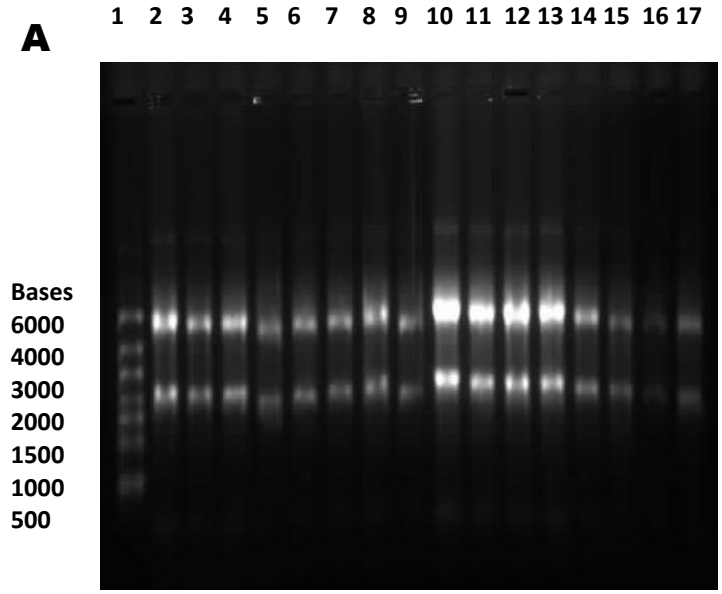


B

Lanes on gel	Sample ID	Conc.	Dilution	260/280	260/230
1	Riboruler	-		-	-
2	WT1 1 h	2707.2 ng/µL	1:1	2.13	2.22
3	DM1 1 h	1835.9 ng/µL	1:1	2.13	2.19
4	HC1 1 h	1704.5 ng/µL	1:1	2.14	2.21
5	MC1 1 h	1404.4 ng/µL	1:1	2.13	2.21

Fig. 2: RNA quality control of samples (1 h biological experiment 1) qRT-PCR. MOPS gel electrophoresis (A) and sample loading information and nanodrop readings.

A

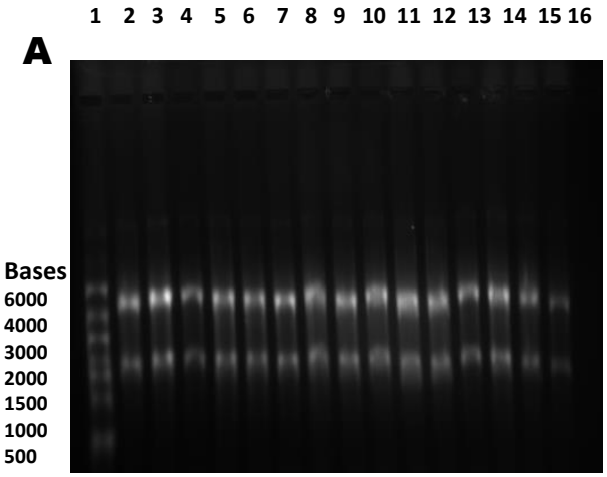


B

Lanes on gel	Sample ID	Conc.	Dilution	260/280	260/230
1	Riboruler	-		-	-
2	WT2 2 h	1231.7 ng/µL	1:1	2.13	2.13
3	DM2 2 h	798.9 ng/µL	1:1	2.11	2.15
4	HC2 2 h	970.8 ng/µL	1:1	2.12	2.15
5	MC2 2 h	708.7 ng/µL	1:1	2.12	2.22
6	WT2 1 h	658.2 ng/µL	1:1	2.09	1.91
7	DM2 1 h	676.6 ng/µL	1:1	2.10	2.19
8	HC2 1 h	1149.2 ng/µL	1:1	2.11	2.19
9	MC2 1 h	820.6 ng/µL	1:1	2.13	1.78
10	WT1 4 h	2370.8 ng/µL	1:1	2.10	2.12
11	DM1 4 h	1711.8 ng/µL	1:1	2.12	2.19
12	HC1 4 h	1586.6 ng/µL	1:1	2.12	2.03
13	MC1 4 h	1497.0 ng/µL	1:1	2.12	2.07
14	WT1 2 h	795.0 ng/µL	1:1	2.12	2.17
15	DM1 2 h	417.8 ng/µL	n/a	2.14	1.64
16	HC1 2 h	489.8 ng/µL	n/a	2.12	1.94
17	MC1 2 h	345.9 ng/µL	n/a	2.13	1.92

Fig. 3: RNA quality control of samples (2 h biological experiment 2, 1 h biological experiment 2, 4 h biological experiment 1 and 2 h biological experiment 1) qRT-PCR. MOPS gel electrophoresis (A) and sample loading information and nanodrop readings (B).

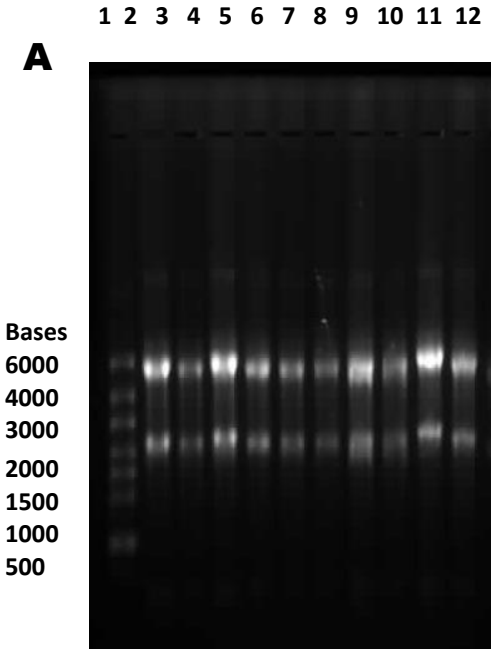
A



B

Lanes on gel	Sample ID	Conc.	Dilution	260/280	260/230
1	Riboruler	-		-	-
2	WT2 4 h	684.8 ng/µL	1:1	2.11	2.00
3	DM2 4 h	884.5 ng/µL	1:1	2.09	2.22
4	MC2 4 h	508.3 ng/µL	1:1	2.09	2.13
5	WT3 4 h	464.4 ng/µL	1:1	2.12	2.04
6	DM3 4 h	457.0 ng/µL	1:1	2.11	2.14
7	HC3 4 h	630.9 ng/µL	1:1	2.11	2.25
8	MC3 4 h	478.6 ng/µL	1:1	2.11	2.15
9	WT3 1 h	406.3 ng/µL	1:1	2.14	1.01
10	DM3 1 h	595.1 ng/µL	1:1	2.12	2.18
11	HC3 1 h	664.1 ng/µL	1:1	2.14	1.96
12	MC3 1 h	471.5 ng/µL	1:1	2.12	2.06
13	WT3 2 h	529.7 ng/µL	1:1	2.05	2.00
14	DM3 2 h	474.2 ng/µL	1:1	2.10	1.99
15	HC3 2 h	444.9 ng/µL	1:1	2.10	2.15
16	MC3 2 h	332.0 ng/µL	1:1	2.10	2.10

Fig. 4: RNA quality control of samples (4 h biological experiment 2, 4 h biological experiment 3, 1 h biological experiment 3 and 2 h biological experiment 3) qRT-PCR. MOPS gel electrophoresis (A) and sample loading information and nanodrop readings (B).



B

Lanes on gel	Sample ID	Conc.	Dilution	260/280	260/230
2	Riboruler	-		-	-
3	N2 2 h	1128.62 ng/µL	1:1	2.12	1.98
4	N3 2 h	635.13 ng/µL	1:1	2.05	2.19
5	N1 4 h	1618.16 ng/µL	1:1	2.10	2.16
6	N2 4 h	831.67 ng/µL	1:1	2.12	2.03
7	N1 2 h	591.8 ng/µL	1:1	2.10	2.15
8	N3 4 h	634.8 ng/µL	1:1	2.10	2.01
9	N3 1 h	828.0 ng/µL	1:1	2.12	2.16
10	HC2 4 h	671.3 ng/µL	1:1	2.10	2.16
11	N1 1 h	1792.9 ng/µL	1:1	2.13	2.17
12	N2 1 h	1007.8 ng/µL	1:1	2.14	2.00

Fig. 5: RNA quality control of samples (uninfected (N) for 1 h, 2 h and 4 h and HC2 4 h) qRT-PCR. MOPS gel electrophoresis (A) and sample loading information and nanodrop readings (B).

Appendix 4: Bioinformatics analysis

1. Forward and reverse raw sequencing reads were downloaded from the ARC server for duplicate samples of WT, DM and N. Files were concatenated using the following template command:
   cat DM1_ATCACG_L002_R1_001.fastq.gz DM1_ATCACG_L002_R1_002.fastq.gz >DM1_R1.fastq.gz

2. Raw reads were subsequently trimmed using trimmomatic. Trimmomatic was downloaded using the following command: **sudo apt install trimmomatic** and template script was:
   java -jar trimmomatic-0.36.jar PE DM1_R1.fastq.gz DM1_R2.fastq.gz TDM1_R1_forward_paired_trimmed.fastq.gz TDM1_R1_trimmed_forward_unpaired.fastq.gz TDM1_R2_reverse_paired.fastq.gz TDM1_R2__reverse_unpaired.fq.gz ILLUMINACLIP:/home/ub2/Documents/Suventha/working/cat_raw_data/Su_RNA_Seq_data/raw_data/Trimmomatic-0.36/adapters/TruSeq3-PE-2.fa:2:30:10 LEADING:3 TRAILING:3 MINLEN:36

3. Trimmed reads were assessed for quality control using FastQC software (version 0.11.5).

4. Trimmed and quality approved reads were uploaded onto server and analysed using previously installed Tophat2 (version 2.1.0) integrated with Bowtie2 (version 2.2.1) and Cuffdiff (version 2.2.1) of the RNA Tuxedo suite of tools.

5. The reference genome was downloaded using the following command:
   wget ftp://igenome:G3nom3s4u@ussd- ftp.illumina.com/ Homo_sapiens /UCSC/ hg38/ Homo_sapiens _UCSC_ hg38.tar.gz

6. The reference genome was unzipped using the following command:
   tar -zxvf downloaded_ref_genome.tar.gz

7. Trimmed reads were aligned to Hg38 reference genome using Tophat2 using the following template tophat run file and script:

Run file example:

#!/bin/sh

#SBATCH --nodes=1
#SBATCH --ntasks-per-node=20
#SBATCH --mem=64000
#SBATCH --time=24:00:00

TMPDIR=/tmp

./tophat_trimmed_DM1_B1_T.sh

Script example:

#!/bin/sh

/home/alecia/installs/bioinformatics/tophat/tophat-2.1.0.Linux_x86_64/tophat2 -p 20 -o /data/georgina/Suventha/DM1_B1_tophat_trimmed_T -G

/data/georgina/Ref_genomes/Homo_sapiens/UCSC/hg38/Annotation/Genes/genes.gtf --transcriptome-index=known/mrna /data/georgina/Ref_genomes/Homo_sapiens/UCSC/hg38/Sequence/Bowtie2Index/genome /data/georgina/Suventha/Trimmed_RNA_Seq_data/TDM1_R1_forward_paired_trimmed.fastq.gz /data/georgina/Suventha/Trimmed_RNA_Seq_data/TDM1_R2_reverse_paired.fastq.gz

- **/data/georgina/Suventha:** an output directory where all the Tophat outputs
- **/data/georgina/Ref_genomes/Homo_sapiens/UCSC/hg38/Sequence/Bowtie2Index/genome**: location of reference genome
- **/data/georgina/Suventha/Trimmed_RNA_Seq_data/TDM1_R1_forward_paired_trimmed.fastq, /data/georgina/Suventha/Trimmed_RNA_Seq_data/TDM1_R2_reverse_paired.fastq.gz :**location of forward and reverse reads.

8. Differential expression analysis of WT and DM infection, relative to uninfected (N) macrophages was carried out using the accepted_hits.bam files generated by tophat using the following template cuffdiff run file and script:

Run file:

#!/bin/sh

#SBATCH --nodes=1
#SBATCH --ntasks-per-node=20
#SBATCH --mem=64000
#SBATCH --time=24:00:00

TMPDIR=/tmp

./cuff_N_vs_DM.sh

SCRIPT file:

#!/bin/sh

/home/alecia/installs/bioinformatics/cufflinks/cufflinks-2.2.1.Linux_x86_64/cuffdiff -p 20 -o cuffdiff_trial_N_vs_DM -L Uninfected,hbhA-mtp -b /data/georgina/Ref_genomes/Homo_sapiens/UCSC/hg38/Sequence/WholeGenomeFasta/genome.fa /data/georgina/Ref_genomes/Homo_sapiens/UCSC/hg38/Annotation/Genes/genes.gtf /data/georgina/Suventha/tophat_trimmed_N1_genome/accepted_hits.bam,/data/georgina/Suventha/tophat_trimmed_N2_genome/accepted_hits.bam /data/georgina/Suventha/tophat_trimmed_DM1_L/accepted_hits.bam,/data/georgina/Suventha/tophat_trimmed_DM2_genome/accepted_hits.bam

9. The generated genes_exp.diff file was downloaded from server and used for subsequent downstream analysis.
10. Two different downstream pipelines:
    A. Biological adhesion pipeline
    B. Significantly differentially expressed genes pipeline

A. *Biological adhesion pipeline*

1. Cuffdiff outputs for uninfected vs WT and uninfected vs DM were filtered according to the Gene Ontology biological adhesion dataset sourced from EntrezGeneIds using the molecular signatures database (http://software.broadinstitute.org/gsea/msigdb/index.jsp).
2. The genes found were subsequently filtered for genes with a False Discovery Rate q-value <1.
3. Generated datasets were subsequently analysed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA). Canonical pathway and biofunction enrichment analysis were carried out with IPA.

B. *Significantly differentially expressed genes pipeline*

1. Cuffdiff outputs for uninfected vs WT and uninfected vs DM were filtered significantly differentially expressed genes (SDEGs) with a False Discovery Rate q-value <0.05.

2. Generated datasets were subsequently analysed with the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, USA). Canonical pathway, upstream regulators and biofunction enrichment analysis were carried out with IPA.

Table 1: Number of reads and mapping rate to Hg38 genomes.

