A transcriptomic approach for studying the activation of dendritic cells in response to mycobacterial infections

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This is to certify that the thesis comprises of original work of the author and all due acknowledgement has been made in the text to all other materials used.

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Abstract

Mycobacterium tuberculosis (M. tuberculosis) is responsible for 2 million deaths annually. In recent years co-infection with HIV and drug-resistant strains has led to the increase in clinical cases of tuberculosis. BCG is the only vaccine currently available but is ineffective against adult pulmonary forms of the disease. Understanding the principle components of the host immune response against the pathogen will aid in the design of a better vaccine. Dendritic cells are potent antigen presenting cells that play a key role in priming naïve T cells. Effective T cell priming is necessary for a successful protective immune response in the host against the pathogen.

I have been investigating the interaction of dendritic cells with *M. tuberculosis,* with a view to better understanding the signalling pathways affecting priming of anti-mycobacterial T cells by dendritic cells. Using DNA microarray and bio-informatics, I have been able to study early transcriptional signatures of bone marrow-derived dendritic cells (BMDCs) in response to *M. tuberculois* infection. Interferon Regulatory Factors (IRFs) and NFκB (Nuclear Factor kappa B) appear to be the principal transcription factors involved in regulating the cellular responses in *M. tuberculosis* infected BMDCs. It has been found that IRFs can function independent of the adaptor MyD88 (Myeloid Differentiation Factor 88) in *M. tuberculosis* infected BMDCs, which is surprising considering the important role played by MyD88 in the Toll mediated signalling pathway. Ex-vivo experiments also show that MyD88 may not be absolutely essential for mounting anti-mycobacterial T cell responses. Hence immune responses generated by *M. tuberculosis* infected BMDCs appear to be mediated via the MyD88 dependent and independent pathways.

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List of abbreviations

AP-1	Activator protein-1
ATP	Adenosine triphosphate
BCG	Bacille Calmette Guérin
BMDCs	Bone marrow derived dendritic cells
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CFP-10	Culture filtrate protein-10
CFU	Colony forming units
CpG	Deoxy-cytidylate-phosphate-deoxy-guanylate
CR	Complement receptor
C-rel	Reticuloendotheliosis oncogene
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ESAT-6	Early secreted antigen-6
Fc	Fragment crystallisable
Foxp3	Forkhead box P3
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS	IFN-γ activated sequence
GCRMA	GC-Robust Multiarray Analysis
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G.O.I	Gene of interest
HIV	Human immunodeficiency virus
HKG	House keeping gene
HRP	Horseradish peroxidase enzyme
ICAM	Intercellular adhesion molecule
IFNAR	Interferon (alpha and beta) receptor

IFNGR	Interferon gamma receptor
IFNs	Interferons
IKK	Inhibitor of nuclear factor kappa-B kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1-receptor-associated kinase
IRF	Interferon regulatory factor
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
JAK	Janus kinase
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
M. tuberculosis	Mycobacterium tuberculosis
M.O.I	Multiplicity of infection
MAL	MyD88 adapter like
ManLAM	Mannosylated lipoarabinomannan
MDR-TB	Multi-drug resistant tuberculosis
MHC	Major histocompatibility complex
Mx	Myxovirus (influenza virus) resistance
MyD88