Sample	Number of Reads	Overall read mapping rate (%)
WT1	24096527	93.8
WT2	31555387	92.8
DM1	19580870	91.1
DM2	18062023	90.0
N1	21923049	90.2
N2	19186660	89.4

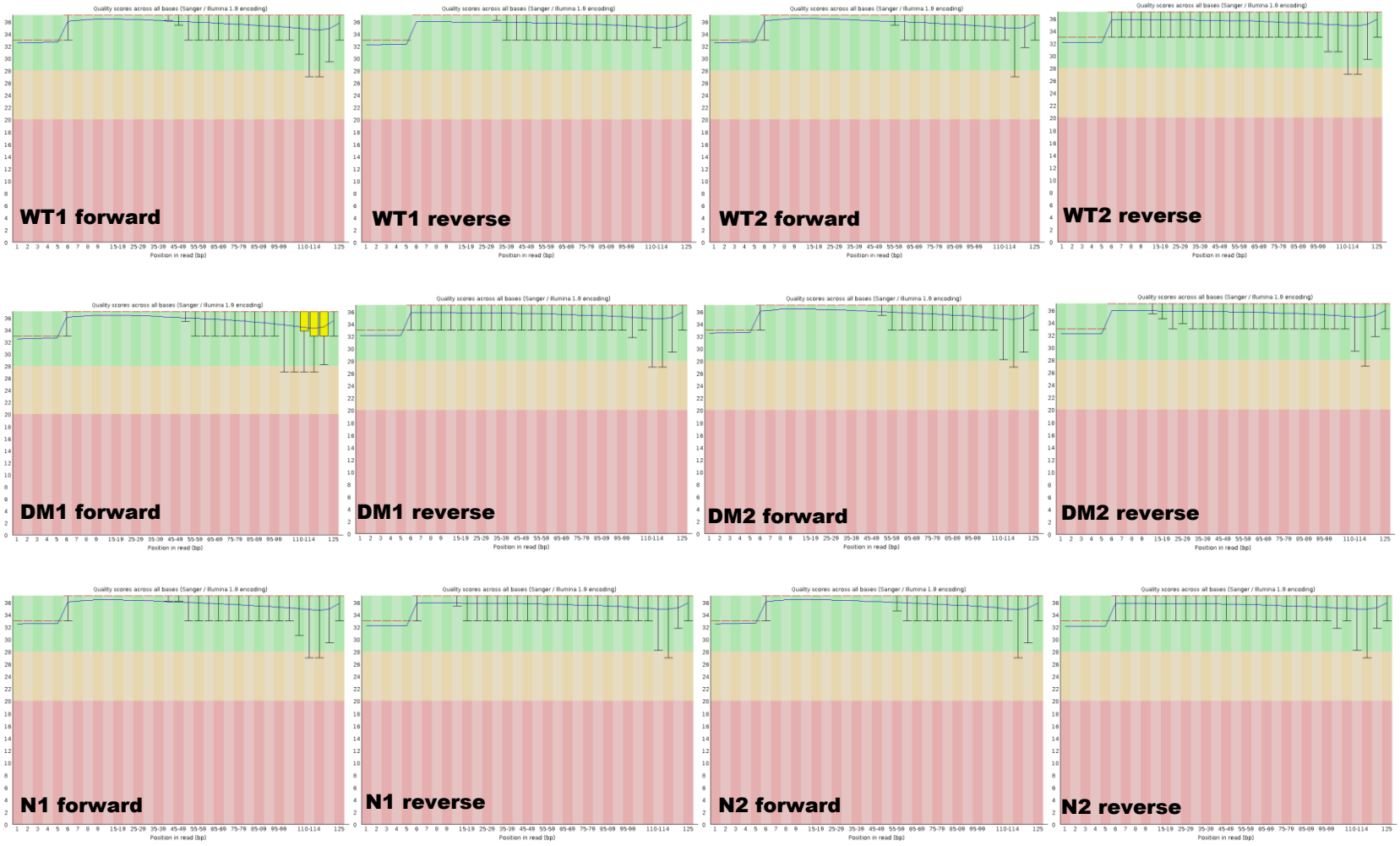


Fig. 6: FastQC per base quality scores of trimmed reads for all samples.

Table 2: Genes associated with biological adhesion elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
AATF	0,064552	**CLDN1**	2,0815	**ICAM1**	2,29404	**OMG**	1,51085	**SIGLEC9**	-1,1449
ABL1	0,078355	**CLDN12**	0,111013	**ICAM2**	0,945536	**PARVB**	0,157881	**SIRPA**	0,462639
ABL2	1,11659	**CLDN15**	0,460623	**ICAM3**	-0,54116	**PARVG**	-0,46847	**SLAMF7**	0,760996
ACTB	-0,21333	**CLDN22**	0,756751	**ICAM4**	0,746006	**PATZ1**	-0,25304	**SLC11A1**	0,013086
ACTG1	-0,06163	**CLDN23**	-0,39585	**ICAM5**	0,964413	**PCDH20**	0,504195	**SLC7A11**	1,56773
ACTN1	-0,11895	**CLDN7**	-1,55319	**ICOSLG**	2,20422	**PCDH7**	-1,00415	**SMAD3**	1,38204
ADA	0,898843	**CLEC7A**	0,728814	**ID1**	-1,43581	**PCDHAC2**	0,117687	**SMAD6**	-0,06646
ADAM15	-0,31412	**CLIC1**	0,034601	**IFNAR1**	0,192154	**PCDHB2**	-0,12457	**SMAGP**	-0,06554
ADAM17	0,445263	**CLSTN1**	-0,44817	**IFNB1**	2,72364	**PCDHB5**	-0,5083	**SORBS3**	-0,32998
ADAM8	0,293956	**CLSTN2**	-0,37757	**IGFBP7**	-0,3981	**PCDHGA10**	-0,3821	**SOX4**	-0,40911
ADAM9	0,145565	**CLSTN3**	-0,34778	**IL18R1**	5,8568	**PCDHGA11**	-0,35694	**SP3**	0,057897
AGGF1	0,034419	**COL6A1**	1,40891	**IL1B**	2,52728	**PCDHGB4**	-0,25581	**SPECC1L**	-0,07389
AIMP1	0,088911	**COL6A2**	1,49293	**IL23A**	3,84271	**PCDHGC3**	-0,39624	**SPG7**	0,224384
AKIP1	-0,27457	**COL7A1**	0,359748	**IL32**	0,927844	**PCDHGC5**	-0,06612	**SPN**	-0,78631
ALCAM	0,28395	**CORO1A**	-0,70653	**IL7R**	1,36284	**PDPK1**	-0,0635	**SPOCK1**	0,499297
AMIGO2	-0,11782	**CREBBP**	0,552259	**ILK**	-0,14402	**PDPN**	-0,44228	**SPON2**	1,16808
AMIGO3	1,15868	**CSF3R**	-0,0799	**INPPL1**	-0,23956	**PEAK1**	1,44579	**SPP1**	-0,02616
ANTXR1	-1,61171	**CSRP1**	-0,39591	**IRF1**	1,48923	**PECAM1**	-0,43541	**SRC**	2,15025
ANXA1	0,253397	**CSTA**	-0,54944	**ITGA3**	-0,36047	**PERP**	1,24919	**SRF**	-0,09848
APBB1IP	-0,46807	**CTNNA1**	0,143487	**ITGA4**	-0,81346	**PIK3CB**	-0,32534	**SRGAP2**	0,005217
APC	0,18478	**CTNNAL1**	-0,23746	**ITGA5**	0,671119	**PIK3CD**	-0,32839	**SRPX2**	0,199341
APP	-0,1149	**CTNNB1**	0,366176	**ITGA6**	0,923171	**PIK3CG**	0,19614	**SSX2IP**	-0,50937
ARF6	-0,43434	**CTNND1**	-0,08528	**ITGA7**	-0,68686	**PIP5K1A**	-0,04202	**STAB1**	-1,02638
ARHGEF7	0,182454	**CTTN**	0,711256	**ITGAE**	0,218108	**PIP5K1C**	0,108177	**STAT5B**	0,135231
ATG5	0,189395	**CX3CR1**	-1,66023	**ITGAL**	-0,55362	**PKD1**	0,433808	**STAT6**	-0,0377

Table 2: Genes associated with biological adhesion elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
ATP1B1	0,946732	**CXCR3**	0	**ITGAM**	-0,26569	**PKN2**	0,04408	**STK10**	0,341053
ATP2A2	0,239152	**CXADR**	-1,24751	**ITGAV**	0,70214	**PKNOX1**	-0,01276	**STK11**	0,116392
ATP2C1	0,207631	**CYP1B1**	-0,33366	**ITGAX**	-0,01419	**PKP4**	-0,24483	**STOML2**	0,189313
ATP7A	-0,32547	**DAG1**	0,372543	**ITGB1**	0,014797	**PLEK**	1,47571	**STXBP1**	0,128131
AXL	-0,16109	**DDOST**	0,047638	**ITGB1BP1**	0,254166	**PLXNC1**	-0,08131	**STXBP3**	-0,07582
AZI2	0,203345	**DGCR2**	-0,38961	**ITGB2**	0,186804	**PNN**	-0,03824	**SYK**	-0,60503
B2M	0,27872	**DLG1**	-0,17522	**ITGB3**	0,612063	**PODXL**	0,542677	**SYMPK**	0,265474
B4GALT1	1,1136	**DLG5**	-0,31557	**ITGB3BP**	0,061997	**PODXL2**	0,170176	**TAOK2**	0,240864
BATF	-0,71362	**DLL4**	4,16847	**ITGB5**	-0,28828	**PPARD**	0,4545	**TESK2**	-0,21135
BAX	0,054172	**DNAJA3**	0,1715	**ITGB7**	-0,33891	**PPFIA1**	0,001024	**TGFB1**	-0,10754
BCAR1	0,842598	**DNAJB6**	0,325849	**ITGB8**	2,34665	**PPFIBP1**	-0,13046	**TGFB1I1**	-0,59909
BCL2	0,047669	**DOCK2**	0,034734	**ITPKB**	-0,94309	**PPP3CA**	-0,07674	**TGFBI**	-0,02083
BCL2L11	-0,49936	**DOCK8**	-0,40491	**JAG2**	-1,16349	**PPP3CB**	0,06113	**THBS2**	-1,01276
BCL3	1,8695	**DSC2**	-0,5641	**JAM2**	0	**PREX1**	-0,05215	**THBS3**	-0,11294
BRAF	0,264107	**DST**	1,137	**JMJD6**	0,348673	**PRKCA**	0,248314	**TLN1**	0,119734
BTN3A1	-0,10325	**EBI3**	3,25167	**JUP**	-0,89575	**PRKD2**	0,126366	**TLN2**	0,028607
BVES	-0,0918	**EFNB1**	-0,56621	**KIAA1462**	-0,75252	**PRKDC**	0,227374	**TMEM8A**	-0,16094
BYSL	0,166746	**EFNB2**	3,3038	**KIF13B**	0,1349	**PRKX**	-0,49692	**TNF**	3,53964
C10orf54	-1,05505	**EGFL7**	-0,20359	**KIFC3**	0,039074	**PSEN1**	0,25296	**TNFAIP6**	5,80764
CADM1	0,308636	**EGR1**	-0,00277	**KITLG**	1,41664	**PSMB10**	0,093059	**TNFRSF12A**	-0,74579
CADM4	0,253924	**EIF2AK4**	-0,25067	**LAMA3**	-0,66767	**PSTPIP1**	-0,56115	**TNFSF14**	-0,83095
CASP8	-0,32031	**ELF4**	0,291037	**LAMB3**	-0,04117	**PTGER4**	1,30071	**TNFSF9**	0,743596
CASS4	-0,0337	**ELMO2**	-0,12948	**LAMC1**	-0,06333	**PTK2B**	-0,68934	**TNIP1**	0
CBLL1	0,165045	**EMILIN2**	-0,54012	**LAT**	0,284575	**PTPN2**	0,311938	**TOR1A**	-0,35606
CCL19	5,49719	**ENG**	0,02946	**LCP1**	0,185892	**PTPN22**	-0,2357	**TPBG**	-0,63569
CCL2	2,00344	**ENTPD1**	-0,24351	**LEPR**	0,047097	**PTPN6**	-0,00655	**TRIP6**	0,017918
CCL21	-0,77537	**EOMES**	-0,41438	**LFNG**	1,42531	**PTPRC**	-0,13825	**TRPM7**	-0,08505

Table 2: Genes associated with biological adhesion elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CCL4	6,38722	**EPDR1**	-1,29505	**LGALS3BP**	-0,00777	**PTPRF**	-0,49782	**TSC1**	0,128141
CCL5	0,115175	**EPHA1**	-1,51818	**LIG4**	0,225262	**PTPRK**	1,31971	**TSPAN32**	-0,95271
CCND3	-0,78909	**EPHB3**	-1,64542	**LMLN**	0,081417	**PTPRS**	-0,24005	**TSTA3**	-0,13715
CCR1	-0,16363	**ERBB2IP**	0,215217	**LPP**	0,540076	**PVR**	0,961281	**TYRO3**	-0,29196
CD151	-0,03313	**ESAM**	-0,20227	**LPXN**	0,914924	**PVRL1**	0,063793	**VAMP3**	-0,05152
CD164	-0,30686	**EZR**	-0,42658	**LRFN3**	-0,25574	**PVRL2**	0,062885	**VAV1**	-0,09896
CD1D	-1,77429	**F11R**	-0,40886	**LRP6**	0,107597	**PVRL3**	1,22339	**VCAM1**	4,99245
CD209	-0,60777	**FADD**	-0,19946	**MAEA**	0,155379	**PXN**	-0,03893	**VCL**	-0,8168
CD22	-0,13589	**FARP2**	0,043047	**MAFB**	0,317419	**RAB1A**	0,218499	**VEZT**	0,052672
CD226	0,668441	**FAT1**	0,016212	**MALT1**	-0,37472	**RAB27A**	-0,96216	**VMP1**	0,313128
CD276	0,127137	**FCER1G**	-0,77335	**MARCKS**	3,07282	**RAC1**	-0,15814	**VWF**	-0,49319
CD2AP	-0,06931	**FER**	0,001759	**MERTK**	-0,41898	**RAC2**	-0,01641	**WAS**	-0,25951
CD300A	0,019975	**FERMT2**	-1,04422	**METAP1**	-0,65079	**RAP2B**	-0,69323	**WHAMM**	-0,08526
CD33	-0,32542	**FERMT3**	0,139151	**MFGE8**	-0,13733	**RAPGEF1**	0,595071	**ZBTB1**	0,249592
CD36	0,307757	**FEZ1**	1,41842	**MICA**	-0,04841	**RC3H1**	0,325779	**ZFP36L1**	-0,68431
CD4	-0,5414	**FGFRL1**	-0,1217	**MICALL2**	0,029038	**RC3H2**	0,188984	**ZFP36L2**	-0,56866
CD44	1,07177	**FIBP**	-0,23209	**MICB**	-0,68562	**RCC2**	0,119714	**ZFPM1**	-0,09206
CD47	-0,34417	**FKBP1A**	-0,08673	**MKLN1**	0,129094	**RELB**	1,83382	**ZYX**	-0,15868
CD58	0,751088	**FLNA**	0,922333	**MLLT4**	-0,17679	**RET**	-0,90886		
CD63	-0,07279	**FLOT2**	-0,46151	**MPZL3**	-0,27783	**RGMB**	-0,46413		
CD72	-0,73058	**FLRT2**	0,136595	**MSN**	-0,08896	**RHOB**	0,010701		
CD74	0,097381	**FNDC3A**	-0,62688	**MTSS1**	0,630534	**RHOH**	0,969676		
CD80	3,55075	**FOXP1**	0,080613	**MYBPC3**	1,38602	**RIC8A**	0,044791		
CD81	-0,04759	**FUT7**	-1,61015	**MYBPH**	-0,25502	**RIPK2**	0,855225		
CD84	-0,4169	**FYN**	-0,06017	**MYH9**	0,010648	**RIPK3**	-0,35342		
CD86	0,011153	**FZD5**	0,210417	**MYL12A**	-0,22678	**RND3**	1,69267		
CD9	-0,35719	**FZD7**	-0,90003	**MYL9**	-0,61353	**ROBO1**	1,31012		

Table 2: Genes associated with biological adhesion elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CD93	0,354043	**GAS6**	-0,24606	**MYO1G**	-0,70735	**ROCK1**	-0,24142		
CD99	-0,3009	**GCNT1**	-1,65324	**NCAM2**	-0,42706	**RPL22**	-0,12855		
CD99L2	-0,53374	**GNAS**	0,111768	**NCK1**	-0,13304	**RPS6**	0,189772		
CDC42	-0,04481	**GNE**	-0,29353	**NCK2**	0,330261	**RPSA**	0,233968		
CDH23	0,958303	**GOLPH3**	-0,22825	**NCR3**	-0,35665	**RSAD2**	1,41974		
CDH24	-0,55418	**GP1BB**	0,236467	**NCSTN**	-0,007	**RUNX2**	0,141017		
CDK5	-0,48367	**GP9**	-0,63645	**NEDD4**	0,008239	**S100A9**	0,594467		
CDK5R1	-0,19639	**GPNMB**	-0,52654	**NEDD9**	0,02386	**SATB1**	-0,20611		
CDK6	0,33746	**HAPLN3**	0	**NHEJ1**	0,120293	**SCARB1**	-0,04506		
CDON	-0,81175	**HBB**	2,15919	**NID1**	0,14351	**SCRIB**	-0,01598		
CEBPB	2,2375	**HCK**	0,801558	**NINJ1**	1,51239	**SDC3**	0,057249		
CELSR1	0,197075	**HES1**	0,440764	**NINJ2**	-0,51681	**SDK1**	0,010896		
CEP41	-0,16805	**HLA-E**	0,297122	**NKAP**	0,060261	**SELPLG**	0,185831		
CERCAM	-0,3517	**HMGB1**	-0,19254	**NLGN4Y**	1,59209	**SEMA4A**	0,743131		
CFDP1	-0,07475	**HPSE**	-1,30499	**NME2**	0,127805	**SEMA4D**	-0,62095		
CGREF1	0,091668	**HSH2D**	0	**NPHP4**	0,620031	**SHC1**	0,285747		
CHD7	-0,33907	**HSP90AB1**	0,342514	**NPTN**	0,294054	**SIGLEC1**	0,078707		
CHST10	-0,6732	**HSPB1**	-0,54887	**NT5E**	1,00996	**SIGLEC11**	-0,01163		
CIB1	0,161503	**HSPD1**	0,067616	**OLR1**	1,23749	**SIGLEC14**	-0,37604		

Table 3: Genes associated with biological adhesion elicited by DM infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
AATF	-0,00891	**CLDN12**	0,039607	**ICAM3**	-0,28589	**PATZ1**	-0,46904	**SLC7A11**	1,57238
ABL1	-0,06392	**CLDN15**	0,354061	**ICAM4**	0,795689	**PCDH20**	-0,0017	**SMAD3**	0,90996

Table 3: Genes associated with biological adhesion elicited by DM infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
ABL2	1,26707	**CLDN22**	0	**ICAM5**	1,42976	**PCDH7**	-0,87108	**SMAD6**	-0,55215
ACTB	-0,27331	**CLDN23**	0,398851	**ICOSLG**	2,35262	**PCDHAC2**	0	**SMAGP**	-0,29367
ACTG1	-0,24198	**CLDN7**	-0,75537	**ID1**	-1,95357	**PCDHB2**	-0,22251	**SORBS3**	0,540753
ACTN1	-0,12179	**CLEC7A**	0,530191	**IFNAR1**	0,147478	**PCDHB5**	-1,43014	**SOX4**	-0,72927
ADA	0,798805	**CLIC1**	-0,0347	**IFNB1**	4,24164	**PCDHGA10**	-0,47483	**SP3**	-0,1111
ADAM15	-0,32387	**CLSTN1**	-0,53369	**IGFBP7**	-0,87936	**PCDHGA11**	-0,24352	**SPECC1L**	-0,21146
ADAM17	0,168002	**CLSTN2**	-0,43979	**IL18R1**	6,32373	**PCDHGB4**	-0,18294	**SPG7**	0,054024
ADAM8	0,741468	**CLSTN3**	-0,20025	**IL1B**	2,09382	**PCDHGC3**	-0,10086	**SPN**	-0,82543
ADAM9	-0,10717	**COL6A1**	1,6225	**IL23A**	4,00216	**PCDHGC5**	0	**SPOCK1**	-0,38443
AGGF1	-0,19946	**COL6A2**	1,63086	**IL32**	1,72168	**PDPK1**	-0,19626	**SPON2**	1,38018
AIMP1	-0,38454	**COL7A1**	0,845836	**IL7R**	1,16117	**PDPN**	-0,10713	**SPP1**	-0,22137
AKIP1	-0,53954	**CORO1A**	-0,61168	**ILK**	-0,24891	**PEAK1**	1,63547	**SRC**	2,74771
ALCAM	0,024153	**CREBBP**	0,683915	**INPPL1**	-0,15466	**PECAM1**	-0,69829	**SRF**	-0,13684
AMIGO2	-1,29107	**CSF3R**	-0,01901	**IRF1**	2,18042	**PERP**	1,28643	**SRGAP2**	0,244476
AMIGO3	0,996279	**CSRP1**	-0,5021	**ITGA3**	-0,65122	**PIK3CB**	-0,30974	**SRPX2**	-0,00231
ANTXR1	-1,94365	**CSTA**	-0,37077	**ITGA4**	-0,92333	**PIK3CD**	-0,35678	**SSX2IP**	-0,85399
ANXA1	-0,18695	**CTNNA1**	-0,02629	**ITGA5**	0,399748	**PIK3CG**	-0,16939	**STAB1**	-1,13184
APBB1IP	-0,74495	**CTNNAL1**	-0,38262	**ITGA6**	0,825857	**PIP5K1A**	0,099261	**STAT5B**	0,122647
APC	0,121416	**CTNNB1**	-0,07536	**ITGA7**	-0,54552	**PIP5K1C**	0,396964	**STAT6**	0,07626
APP	-0,28446	**CTNND1**	0,034547	**ITGAE**	0,016922	**PKD1**	0,514184	**STK10**	0,127318
ARF6	-0,38936	**CTTN**	0,488434	**ITGAL**	-0,47635	**PKN2**	-0,1034	**STK11**	-0,03914
ARHGEF7	0,032778	**CX3CR1**	-1,46054	**ITGAM**	-0,45547	**PKNOX1**	-0,12684	**STOML2**	-0,011
ATG5	-0,06056	**CXCR3**	0,97712	**ITGAV**	0,349469	**PKP4**	-0,37553	**STXBP1**	0,129251
ATP1B1	0,928915	**CXADR**	0	**ITGAX**	0,12417	**PLEK**	1,24909	**STXBP3**	-0,40109
ATP2A2	0,239617	**CYP1B1**	-0,44617	**ITGB1**	-0,15896	**PLXNC1**	0,206115	**SYK**	-0,87882
ATP2C1	0,298161	**DAG1**	0,109544	**ITGB1BP1**	-0,04307	**PNN**	-0,06678	**SYMPK**	0,258281
ATP7A	-0,42565	**DDOST**	-0,07224	**ITGB2**	0,141546	**PODXL**	0	**TAOK2**	0,281762

Table 3: Genes associated with biological adhesion elicited by DM infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
AXL	0,115392	**DGCR2**	-0,55748	**ITGB3**	-0,07349	**PODXL2**	-0,05661	**TESK2**	-0,28963
AZI2	0,113182	**DLG1**	-0,15651	**ITGB3BP**	-0,48873	**PPARD**	0,471538	**TGFB1**	0,062235
B2M	0,081488	**DLG5**	-0,60701	**ITGB5**	-0,5714	**PPFIA1**	-0,16432	**TGFB1I1**	0,343722
B4GALT1	0,747909	**DLL4**	4,61804	**ITGB7**	-0,54615	**PPFIBP1**	-0,17062	**TGFBI**	0,167056
BATF	-0,86036	**DNAJA3**	-0,08149	**ITGB8**	1,8526	**PPP3CA**	-0,22881	**THBS2**	-1,27481
BAX	-0,06356	**DNAJB6**	0,414396	**ITPKB**	-0,94579	**PPP3CB**	-0,10132	**THBS3**	-0,22624
BCAR1	1,26397	**DOCK2**	-0,16125	**JAG2**	-1,14071	**PREX1**	-0,36012	**TLN1**	0,096169
BCL2	-0,09755	**DOCK8**	-0,54342	**JAM2**	1,31857	**PRKCA**	0,102829	**TLN2**	0,039271
BCL2L11	-0,98519	**DSC2**	-0,79713	**JMJD6**	0,105646	**PRKD2**	0,265086	**TMEM8A**	-0,13941
BCL3	2,38907	**DST**	1,35092	**JUP**	-0,81199	**PRKDC**	0,134517	**TNF**	3,93477
BRAF	0,126763	**EBI3**	3,60565	**KIAA1462**	-1,4197	**PRKX**	-0,67361	**TNFAIP6**	5,47481
BTN3A1	-0,16305	**EFNB1**	-0,81047	**KIF13B**	0,009744	**PSEN1**	0,229044	**TNFRSF12A**	-0,22466
BVES	0,084328	**EFNB2**	0	**KIFC3**	-0,19393	**PSMB10**	-0,06559	**TNFSF14**	-0,86773
BYSL	0,006508	**EGFL7**	-0,04016	**KITLG**	1,23425	**PSTPIP1**	-0,13026	**TNFSF9**	0
C10orf54	-1,12307	**EGR1**	0,07756	**LAMA3**	-1,25706	**PTGER4**	1,14902	**TNIP1**	2,02672
CADM1	0,080758	**EIF2AK4**	-0,4776	**LAMB3**	0,013609	**PTK2B**	-0,56655	**TOR1A**	-0,53538
CADM4	0,426816	**ELF4**	0,184667	**LAMC1**	-0,2166	**PTPN2**	0,211605	**TPBG**	-0,72307
CASP8	-0,42423	**ELMO2**	-0,14169	**LAT**	0,345069	**PTPN22**	-0,6765	**TRIP6**	0,017726
CASS4	-0,15801	**EMILIN2**	-0,3639	**LCP1**	0,02936	**PTPN6**	-0,00279	**TRPM7**	-0,2495
CBLL1	-0,20794	**ENG**	0,144683	**LEPR**	-0,4603	**PTPRC**	-0,23115	**TSC1**	0,031915
CCL19	5,65255	**ENTPD1**	-0,28492	**LFNG**	1,18626	**PTPRF**	-0,5354	**TSPAN32**	-0,88761
CCL2	2,6028	**EOMES**	-0,30436	**LGALS3BP**	-0,2652	**PTPRK**	1,42425	**TSTA3**	-0,1951
CCL21	-0,8275	**EPDR1**	-1,09308	**LIG4**	-0,04969	**PTPRS**	-0,03767	**TYRO3**	-0,30301
CCL4	6,45048	**EPHA1**	-1,27804	**LMLN**	-0,27392	**PVR**	1,04982	**VAMP3**	-0,09411
CCL5	0,277887	**EPHB3**	0	**LPP**	0,349765	**PVRL1**	0,468949	**VAV1**	-0,20347
CCND3	-0,78471	**ERBB2IP**	0,168596	**LPXN**	0,724012	**PVRL2**	0,286398	**VCAM1**	5,0056

Table 3: Genes associated with biological adhesion elicited by DM infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CCR1	0,093486	**ESAM**	0,285186	**LRFN3**	0,088919	**PVRL3**	1,04929	**VCL**	-1,02919
CD151	-0,15588	**EZR**	-0,37981	**LRP6**	-0,05679	**PXN**	0,036429	**VEZT**	-0,21171
CD164	-0,68054	**F11R**	-0,50263	**MAEA**	0,062577	**RAB1A**	0,005822	**VMP1**	0,49813
CD1D	-2,284	**FADD**	-0,49194	**MAFB**	0,200392	**RAB27A**	-0,9391	**VWF**	-0,65854
CD209	-0,69157	**FARP2**	-0,19371	**MALT1**	-0,35074	**RAC1**	-0,19509	**WAS**	-0,23965
CD22	-0,50578	**FAT1**	0,256985	**MARCKS**	3,02788	**RAC2**	0,115051	**WHAMM**	-0,31978
CD226	0,30137	**FCER1G**	-0,69766	**MERTK**	-0,78012	**RAP2B**	-0,702	**ZBTB1**	0,057716
CD276	0,310873	**FER**	0,45348	**METAP1**	-0,64961	**RAPGEF1**	0,464174	**ZFP36L1**	-0,69501
CD2AP	-0,20049	**FERMT2**	-1,12909	**MFGE8**	-0,20083	**RC3H1**	0,261917	**ZFP36L2**	-0,25346
CD300A	-1,48089	**FERMT3**	0,223884	**MICA**	0,281897	**RC3H2**	0,181016	**ZFPM1**	0
CD33	-0,53241	**FEZ1**	1,37791	**MICALL2**	0,17163	**RCC2**	0,093815	**ZYX**	-0,10955
CD36	-0,20711	**FGFRL1**	0,001713	**MICB**	-0,78431	**RELB**	1,85855		
CD4	-0,59736	**FIBP**	-0,35023	**MKLN1**	0,092447	**RET**	-0,49831		
CD44	1,3518	**FKBP1A**	-0,11686	**MLLT4**	-0,02515	**RGMB**	-0,03137		
CD47	-0,31127	**FLNA**	0,195239	**MPZL3**	0	**RHOB**	-0,25879		
CD58	0,747914	**FLOT2**	-0,61859	**MSN**	-0,15871	**RHOH**	0,376472		
CD63	0,099498	**FLRT2**	0,133612	**MTSS1**	0,716254	**RIC8A**	0,090681		
CD72	-0,1332	**FNDC3A**	-0,66618	**MYBPC3**	0	**RIPK2**	0,801641		
CD74	0,015868	**FOXP1**	-0,25275	**MYBPH**	-0,28777	**RIPK3**	-0,6198		
CD80	4,08467	**FUT7**	-1,47076	**MYH9**	-0,03309	**RND3**	1,308		
CD81	0,07387	**FYN**	-0,06366	**MYL12A**	-0,25938	**ROBO1**	1,40397		
CD84	-0,57277	**FZD5**	0,660232	**MYL9**	-0,57051	**ROCK1**	-0,28371		
CD86	0,441844	**FZD7**	-1,27118	**MYO1G**	-0,5785	**RPL22**	-0,10106		
CD9	-0,92524	**GAS6**	-0,06567	**NCAM2**	-0,67961	**RPS6**	-0,16418		
CD93	-0,51114	**GCNT1**	-1,86427	**NCK1**	-0,30205	**RPSA**	0,03759		
CD99	-0,24164	**GNAS**	-0,05174	**NCK2**	-0,04463	**RSAD2**	1,82799		
CD99L2	-0,55136	**GNE**	-0,26948	**NCR3**	0,003719	**RUNX2**	-0,30445		

Table 3: Genes associated with biological adhesion elicited by DM infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CDC42	-0,13226	**GOLPH3**	-0,42542	**NCSTN**	0,012615	**S100A9**	-0,73309		
CDH23	0,753798	**GP1BB**	-2,91471	**NEDD4**	-0,05259	**SATB1**	-0,3271		
CDH24	-0,24478	**GP9**	-2,629	**NEDD9**	-0,24557	**SCARB1**	-0,31943		
CDK5	-0,47717	**GPNMB**	-0,46396	**NHEJ1**	0,516753	**SCRIB**	-0,03989		
CDK5R1	-0,66656	**HAPLN3**	2,56765	**NID1**	0,405194	**SDC3**	-0,36446		
CDK6	0,261583	**HBB**	1,19171	**NINJ1**	1,38117	**SDK1**	-0,10603		
CDON	0	**HCK**	0,78546	**NINJ2**	-0,61983	**SELPLG**	0,634882		
CEBPB	1,70697	**HES1**	0,182222	**NKAP**	-0,21141	**SEMA4A**	1,03515		
CELSR1	0,186946	**HLA-E**	0,3773	**NLGN4Y**	1,61471	**SEMA4D**	-0,59031		
CEP41	-0,31708	**HMGB1**	-0,4537	**NME2**	0,138523	**SHC1**	0,32777		
CERCAM	-0,33424	**HPSE**	-1,49519	**NPHP4**	0,373308	**SIGLEC1**	0,534376		
CFDP1	-0,1666	**HSH2D**	0,800316	**NPTN**	0,248458	**SIGLEC11**	-0,8664		
CGREF1	-0,11766	**HSP90AB1**	0,196255	**NT5E**	0,336649	**SIGLEC14**	-0,52901		
CHD7	-0,23976	**HSPB1**	-0,2429	**OLR1**	0,790683	**SIGLEC9**	-0,60265		
CHST10	-0,96984	**HSPD1**	-0,0182	**OMG**	1,11398	**SIRPA**	0,505908		
CIB1	-0,11203	**ICAM1**	2,22881	**PARVB**	-0,12627	**SLAMF7**	0,9861		
CLDN1	2,35661	**ICAM2**	0,87747	**PARVG**	-0,48763	**SLC11A1**	-0,12799		

Table 4: Significantly differentially expressed Genes elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
ABCA1	3,80	**CD274**	4,16	**ELOVL7**	2,59	**IER3**	3,61	**MPZL1**	1,92	**PPP1R15A**	1,20	**SLC43A2**	2,98
ABHD16B	1,40	**CD40**	2,35	**ETS2**	0,93	**IFIT1**	1,71	**MRAS**	0,98	**PRDM1**	1,33	**SLC5A3**	1,28
ABL2	1,12	**CD44**	1,07	**ETV3**	1,37	**IFIT2**	2,44	**MREG**	2,07	**PRR5L**	-1,99	**SLC7A11**	1,57
ABTB2	3,23	**CD83**	2,10	**ETV3L**	2,08	**IFIT3**	1,83	**MSANTD3**	1,36	**PSTPIP2**	1,73	**SLFN11**	-1,10
ACSL1	2,07	**CEBPB**	2,24	**ETV5**	-1,14	**IFIT5**	1,97	**MSC**	1,43	**PTGER4**	1,30	**SMAD3**	1,38
ACSL5	1,51	**CEBPD**	1,16	**FABP4**	2,24	**IGFBP3**	2,86	**MYB**	-1,56	**PTGS2**	2,20	**SMYD3**	-1,16
ADAMTS3	1,86	**CFLAR**	1,57	**FAM102B**	-1,07	**IL1A**	5,37	**MYO10**	1,36	**PTP4A3**	-1,27	**SNORD3A**	1,58
ADCY7	-1,29	**CHST2**	1,79	**FAM105A**	-1,34	**IL1B**	2,53	**NAMPT**	1,91	**PTPRK**	1,32	**SOCS3**	1,85
ADRB1	-1,53	**CHST7**	1,58	**FAM186B**	3,94	**IL1RN**	1,35	**NBPF10**	1,20	**PTX3**	2,26	**SOD2**	4,74
AGO2	1,07	**CLCN4**	-1,48	**FAM212A**	-1,78	**IL23A**	3,84	**NBPF14**	1,14	**PVRL3**	1,22	**SPAG9**	1,02
AHR	1,00	**CLDN1**	2,08	**FAM46C**	-2,20	**IL7R**	1,36	**NEAT1**	1,35	**RAI14**	1,11	**SPHK1**	1,09
AKAP2	1,13	**CLEC2D**	1,40	**FBRS**	1,03	**IPCEF1**	-1,25	**NEK10**	1,99	**RASGEF1B**	2,32	**SPOCD1**	1,00
AKNA	-0,98	**CMPK2**	1,24	**FCAR**	1,44	**IRAK2**	3,67	**NFE2L2**	1,18	**RASSF5**	1,19	**SPSB1**	0,99
ALOX5	-1,72	**COL6A1**	1,41	**FEZ1**	1,42	**IRF1**	1,49	**NFKB1**	1,58	**RCAN1**	1,51	**SQSTM1**	1,83
ALPP	2,43	**COL6A2**	1,49	**FGD4**	-1,69	**IRF8**	-1,12	**NFKB2**	1,64	**REL**	1,62	**SRC**	2,15
AMPD3	2,59	**CRIM1**	2,70	**FKBP5**	1,44	**ITGB8**	2,35	**NFKBIA**	2,24	**RELB**	1,83	**SRXN1**	0,96
ANTXR1	-1,61	**CSF1**	1,58	**FMNL3**	1,12	**JAG2**	-1,16	**NFKBIZ**	2,97	**RFTN1**	1,03	**STAT5A**	1,81
APAF1	-1,27	**CSPG4**	-1,17	**FOSL2**	1,21	**KCNG1**	1,43	**NINJ1**	1,51	**RGL1**	1,72	**STX11**	1,56
APOC1	1,21	**CX3CR1**	-1,66	**FRAT2**	-1,10	**KDM6B**	2,25	**NLRP3**	1,39	**RGS14**	-1,50	**SUSD6**	1,17

Table 4: Significantly differentially expressed Genes elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
AREG	1,94	**CXCL1**	4,61	**G0S2**	3,52	**KITLG**	1,42	**NOTCH2NL**	1,17	**RGS16**	1,32	**TANK**	1,10
ARHGAP31	1,31	**CXCL10**	3,73	**GADD45B**	1,40	**KLF3**	1,03	**NPTX1**	-1,41	**RGS18**	-1,47	**TDO2**	1,09
ATF3	1,14	**CXCL2**	4,83	**GBP1**	1,89	**KYNU**	1,87	**NR4A3**	2,19	**RHOBTB3**	1,95	**TEC**	-1,33
ATP10A	-1,06	**CXCL3**	5,37	**GBP2**	1,32	**LFNG**	1,43	**NRG1**	3,67	**RND3**	1,69	**TFEC**	1,04
ATP2B1	1,73	**CXCL6**	4,12	**GBP4**	2,13	**LINC00622**	2,52	**OASL**	1,46	**RNF125**	-1,73	**TGFBR1**	-0,98
B4GALT1	1,11	**CXCL8**	4,94	**GBP5**	2,58	**LINC01268**	1,58	**OGFRL1**	1,63	**RNF19B**	1,46	**TGM5**	-2,61
B4GALT5	1,21	**CXCR4**	1,90	**GCH1**	1,78	**LMNB1**	-1,10	**OLR1**	1,24	**RNU4-1**	-1,23	**THBD**	1,13
BASP1	1,10	**CYSLTR1**	-1,21	**GCLM**	1,05	**LMO2**	-1,21	**OSBPL5**	-1,19	**RNU4-2**	-1,08	**THSD7A**	-1,38
BATF2	1,61	**DDIT4L**	-1,40	**GCNT1**	-1,65	**LOC374443**	1,24	**OSGIN1**	1,52	**ROBO1**	1,31	**TLR2**	1,17
BCAR3	1,31	**DDX58**	1,25	**GDF15**	1,96	**LOC729348**	1,37	**P2RY8**	-1,12	**RSAD2**	1,42	**TMEM154**	-1,21
BCL2A1	1,36	**DENND5A**	1,52	**GEM**	2,94	**LRFN5**	2,17	**P3H2**	2,34	**RTP4**	1,47	**TMEM173**	-1,30
BCL3	1,87	**DGAT2**	1,52	**GFI1**	-1,34	**LRMP**	-1,07	**PARM1**	1,17	**S1PR3**	1,50	**TNF**	3,54
BCL6	0,98	**DHRS9**	-1,45	**GIMAP8**	2,42	**LRP12**	1,20	**PDE3A**	-1,99	**SAT1**	1,93	**TNFAIP2**	2,35
BCL9L	1,75	**DLL4**	4,17	**GPR132**	1,14	**LRRC8C**	-1,13	**PDE4B**	3,37	**SDC4**	1,41	**TNFAIP3**	2,14
BIRC3	3,91	**DLX3**	-2,90	**GPR35**	1,69	**LYN**	0,96	**PDE4DIP**	1,48	**SEMA3C**	2,45	**TNFAIP6**	5,81
BMP8B	-1,48	**DMXL2**	0,96	**GPX3**	3,27	**LYZ**	-1,06	**PEAK1**	1,45	**SERPINB2**	2,57	**TNFAIP8**	1,73
BTG1	1,30	**DRAM1**	2,55	**GRAMD3**	1,57	**MAFF**	2,20	**PERP**	1,25	**SERPINE2**	4,18	**TNFRSF11A**	-1,90
C17orf96	1,97	**DSE**	1,59	**GSAP**	1,32	**MAML2**	1,02	**PIK3R5**	1,36	**SESN1**	-1,16	**TNFRSF9**	1,49
C7orf60	1,17	**DST**	1,14	**GUCY1A2**	1,19	**MAP2K3**	1,05	**PIM2**	1,57	**SESN2**	1,47	**TNFSF10**	1,76

Table 4: Significantly differentially expressed Genes elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CASP2	-1,03	**DTX4**	2,47	**HBB**	2,16	**MAP3K8**	1,62	**PIM3**	1,37	**SGIP1**	1,17	**TNFSF13B**	2,03
CCDC57	1,49	**DUSP1**	1,90	**HBEGF**	1,23	**MARCKS**	3,07	**PKDCC**	-1,37	**SGK1**	1,16	**TNFSF15**	2,35
CCL2	2,00	**DUSP10**	1,13	**HERC5**	1,36	**MCOLN2**	2,35	**PLAC8**	-1,35	**SGTB**	1,20	**TNIP1**	2,19
CCL20	5,47	**DUSP16**	1,84	**HIC1**	3,64	**MCTP1**	1,01	**PLCL1**	1,81	**SH2D3C**	-1,57	**TNIP2**	1,15
CCL3	2,98	**DUSP8**	2,22	**HIVEP2**	4,25	**MFSD2A**	1,07	**PLEK**	1,48	**SHANK3**	1,72	**TPRA1**	1,23
CCL4	6,39	**EBF1**	2,74	**HIVEP3**	1,21	**MGLL**	4,21	**PLEKHM3**	1,41	**SHB**	1,07	**TRAF1**	4,17
CCL4L2	5,24	**EBI3**	3,25	**HPSE**	-1,30	**MIR155HG**	1,69	**PLEKHO1**	1,18	**SIK3**	1,12	**TRIM58**	-1,22
CCR2	-2,07	**ECE1**	3,04	**HSD3BP4**	2,61	**MLLT6**	1,60	**PLIN2**	2,02	**SLC11A2**	1,31	**TTLL4**	1,39
CCRL2	1,59	**EFNB2**	3,30	**HSPA6**	2,84	**MLXIPL**	1,34	**PLK3**	1,92	**SLC2A6**	3,25	**TUBA1A**	-1,10
CD180	-1,09	**EHD1**	3,06	**HUWE1**	1,23	**MMP9**	2,01	**PLXNA1**	0,96	**SLC39A14**	1,14	**TWIST1**	1,51
CD1D	-1,77	**ELL2**	1,38	**ICAM1**	2,29	**MOB3C**	1,30	**POU2F2**	1,56	**SLC41A2**	1,42	**TXNRD1**	0,99
												UBTD2	1,09
												USP12	1,37
												UXS1	1,07
												VSIG4	-1,56
												WTAP	1,37
												ZBTB10	1,29
												ZC3H12A	1,81
												ZC3H12C	2,14
												ZC3H12D	1,04
												ZCCHC2	1,01
												ZMIZ2	1,23
												ZNF618	-1,48

Table 4: Significantly differentially expressed Genes elicited by WT infection continued…

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
												ZNF697	1,31
												ZSWIM4	1,28

Table 5: Significantly differentially expressed Genes elicited by DM infection continued ….

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
ABCA1	1,69984	**CSRNP1**	0,93573	**HIST1 h1B**	-1,38115	**MEF2C**	-0,9825	**PYCARD**	-1,07352	**TICAM1**	1,10659
ABHD10	-1,17252	**CX3CR1**	-1,46054	**HIST1 h1C**	-1,03829	**METTL7A**	-0,99815	**PYROXD2**	-1,80432	**TK2**	-1,03786
ABL2	1,26707	**CXCL1**	4,54147	**HIST1 h2BL**	-1,55847	**METTL9**	-0,98515	**QPRT**	-0,91744	**TLR2**	0,996172
ABTB2	3,25584	**CXCL10**	4,7508	**HIVEP1**	0,979742	**MFSD2A**	1,0718	**RAB11FIP5**	-1,28103	**TMEM154**	-1,42064
ACSL1	1,50432	**CXCL11**	4,67412	**HIVEP2**	3,77435	**MGLL**	3,98013	**RAB29**	-0,8974	**TMEM156**	-1,34104
ACSL5	1,10292	**CXCL2**	4,63262	**HIVEP3**	1,03763	**MIR155HG**	1,1142	**RAB3D**	-2,13153	**TMEM158**	-0,88213
ADAMTS1	-1,24916	**CXCL3**	5,13881	**HLTF**	-1,10941	**MLLT6**	1,66207	**RASGEF1B**	2,51014	**TMEM173**	-1,24212
ADAMTS3	1,27668	**CXCL6**	3,80182	**HMGB2**	-1,06711	**MMP9**	2,6258	**RASSF5**	1,18502	**TMEM87B**	-0,89326
ADCK3	-1,03032	**CXCL8**	4,44107	**HPSE**	-1,49519	**MOB3C**	1,5774	**RBM43**	1,18913	**TNF**	3,93477
ADCY7	-1,23307	**CXCR4**	2,16579	**HSD17B11**	-0,85759	**MPZL1**	1,72237	**REL**	1,69598	**TNFAIP2**	2,54352
ADGRE5	0,86527	**CYP27B1**	1,34752	**HSD3BP4**	2,74729	**MREG**	2,39649	**RELB**	1,85855	**TNFAIP3**	2,22372
ADORA3	-2,0646	**CYSLTR1**	-1,33558	**HSPA6**	2,70095	**MSANTD3**	1,23507	**RFTN1**	0,974016	**TNFAIP6**	5,47481
ADRB1	-2,15261	**DCP1A**	0,865755	**HUWE1**	1,27277	**MSC**	1,55081	**RGL1**	2,35885	**TNFAIP8**	1,59196
AGAP3	0,880844	**DDIT4L**	-1,79253	**ICAM1**	2,22881	**MSR1**	-0,9969	**RGS14**	-1,10062	**TNFRSF9**	1,30777
AGO2	0,92383	**DDX58**	1,99364	**ICOSLG**	2,35262	**MSRB2**	-1,47325	**RGS16**	1,57197	**TNFSF10**	2,46203
AKAP2	0,896739	**DEF6**	-1,13302	**ID1**	-1,95357	**MTM1**	-1,16289	**RGS18**	-1,64591	**TNFSF13B**	2,0201
AKNA	-0,829719	**DENND1B**	-1,20021	**IDH1**	-0,95837	**MTUS1**	-1,56676	**RHOBTB1**	-1,6636	**TNFSF15**	2,23714
AKT1S1	0,976855	**DENND5A**	1,36525	**IER3**	3,58766	**MUC1**	1,65953	**RICTOR**	0,835964	**TNIP1**	2,02672
ALOX5	-1,96962	**DGAT2**	0,907161	**IER5L**	-1,00056	**MURC**	1,83773	**RND3**	1,308	**TNIP2**	1,0926

Table 5: Significantly differentially expressed Genes elicited by DM infection continued ….

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
ALPP	2,05726	**DHRS9**	-1,58858	**IFI16**	0,994513	**MX2**	1,0673	**RNF125**	-2,29787	**TOP2A**	-1,00216
AMPD3	2,58904	**DHTKD1**	-0,94704	**IFI44**	1,15114	**MYB**	-1,72866	**RNF166**	-0,90274	**TPRA1**	1,07001
ANTXR1	-1,94365	**DLL4**	4,61804	**IFI44L**	1,62668	**MYEOV**	2,01443	**RNF168**	-0,96966	**TRAF1**	4,07218
APAF1	-1,36446	**DLX3**	-2,49898	**IFIH1**	1,35166	**MYO10**	1,4724	**RNF19B**	1,88492	**TRAM2**	-0,89734
APOL6	1,21592	**DMRT2**	-0,92524	**IFIT1**	2,25421	**N4BP1**	0,899606	**ROBO1**	1,40397	**TRIM22**	1,25689
AREG	2,08559	**DMWD**	1,51219	**IFIT2**	3,53478	**N4BP2L1**	1,49233	**RSAD2**	1,82799	**TRIM25**	0,906054
ARHGAP18	-0,84066	**DMXL2**	0,831496	**IFIT3**	2,44045	**NAMPT**	1,94892	**RTN1**	1,23355	**TRIM56**	1,17286
ARHGAP31	1,54922	**DOK2**	-0,91262	**IFIT5**	2,23138	**NCOA7**	1,2516	**RTP4**	1,87876	**TRIM58**	-1,33823
ARHGEF6	-1,05165	**DRAM1**	2,11029	**IFNGR1**	-0,9772	**NEAT1**	1,66116	**S100A4**	-0,81209	**TRIM59**	-1,14694
ARL5B	0,963633	**DSE**	1,00489	**IGFBP3**	3,17708	**NEMP1**	0,912349	**S1PR3**	1,50409	**TRIO**	1,16389
ASAP1	1,07565	**DST**	1,35092	**IL10RA**	1,10511	**NFE2L2**	1,08444	**SAMD9**	1,25076	**TSPAN14**	-1,20236
ATF3	1,42168	**DTX4**	2,75907	**IL15RA**	1,52789	**NFE2L3**	1,48589	**SAMD9L**	1,301	**TTLL4**	1,29403
ATP10A	-1,00812	**DUSP1**	1,77754	**IL1A**	4,43169	**NFKB1**	1,5189	**SASH3**	-0,92415	**TUBA1A**	-1,14132
ATP2B1	1,57788	**DUSP10**	1,06673	**IL1B**	2,09382	**NFKB2**	1,92816	**SAT1**	2,11234	**TWIST1**	1,29711
B3GALNT1	-1,3777	**DUSP16**	1,74636	**IL1RN**	1,04886	**NFKBIA**	2,13277	**SCAMP5**	-1,54989	**UBTD2**	0,954115
B4GALT5	1,1818	**DUSP5**	1,06329	**IL23A**	4,00216	**NFKBIE**	0,881961	**SDC4**	1,2037	**UCN2**	1,17763
BASP1	1,32968	**DUSP8**	2,42443	**IL4I1**	1,26905	**NFKBIZ**	2,88687	**SEMA3C**	1,65754	**USP12**	1,29068
BATF2	2,58247	**EBF1**	2,8863	**IL7R**	1,16117	**NINJ1**	1,38117	**SEMA4A**	1,03515	**UXS1**	0,882822
BCAR1	1,26397	**EBF3**	-1,49159	**ING2**	1,12585	**NIPAL3**	-1,15015	**SEPHS2**	-0,94445	**VANGL1**	-1,10028
BCL2A1	1,33083	**EBI3**	3,60565	**INHBA**	1,90307	**NOL4L**	1,43231	**SERPINE1**	1,53579	**VCL**	-1,02919
BCL3	2,38907	**ECE1**	3,11708	**IPCEF1**	-1,09518	**NPTX1**	-1,46668	**SERPINE2**	3,73945	**VSIG4**	-1,80128
BCL9L	1,74876	**EGLN1**	-0,95693	**IRAK1BP1**	-2,26779	**NR4A3**	2,08848	**SESN1**	-1,42267	**WTAP**	1,26113
BIRC3	3,90228	**EHD1**	3,0374	**IRAK2**	3,455	**NRG1**	3,80116	**SESN2**	1,0945	**XAF1**	1,1472
BMP8B	-1,55216	**EHHADH**	-1,41872	**IRF1**	2,18042	**OAS3**	0,976597	**SGK1**	1,40637	**XIRP1**	0,98527
BTG1	0,931801	**ELL2**	1,24077	**IRF7**	0,97759	**OASL**	2,11937	**SH2D3C**	-1,70147	**ZADH2**	-0,95179
BTG2	1,12858	**ELOVL7**	2,08613	**IRF8**	-1,08611	**OGFRL1**	1,40398	**SH2D4A**	-1,12948	**ZBTB10**	1,28746
C11orf21	-1,24906	**ENAH**	-1,30324	**ISG20**	1,75719	**OLIG2**	1,14413	**SH3PXD2B**	1,06927	**ZC3H12A**	1,89623

Table 5: Significantly differentially expressed Genes elicited by DM infection continued ….

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
C17orf96	2,22962	**EPSTI1**	1,01122	**ITGA4**	-0,92333	**OSBPL5**	-1,36218	**SIK3**	0,991272	**ZC3H12C**	2,08076
C1orf162	-1,39672	**ESYT1**	-0,91471	**ITGA6**	0,825857	**OSGIN1**	1,71423	**SLAMF7**	0,9861	**ZC3H12D**	1,45495
C7orf60	1,10194	**ETS2**	0,894502	**ITGB8**	1,8526	**OTUD1**	1,1972	**SLC11A2**	1,18042	**ZC3HAV1**	1,03315
CA2	-1,13655	**ETV3**	1,51121	**ITPKB**	-0,94579	**P2RY8**	-1,31797	**SLC22A16**	-1,052	**ZCCHC2**	1,31781
CACNA1E	0,899392	**ETV3L**	2,86128	**JAG2**	-1,14071	**P3H2**	2,66437	**SLC2A6**	3,02476	**ZMIZ1-AS1**	1,31194
CACNB4	-1,15323	**ETV5**	-1,20421	**JUNB**	0,92128	**PARM1**	1,21599	**SLC39A14**	1,06967	**ZMIZ2**	1,48977
CAPRIN2	-1,00161	**EVA1B**	2,0606	**KANK2**	-1,09777	**PARP14**	1,01047	**SLC41A2**	1,41104	**ZNF618**	-0,97722
CASP2	-1,09946	**FAM102B**	-1,23653	**KCNA3**	2,48159	**PARP8**	-0,85423	**SLC43A2**	2,82514	**ZNF697**	1,13318
CBLB	1,29021	**FAM105A**	-1,62383	**KCTD12**	-1,21864	**PATL1**	0,947828	**SLC4A4**	1,27513	**ZNFX1**	1,35938
CCDC112	-1,60302	**FAM212A**	-1,47886	**KDM6B**	2,43254	**PBXIP1**	-1,04178	**SLC7A11**	1,57238	**ZSWIM4**	1,50555
CCDC57	1,52158	**FAM213B**	1,79823	**KIAA1462**	-1,4197	**PCM1**	-0,94217	**SLC8A1**	-0,89638	**ZWINT**	-0,96392
CCL2	2,6028	**FAM46C**	-2,21676	**KIF15**	-1,35661	**PDE4B**	3,63789	**SLCO4C1**	-1,52232	**ZYG11B**	-0,85942
CCL20	4,42474	**FAM63B**	-0,96303	**KITLG**	1,23425	**PDE4DIP**	1,12493	**SLFN11**	-1,2541		
CCL3	3,15722	**FAR2**	-1,37367	**KLF4**	-1,60631	**PDE7B**	-1,85447	**SLITRK5**	-1,31727		
CCL4	6,45048	**FBRS**	1,38181	**KMT2D**	0,85234	**PEAK1**	1,63547	**SMOX**	1,02199		
CCL4L2	5,18377	**FCAR**	1,35887	**KYNU**	1,76114	**PERP**	1,28643	**SMTN**	1,1653		
CCL8	3,61311	**FES**	-1,05456	**LBH**	-1,64692	**PHACTR3**	-1,52902	**SMYD3**	-1,31006		
CCR2	-2,45816	**FEZ1**	1,37791	**LDLRAP1**	-1,01074	**PHACTR4**	1,44463	**SNAI3**	-1,66257		
CCRL2	1,76295	**FGD4**	-2,08188	**LFNG**	1,18626	**PIK3C2B**	-1,21247	**SNX18**	-1,13791		
CCSAP	-1,10848	**FKBP5**	1,47894	**LGR4**	-1,16384	**PIK3R5**	1,29823	**SOCS3**	2,48591		
CD180	-1,19165	**FMNL3**	1,07801	**LINC00477**	1,3763	**PIM1**	1,20627	**SOCS6**	-0,984		
CD1D	-2,284	**FOSB**	4,0206	**LINC00599**	-2,2662	**PIM2**	1,62928	**SOD2**	4,63335		
CD244	-1,28527	**FOSL1**	1,10017	**LINC00622**	2,25354	**PIM3**	1,41801	**SORT1**	-1,19187		
CD274	5,04191	**FOSL2**	1,0617	**LINC00641**	1,14833	**PKDCC**	-1,13824	**SPHK1**	1,11293		
CD300A	-1,48089	**FRAT2**	-1,09461	**LINC01004**	1,54585	**PLA2G15**	-1,06537	**SPIN4**	-1,87598		
CD300C	-0,874183	**FZD7**	-1,27118	**LMNB1**	-1,09535	**PLAC8**	-1,18579	**SPN**	-0,82543		
CD40	2,54369	**G0S2**	2,15157	**LMO2**	-1,02893	**PLCL1**	2,05349	**SPRED1**	-1,34778		

Table 5: Significantly differentially expressed Genes elicited by DM infection continued ….

Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)	Gene	Log2(fold-change)
CD44	1,3518	**GADD45B**	1,73765	**LOC100419583**	1,34358	**PLD1**	-1,16827	**SPTBN1**	-1,08013		
CD80	4,08467	**GAL**	1,02353	**LOC100499194**	2,57529	**PLEK**	1,24909	**SQSTM1**	1,58664		
CD83	2,07508	**GATA2**	-1,63311	**LOC100506860**	2,19	**PLEKHA2**	-0,878	**SRC**	2,74771		
CDC42EP2	3,70012	**GBP1**	2,44764	**LOC284454**	1,12698	**PLEKHM3**	1,39148	**SRCAP**	0,855221		
CEBPA	-1,07499	**GBP2**	1,81611	**LOC729348**	1,58594	**PLEKHO1**	1,16625	**SRRM2**	0,861147		
CEBPB	1,70697	**GBP3**	1,05565	**LOXL4**	-1,67261	**PLK3**	1,76097	**SRXN1**	0,955951		
CEBPD	0,915021	**GBP4**	3,07682	**LPAR5**	-2,57093	**PLSCR1**	0,934259	**STAT2**	1,02691		
CFLAR	1,57813	**GBP5**	3,43377	**LRFN5**	2,18703	**PLXNA1**	1,01849	**STAT4**	2,06454		
CHODL	-1,70776	**GCH1**	1,52696	**LRMP**	-1,20491	**PML**	1,35571	**STAT5A**	2,0261		
CHRM3	-1,07672	**GCNT1**	-1,86427	**LRP12**	1,02616	**PNPT1**	0,958708	**STK17A**	0,882049		
CHST2	1,77838	**GDF15**	1,13872	**LRRC8C**	-1,08228	**POU2F2**	1,8102	**STK38L**	-0,86648		
CHST7	1,16671	**GEM**	2,61506	**LRRK2**	-1,2777	**PPIF**	0,896478	**STX11**	1,62642		
CIC	0,961014	**GFI1**	-1,48155	**LYN**	1,07459	**PPM1K**	1,32697	**STX7**	-0,90375		
CITED2	-0,835378	**GIMAP6**	-1,1626	**LYZ**	-1,28225	**PPP1R15A**	1,15223	**SUCNR1**	-1,34346		
CITED4	0,847348	**GIMAP8**	2,50358	**MAFF**	1,86388	**PRDM1**	1,57194	**SUSD6**	1,29486		
CLCN4	-1,98851	**GNAQ**	-1,04536	**MAML2**	1,07066	**PRG2**	-1,82788	**SYK**	-0,87882		
CLDN1	2,35661	**GPR132**	0,961894	**MAP2K3**	0,962911	**PRKACB**	-1,0182	**TEC**	-1,33256		
CLEC2D	1,15265	**GPR35**	1,34406	**MAP3K1**	-1,13973	**PRKD3**	0,973782	**TESC**	-1,18704		
CLTCL1	-1,33872	**GPR82**	-3,22982	**MAP3K8**	1,42878	**PRR5L**	-2,23578	**TEX2**	-0,87068		
CMPK2	1,8986	**GPX3**	3,12156	**MARCKS**	3,02788	**PTGER4**	1,14902	**TFEC**	0,809173		
COL6A1	1,6225	**GSAP**	1,15516	**MASTL**	0,928014	**PTGFRN**	-1,5978	**TGFBR1**	-1,13828		
COL6A2	1,63086	**HBB**	1,19171	**MBOAT1**	-1,25031	**PTGS2**	2,42837	**TGFBR2**	-0,97541		
CPM	0,850024	**HELZ2**	1,36775	**MCEMP1**	-1,18497	**PTP4A3**	-1,30289	**TGM5**	-3,33238		
CRIM1	2,58255	**HERC5**	1,93041	**MCOLN2**	2,45283	**PTPRK**	1,42425	**THBS2**	-1,27481		
CSF1	1,4977	**HESX1**	1,8731	**MCTP1**	0,89511	**PTX3**	2,07955	**THSD7A**	-1,52407		
CSPG4	-0,934991	**HHEX**	-1,90234	**MDFIC**	-0,85871	**PXDC1**	3,02766	**THYN1**	-1,30423		

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Cell movement of hematopoietic cells	3,08292	2,193616
Cell movement of hematopoietic progenitor cells	2,934025	2,021893
Interaction of lymphocytes	2,004336	2,381275
Activation of cells	2,174973	2,057905
Polarization of lymphocytes	2	2,2
Polarization of leukocytes	2	2,2
Binding of lymphocytes	1,883876	2,266091
Migration of hematopoietic progenitor cells	2,575569	1,531419
Adhesion of lymphatic system cells	1,830946	2,252158
Binding of lymphatic system cells	1,619403	2,45896
Replication of virus	-1,62162	-2,45202
Binding of eosinophils	2,265021	1,697245
Quantity of blood cells	1,606834	2,303834
Adhesion of endothelial cells	2,050445	1,858095
Migration of lymphatic system cells	1,92473	1,935105
Binding of lymphoid cells	1,752652	2,078387
Binding of mast cells	1,889822	1,889822
Binding of endothelial cells	2,149385	1,611355
Cell movement of lymphatic system cells	2,056838	1,654661
Polarization of cells	1,82868	1,82868
Interaction of T lymphocytes	1,657458	1,995514
Communication	-1,51579	-2,13447
Communication of cells	-1,51579	-2,13447
Transactivation of RNA	1,248127	2,34546
Quantity of lymphatic system cells	1,100887	2,484053
Adhesion of lymphocytes	1,655108	1,874809
Adhesion of lymphoma cell lines	1,076856	2,435664
Liver lesion	0,759819	2,744801
T cell development	1,605524	1,893841
Interaction of mononuclear leukocytes	1,675604	1,77395
Lymphocyte migration	1,831582	1,604415
Cell movement of sarcoma cell lines	2,263226	1,170634
Interaction of endothelial cells	1,971952	1,443332
Binding of T lymphocytes	1,522264	1,866592
Cell movement of lymphocytes	1,967813	1,325842
Binding of protein binding site	1,136087	2,151829
Adhesion of eosinophils	1,863444	1,378833
Binding of mononuclear leukocytes	1,561768	1,662306
Activation of leukocytes	1,974745	1,245277
Replication of RNA virus	-1,15384	-1,989
Quantity of cells	1,424906	1,716874
Migration of vascular endothelial cells	-0,83498	-2,30384
Cell movement of T lymphocytes	2,191419	0,916228
Cell death of tumor cells	1,996965	1,088525
Differentiation of T lymphocytes	1,381764	1,699622
Cell survival	1,500098	1,552953
Binding of connective tissue cells	1,421806	1,606696

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
T cell migration	1,971828	1,053863
Movement of vascular endothelial cells	-0,80665	-2,2166
Activation of blood cells	1,866188	1,143584
Malignant solid tumor	0,960988	1,995897
Nonhematologic malignant neoplasm	0,960988	1,995897
Viral Infection	-0,54483	-2,37225
Adhesion of mononuclear leukocytes	1,403459	1,503196
Migration of granulocytes	1,519094	1,384615
Cell movement of leukemia cell lines	1,624609	1,230594
Cellular homeostasis	1,984159	0,865434
Advanced malignant tumor	1,372813	1,372813
Metastasis	1,372813	1,372813
Binding of leukocyte cell lines	-1,45415	-1,27789
Development of endothelial tissue	1,670218	-1,05834
Endothelial cell development	1,670218	-1,05834
Cell viability	1,309616	1,416171
Binding of peripheral blood lymphocytes	0,911331	1,813266
Cell proliferation of T lymphocytes	2,217657	0,499909
Activation of mononuclear leukocytes	1,82028	0,881201
T cell homeostasis	1,206241	1,479721
Migration of mononuclear leukocytes	1,537118	1,148508
Cancer	1,835178	0,846332
Proliferation of tumor cells	-0,69278	-1,95785
Binding of peripheral blood leukocytes	0,909889	1,726238
Immune response of phagocytes	1,952562	0,640196
Engulfment of tumor cell lines	0,967551	-1,62274
Cell rolling of blood cells	-0,71058	1,864233
Activation of T lymphocytes	2,304664	0,260931
Cell death of hematopoietic cell lines	0,708131	1,829924
Binding of granulocytes	1,50083	1,024569
Activation of lymphocytes	1,989471	0,519183
Apoptosis of hematopoietic cell lines	0,690312	1,817352
Activation of phagocytes	1,303384	1,189764
Cell death of cancer cells	1,722896	0,753298
Hemorrhagic disease	-1,63299	-0,8165
Migration of leukemia cell lines	1,647604	0,788518
Proliferation of blood cells	1,809916	0,622667
Activation of myeloid cells	1,088762	1,33481
Proliferation of endothelial cells	1,21338	-1,18812
Homing of tumor cell lines	1,335235	1,049892
Development of epithelial tissue	1,732207	-0,60951
Chemotaxis of tumor cell lines	1,385291	0,891885
Hematopoiesis of mononuclear leukocytes	1,215237	1,060608
Cell movement of tumor cell lines	1,484744	0,783459
Digestive system cancer	1,131371	1,131371
Growth of connective tissue	1,194935	1,040481
Migration of myeloid cells	1,2575	0,975067
Adhesion of T lymphocytes	1,036554	1,18285
Binding of lymphoma cell lines	0,69801	1,510364
Interaction of lymphoma cell lines	0,69801	1,510364

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Engulfment of leukocytes	1,771784	0,430463
Lymphocyte homeostasis	1,035153	1,166542
Homeostasis of leukocytes	1,035153	1,166542
Growth of epithelial tissue	-0,11158	-2,07361
Proliferation of immune cells	1,854687	0,318293
Ion homeostasis of cells	0,769837	1,401457
Proliferation of cancer cells	-0,7171	-1,44459
Cell movement of mononuclear leukocytes	1,585371	0,573776
Lymphopoiesis	0,954373	1,19769
Interaction of leukocytes	1,059543	1,084357
Cytotoxicity of lymphocytes	1,887834	0,24203
Cell-cell contact	-0,35371	-1,74884
Phosphorylation of L-amino acid	1,043498	1,043498
Phosphorylation of L-tyrosine	1,043498	1,043498
Flux of Ca2+	0,704744	1,372624
Transcription of RNA	1,001733	1,07071
Interaction of blood cells	1,124932	0,947469
Migration of endothelial cells	0,952564	-1,10987
Cell proliferation of breast cancer cell lines	1,027051	-1,02954
Adhesion of phagocytes	1,361237	0,684493
Cell death of phagocytes	-0,60662	-1,43885
Transmigration of leukocytes	1,657713	0,376759
Endocytosis	1,754476	0,278581
Adhesion of antigen presenting cells	1	1
Degranulation of mast cells	0,6	1,4
Phagocytosis of phagocytes	1,472919	0,518321
Phagocytosis of leukocytes	1,472919	0,518321
Transmigration of cells	1,594394	0,388424
Chemotaxis of neutrophils	-1,1494	-0,83089
Proliferation of lymphocytes	1,779492	0,186824
Chemotaxis of mononuclear leukocytes	1,555486	0,408782
Cell movement of carcinoma cell lines	0,716652	1,245612
Binding of leukocytes	0,96363	0,989384
Adhesion of connective tissue cells	0,863614	1,086482
Cell death of embryonic cell lines	-0,54327	-1,38812
Cell movement of gonadal cell lines	0,659126	-1,26755
Angiogenesis	1,654122	-0,26703
Cell movement of PBMCs	1,281383	0,624887
Cell death of endothelial cells	-1,29246	0,607315
Formation of cell-cell contacts	-0,27735	-1,60863
Binding of blood cells	1,030239	0,853035
Entrance of virus	-0,7503	-1,12401
Endocytosis by eukaryotic cells	1,60674	-0,26665
Cell death of carcinoma cell lines	-1,29598	-0,5695
Immune response of leukocytes	1,585458	0,266466
Apoptosis of endothelial cells	-1,1508	0,694248
Aggregation of cells	0,929661	0,911176
Cytotoxicity of cells	1,497627	0,331954
Adhesion of immune cells	0,922906	0,896953
Cell movement of endothelial cells	0,744339	-1,06247

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Cell viability of tumor cell lines	1,071634	0,704096
Expansion of cells	0,698499	1,029635
Aggregation of phagocytes	-0,86266	-0,86266
Leukopoiesis	0,562098	1,156572
Cell death of epithelial cells	-0,62116	-1,0734
Migration of kidney cell lines	1,381075	0,307701
Cytotoxicity of natural killer cells	1,668952	0,01834
Flux of ion	0,514135	1,171204
Binding of professional phagocytic cells	1,324119	0,353926
Anoikis	-1,60706	0,064282
Cytotoxicity of leukocytes	1,500586	-0,14522
Internalization of cells	0,08681	-1,55344
Sprouting	-0,61293	0,999921
Engulfment of cells	1,13085	-0,45487
Adhesion of granulocytes	0,964917	0,615801
Adhesion of embryonic cells	1,130765	0,428929
Adhesion of blood cells	0,9044	0,64615
Adhesion of vascular endothelial cells	0,67249	0,876791
Cell movement of blood cells	0,90901	0,636499
Release of Ca2+	0,876806	-0,66295
Proliferation of leukemia cell lines	0,815084	-0,71037
Cell death of myeloid cells	-0,42856	-1,09408
Vasculogenesis	1,212155	-0,30558
Cell proliferation of tumor cell lines	-0,40218	-1,11309
Formation of focal adhesions	0,745356	-0,74536
Cell movement of cancer cells	-0,74336	-0,74336
Chemotaxis of phagocytes	-0,75441	-0,72617
Binding of myeloid cells	0,870451	0,605385
Cell death of lymphoma cell lines	-0,75291	-0,71994
Cell death of mononuclear leukocytes	-1,07223	-0,3993
Cell death of breast cancer cell lines	0,822943	0,640275
Cell movement of phagocytes	-0,70787	-0,75376
Formation of lamellipodia	-1,28138	0,17842
Apoptosis of leukocytes	-1,11896	-0,33336
Microtubule dynamics	0,371566	1,073767
Anoikis of tumor cell lines	-0,84	0,6
Mobilization of Ca2+	0,25057	1,160418
Binding of leukemia cell lines	1,023452	-0,38576
Neoplasia of cells	0,506838	0,898764
Formation of skin	0,32323	1,062041
Migration of PBMCs	0,958194	0,425864
Protein kinase cascade	-0,58977	-0,7918
Binding of breast cancer cell lines	-0,11662	-1,26246
Binding of hematopoietic cell lines	-0,68813	-0,68813
Leukocyte migration	0,818755	0,547829
Migration of cells	0,975354	0,390517
Chemotaxis of granulocytes	-0,97533	-0,38723
Immune response of tumor cell lines	-0,756	-0,60171
Apoptosis of mononuclear leukocytes	-1,18005	-0,17023
Cell death of kidney cell lines	-0,53978	-0,79849

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Cell death of epithelial cell lines	-0,22772	-1,09869
Binding of dendritic cells	-0,20777	-1,1144
Formation of filopodia	0,821995	-0,4932
Interaction of antigen presenting cells	-0,25693	-1,02772
Binding of antigen presenting cells	-0,25693	-1,02772
Production of reactive oxygen species	0,03858	1,227621
Formation of plasma membrane	0,052632	-1,21053
Formation of cytoskeleton	-0,61302	0,636516
Invasion of breast cancer cell lines	-0,65738	-0,58933
Phagocytosis	0,640466	-0,59175
Growth of malignant tumor	-0,26812	-0,96326
Growth of tumor	-0,3585	-0,87092
Migration of tumor cell lines	0,922581	0,286888
Phagocytosis of cells	0,454938	-0,75133
Cell rolling	-0,11926	1,086557
Necrosis of prostate cancer cell lines	-0,45696	-0,70999
Differentiation of keratinocytes	0,194257	0,971286
Adhesion of myeloid cells	0,813735	0,350634
Aggregation of tumor cell lines	0,747409	0,415227
Cell death of lymphocytes	-0,92172	-0,23969
Cell movement of dendritic cells	-0,75005	0,409331
Differentiation of myeloid leukocytes	0,029243	1,112184
Expansion of leukocytes	0,624022	0,508899
Binding of monocytes	0,677399	0,455474
Cell movement of epithelial cell lines	0,673542	0,44693
Expansion of blood cells	0,391551	0,727046
Inflammatory response	-0,15132	-0,96404
Cell death of leukemia cell lines	1,059203	-0,04591
Cell-cell adhesion	0,754231	-0,34976
Adhesion of kidney cells	0,851176	-0,2412
Apoptosis of leukemia cell lines	0,746451	-0,34402
Synthesis of reactive oxygen species	-0,21885	0,861002
Cancer of cells	0,862662	0,215666
Cell proliferation of sarcoma cell lines	0,174579	-0,89975
Central nervous system solid tumor	0,298275	-0,77551
Glioma	0,298275	-0,77551
Apoptosis of myeloid cells	-0,40804	-0,65216
Cell movement	0,83361	0,217948
Invasion of cells	0,739899	0,300606
Adhesion of leukemia cell lines	0,99213	0,047804
Transmigration of mononuclear leukocytes	1,018552	-0,02035
Reorganization of actin cytoskeleton	-0,81153	-0,22723
Cell spreading	0,073871	-0,9627
Tubulation of cells	0,648241	-0,38342
Apoptosis of lymphocytes	-1,02237	0,002929
Differentiation of epithelial cells	0,039193	0,979827
Necrosis of epithelial tissue	-0,60994	-0,39124
Formation of actin stress fibers	-0,18396	0,815293
Organization of actin cytoskeleton	-0,49854	-0,49854
Transcription	0,566185	0,419434

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

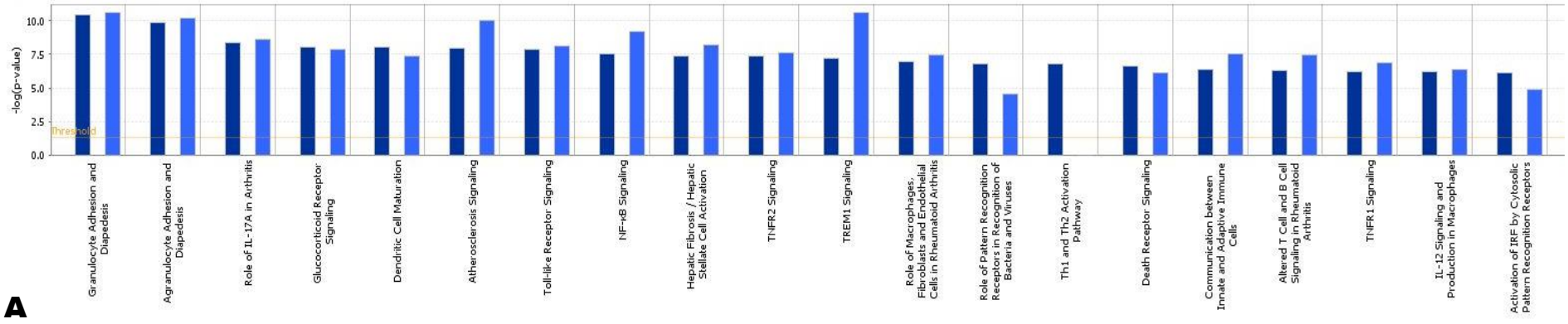
Diseases and Bio Functions	BA WT	BA DM
Shape change of tumor cell lines	-0,55682	-0,41705
Differentiation of phagocytes	-0,23577	0,732592
Interaction of tumor cell lines	-0,08984	-0,87099
Migration of breast cancer cell lines	0,479714	0,481001
Apoptosis of T lymphocytes	-0,73172	-0,22396
Chemotaxis	-0,17667	-0,77362
Inhibition of cells	-0,25073	0,693203
Binding of epithelial cell lines	-0,22471	-0,7177
Binding of fibroblasts	0,543124	0,393297
Reorganization of cytoskeleton	-0,46544	-0,46544
Invasion of tumor cell lines	0,595235	0,335368
Branching of cells	-0,12154	0,807532
Immune response of cells	0,013269	-0,8864
Transmigration of phagocytes	0,805448	-0,08345
Apoptosis of cancer cells	0,002008	-0,88311
Cell proliferation of carcinoma cell lines	-0,68337	0,197718
Cell spreading of tumor cell lines	0,28724	-0,58697
Carcinoma	-0,21822	0,654654
Cell movement of peripheral blood monocytes	0,620651	0,248261
Cell movement of embryonic cell lines	-0,13192	-0,73498
Adhesion of epithelial cells	0,536725	0,326692
Cell movement of tumor cells	-0,19597	-0,66628
Shape change of kidney cell lines	0,018254	-0,83511
Chemotaxis of leukocytes	-0,15607	-0,69648
Attachment of cells	0,775886	-0,07563
Cell movement of monocyte-derived dendritic cells	-0,40158	0,447584
Cell movement of kidney cell lines	0,725682	-0,12276
Interaction of kidney cell lines	0,588161	-0,25599
Proliferation of B lymphocytes	-0,53416	0,300753
Cell movement of neutrophils	-0,42874	-0,40273
Tumorigenesis of tissue	-0,51848	0,311086
Neoplasia of epithelial tissue	-0,51848	0,311086
Apoptosis of tumor cells	0,312687	-0,51186
Adhesion of epithelial cell lines	0,593013	-0,20492
Adhesion of embryonic cell lines	0,593013	-0,20492
Development of cytoplasm	-0,18502	0,610175
Invasion of carcinoma cell lines	-0,7193	0,065208
Proliferation of prostate cancer cell lines	-0,46296	0,317686
Binding of tumor cell lines	0,02987	-0,75009
Cell death of T lymphocytes	-0,53599	-0,23441
Chemotaxis of myeloid cells	-0,26191	-0,50135
Adhesion of kidney cell lines	0,227933	-0,53483
Cell death of neutrophils	-0,27742	-0,47877
Development of neurons	0,377964	-0,37796
Binding of melanoma cell lines	0,369007	0,369007
Adhesion of tumor cell lines	0,679026	0,047431
Binding of vascular endothelial cells	0,514734	0,202478
Lymphohematopoietic neoplasia	0,478091	-0,23905
Hematopoietic neoplasm	0,478091	-0,23905

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Transmigration of myeloid cells	0,656906	0,043794
Cell death of granulocytes	-0,25004	-0,44962
Expansion of mononuclear leukocytes	0,411507	0,278389
Organization of cytoskeleton	0,236755	0,451824
Organization of cytoplasm	0,236755	0,451824
Aggregation of blood cells	-0,48061	-0,1976
Migration of carcinoma cell lines	-0,32023	0,350826
Attachment of tumor cell lines	0,249136	-0,41523
Apoptosis of breast cancer cell lines	0,113004	0,548199
Digestive organ tumor	-0,21822	0,436436
Cell movement of antigen presenting cells	-0,21261	0,434406
Binding of B lymphocytes	7,18E-17	-0,647
Generation of reactive oxygen species	-0,24697	0,397817
Tubulation of endothelial cells	0,240239	-0,38579
Apoptosis	-0,43484	-0,17885
Cell movement of leukocytes	0,539294	0,068669
Cell movement of granulocytes	-0,23909	0,343355
Cell movement of lung cancer cell lines	0,327808	-0,23596
Non-melanoma solid tumor	0,331991	0,221327
Migration of neutrophils	0,357095	0,192913
Homing of cells	0,063344	-0,47856
Tyrosine phosphorylation of protein	0,426722	0,113941
Transmigration of monocytes	0,130931	-0,39279
Cell movement of breast cancer cell lines	0,348424	0,174623
Binding of embryonic cell lines	0,139111	-0,37992
Aggregation of blood platelets	-0,25603	-0,25603
Production of superoxide	0,254685	0,254685
Phosphorylation of protein	0,332	-0,16132
Cell movement of monocytes	0,385564	-0,08895
Formation of cellular protrusions	0,258895	0,212307
Binding of kidney cell lines	0,393837	-0,05032
Cell death of connective tissue cells	0,402856	0,035862
Migration of monocytes	0,388	-0,0485
Cell death	-0,13732	-0,287
Binding of epithelial cells	0,32279	0,101052
Migration of antigen presenting cells	0,10673	0,30963
Invasion of melanoma cell lines	0,055134	0,358374
Migration of phagocytes	0,250043	0,161108
Necrosis	-0,36901	0,015529
Tyrosine phosphorylation	0,187058	-0,11746
Degranulation of cells	0,058074	0,223999
Degranulation	0,058074	0,223999
Cell death of immune cells	-0,20032	-0,08005
Cell movement of myeloid cells	0,203515	-0,06646
Formation of actin filaments	-0,11289	0,146031
Cell death of tumor cell lines	-0,06386	-0,17186
Expansion of T lymphocytes	0,187867	0,033787
Expression of RNA	0,176937	0,041887
Migration of dendritic cells	-0,16855	0,045547
Adhesion of fibroblasts	0,18179	0,020199

Table 6: Biofunctions enriched by biological adhesion associated genes (BA) elicited by WT and DM continued…

Diseases and Bio Functions	BA WT	BA DM
Cell movement of prostate cancer cell lines	-0,02684	-0,16643
Invasion of lung cancer cell lines	-0,07774	-0,07666
Formation of filaments	-0,06273	0,086606
Apoptosis of tumor cell lines	-0,05951	-0,00114



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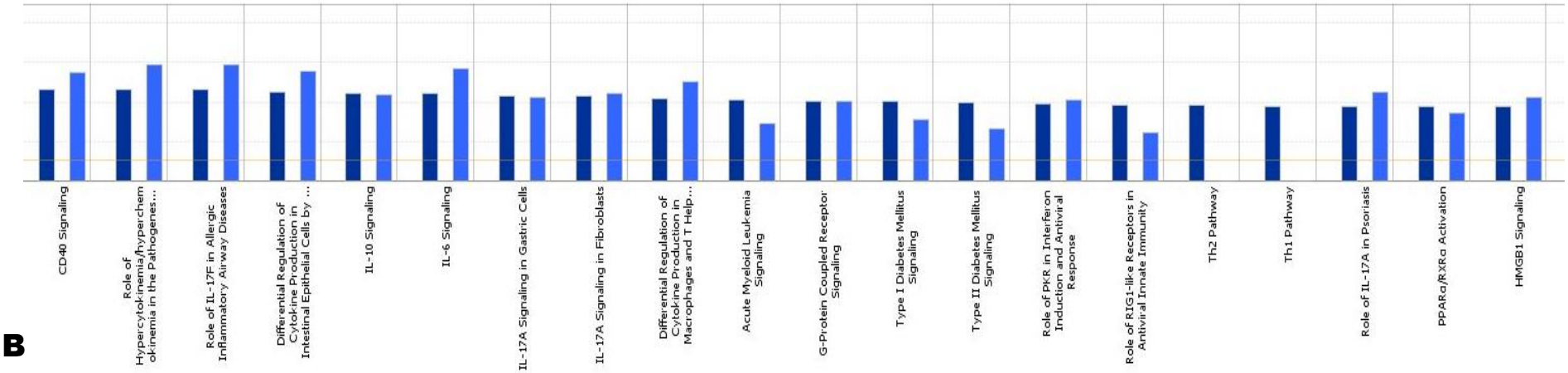

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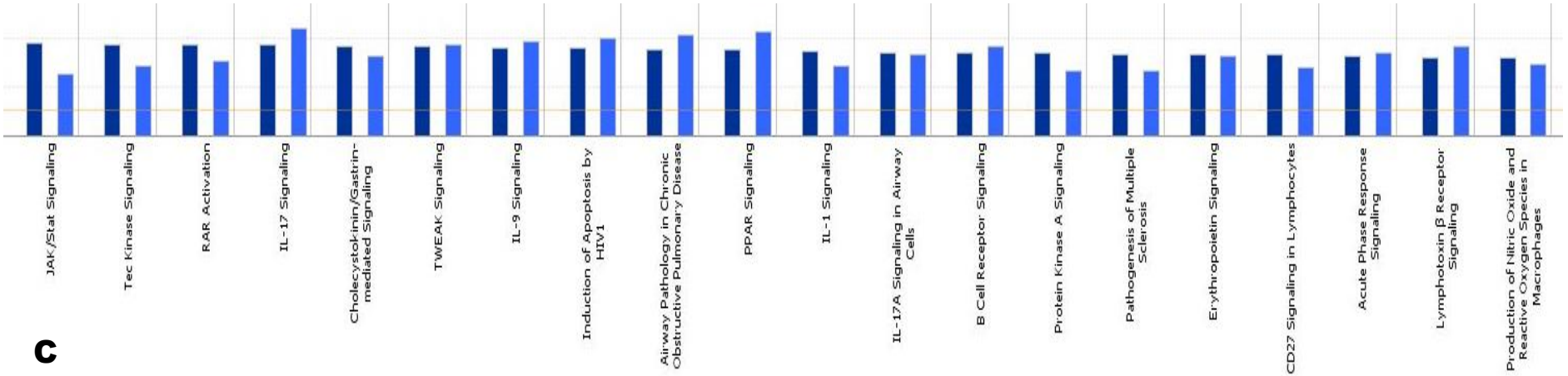

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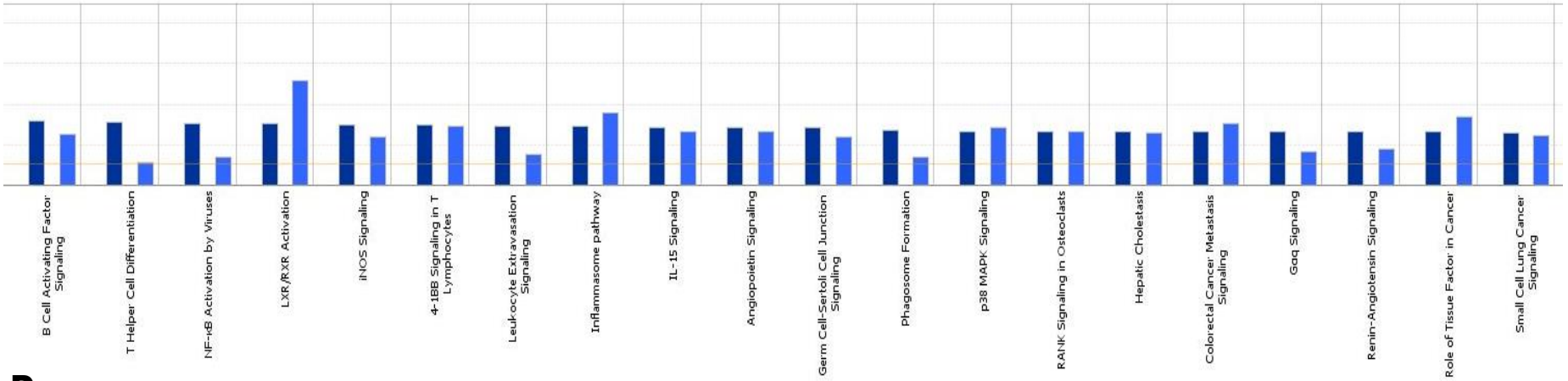

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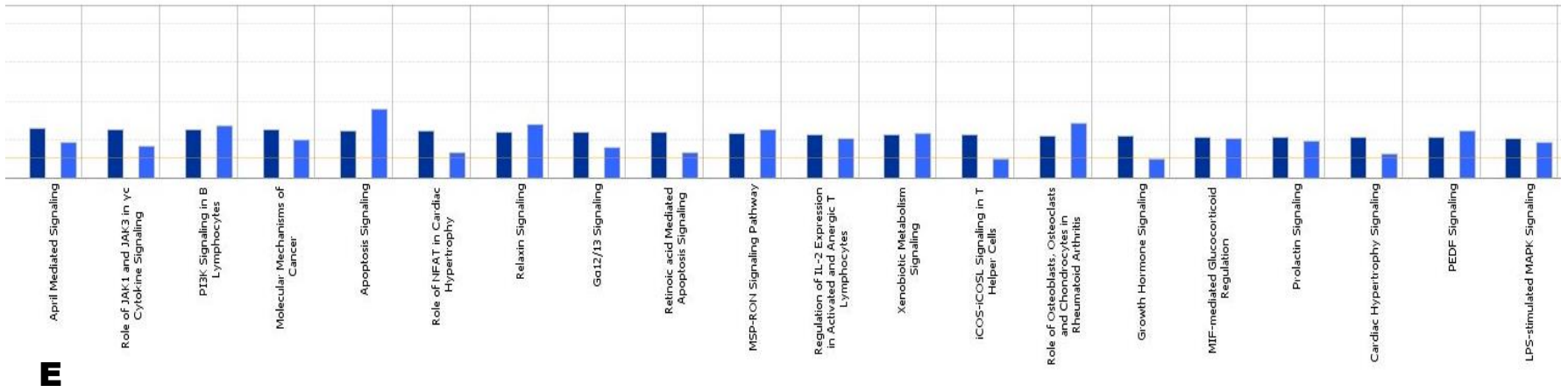

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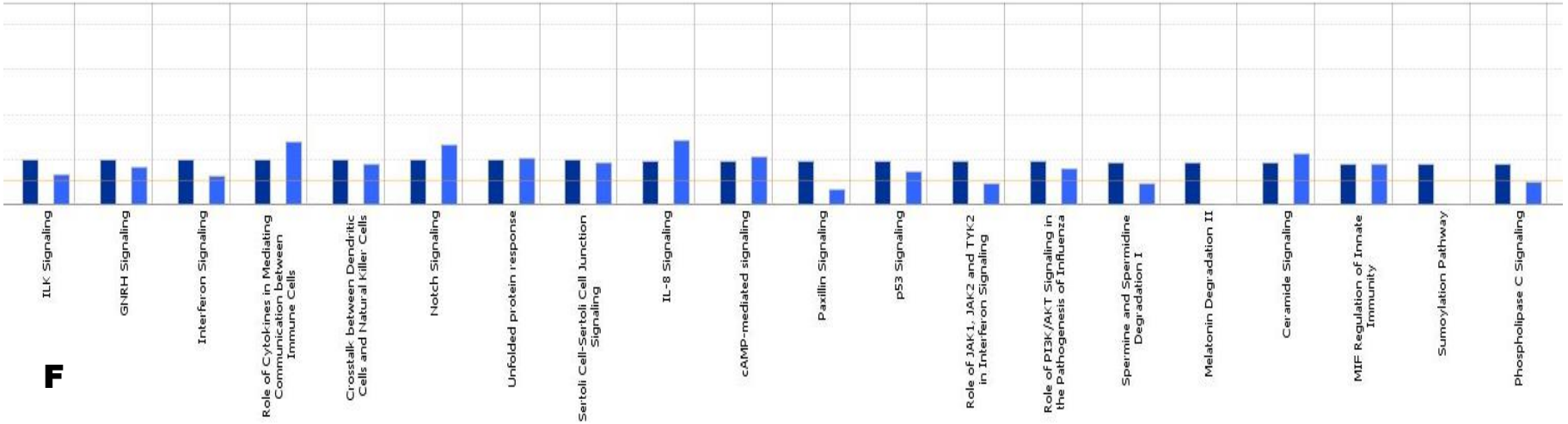

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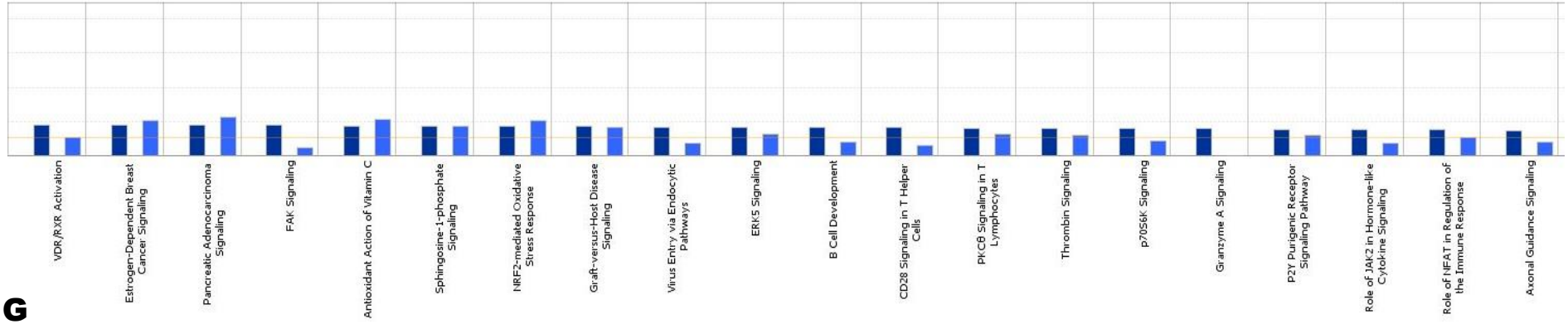

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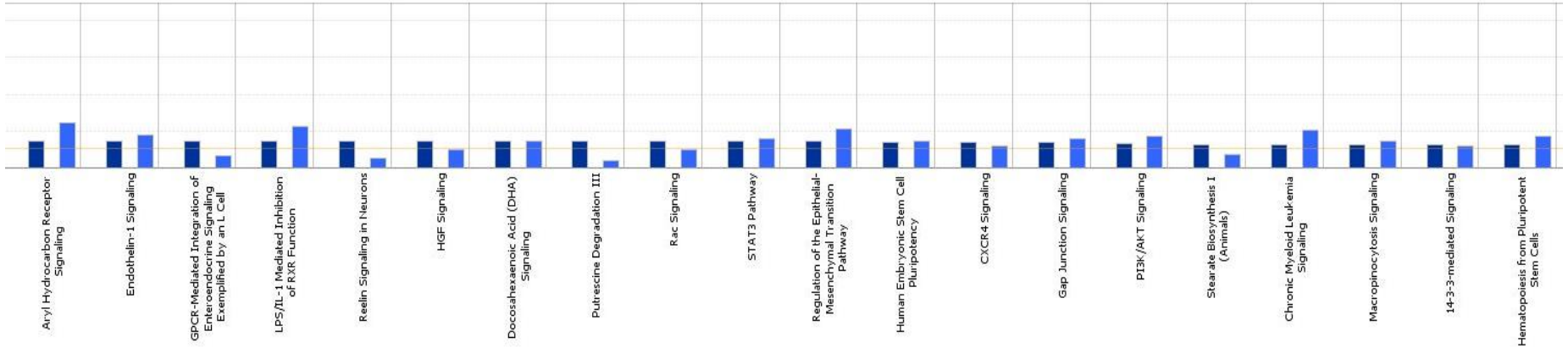

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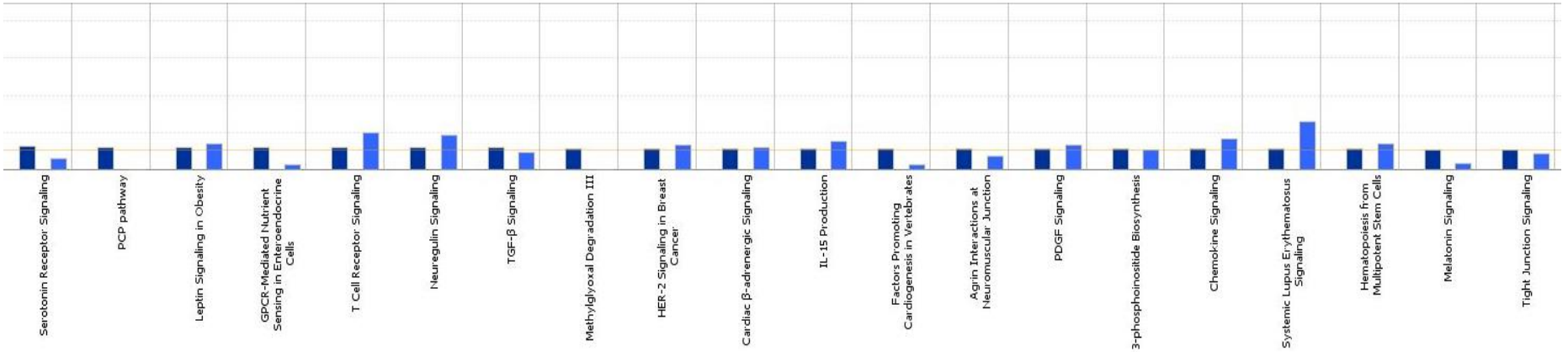

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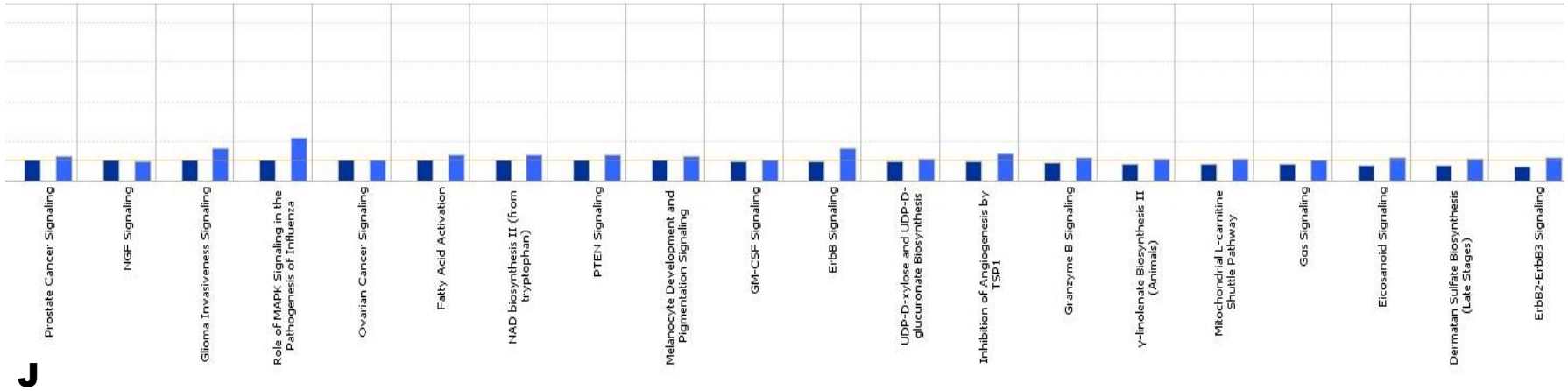

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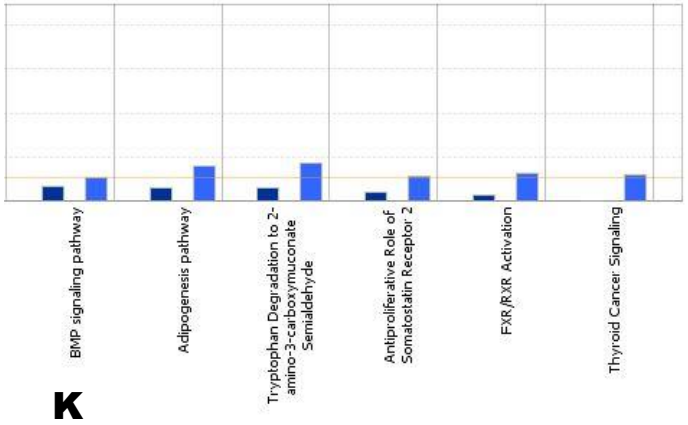


Figure 7: All canonical pathways elicited by SDEGs in WT and DM infections respectively. (A) 1-20 pathways (B) 20-40 pathways. (C) 40-60 pathways. (D) 60-80 pathways elicited by all genes in WT and DM infections respectively. (E) 80-100. (F) 100-120 pathways. (G) 120-140 pathways. (H) 140-160 pathways. (I) 160-180 pathways. (J) 180-200 pathways. (K) 200-206 pathways. Dark blue bars represent DM infection and light blue bars represent WT infection.

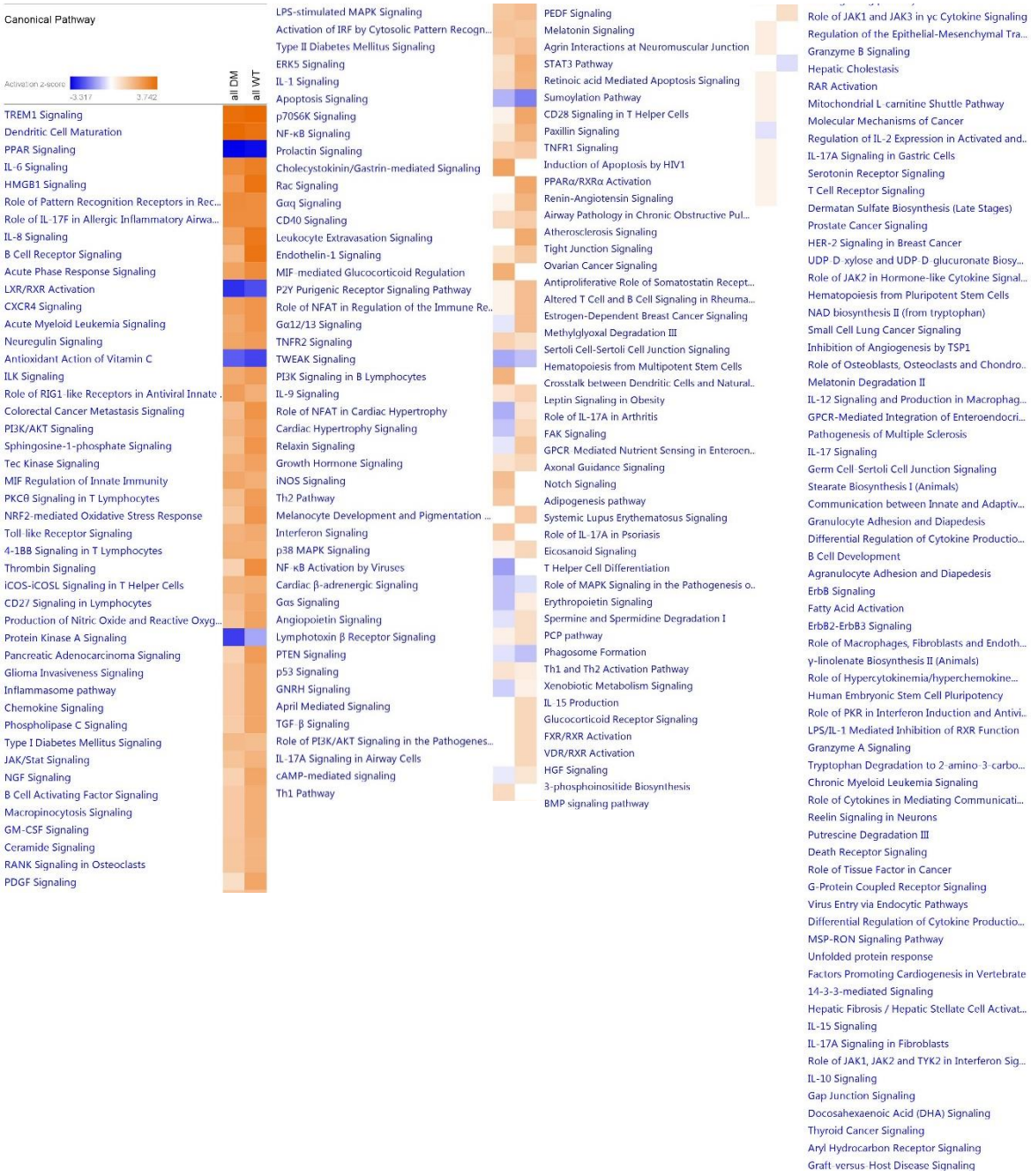


Figure 8: Canonical pathway. Activation z-scores elicited by SDEGs in WT and DM infections respectively.

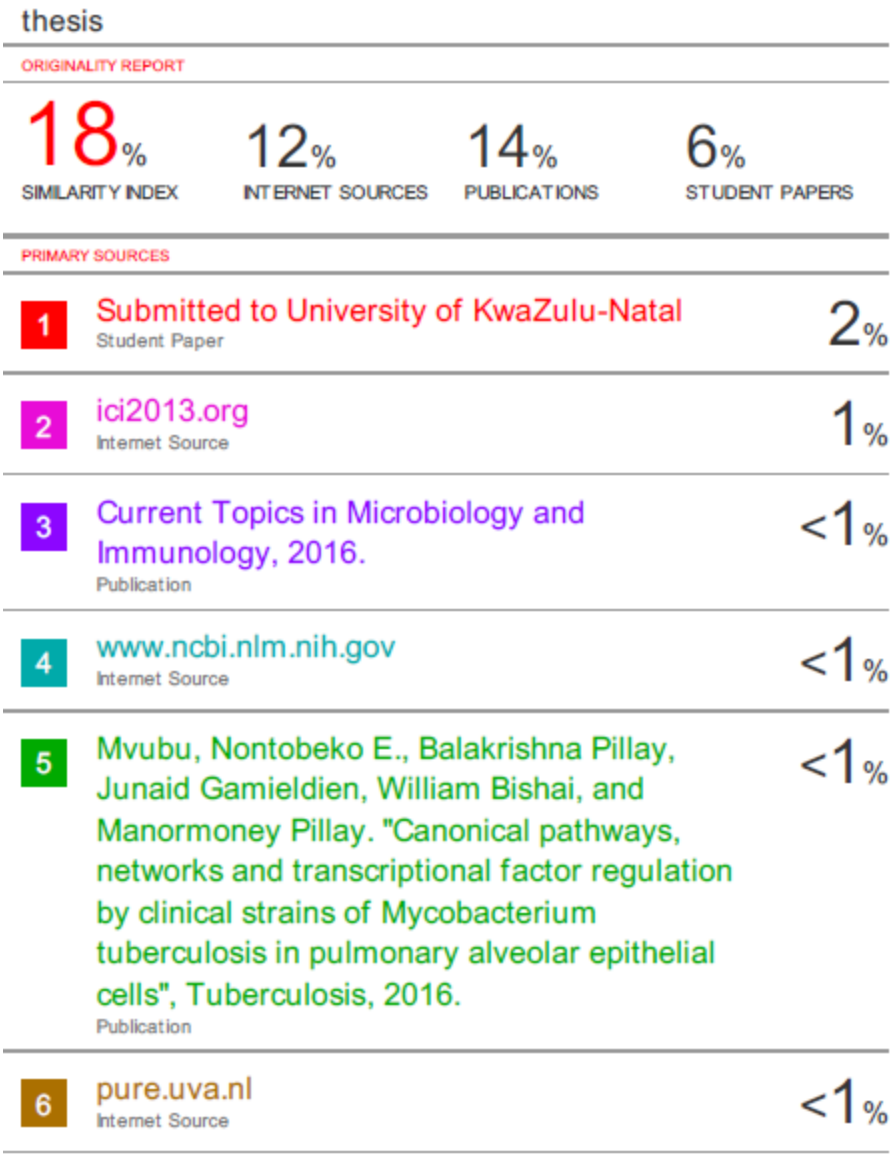
Table 7: Upstream regulators continued...
Activation z-scores elicited by SDEGs in WT and DM infections respectively.

Upstream regulators	all DM	all WT
NFkB (complex)	6,381094	6,160147
RELA	4,298981	4,720937
ECSIT	4,478977	4,032016
FOXL2	3,851703	3,858103
SMARCA4	3,156367	3,554647
EZH2	3,261344	3,19023
NUPR1	3,063165	3,156821
IRF3	3,258144	2,94728
JUN	2,753616	3,160921
FOXO1	2,778211	2,778211
CEBPA	2,548408	2,853217
PPRC1	2,733967	2,561959
NFKB1	2,735878	2,548852
FOXO3	2,706862	2,540491
CEBPB	2,589689	2,589689
HLX	-2,44949	-2,44949
BCL11B	2,432701	2,432701
HMGB1	2,296662	2,296662
POU5F1	2,013996	2,459675
TAL1	-2,13809	-2,33333
ATF2	2,213211	2,213211
TP53	2,615438	1,783756
STAT1	2,117613	2,26022
Ap1	2,156655	2,213211
STAT3	2,373267	1,959395
SP1	1,963961	2,2
IRF4	-2,34442	-1,76273
Hdac	-1,49482	-2,37723
FOS	2,031655	1,789474
MYC	-1,91237	-1,66448
EHF	1,889822	1,632993
HIF1A	1,704868	1,807151
SMAD4	2,215647	1,192079
KLF6	1,610685	1,610685
NFE2L2	1,192079	1,997251
EP300	1,38675	1,664479
PSMD10	1	2
CTNNB1	1,011368	1,762818
BRCA1	-1,22462	-1,22462
BCL6	-1,19208	-1,19208
SATB1	-1,86214	-0,50307
EGR1	1,015866	1,287179
NEUROG1	-2,23607	N/A
WT1	-1,40841	-0,79772
TP63	1,147601	1,021119
RUVBL1	1	1
IRF1	2	N/A

Table 7: Upstream regulators continued...
Activation z-scores elicited by SDEGs in WT and DM infections respectively.

Upstream regulators	all DM	all WT
SPDEF	-2	N/A
ZNF217	N/A	-2
RELB	0,991492	0,991492
NOTCH1	N/A	1,979899
CREB1	1,974383	N/A
SMAD7	-1	-0,92717
ERG	0,377964	1,341641
SOX11	-0,72761	-0,84853
TP73	-0,34062	-1,18046
E2F3	-1	-0,37796
RBPJ	-0,65465	-0,65465
NFATC2	1,09821	N/A
HDAC6	1,06749	N/A
UXT	0,762493	0,27735
CDKN2A	-1	N/A
STAT5a/b	N/A	1
GLI1	-0,06855	0,850265
MDM2	-0,39057	-0,39057
HIC1	-0,44721	N/A
YBX1	0,447214	N/A
NOTCH3	0,447214	N/A

Appendix 5: Turnitin similarity report



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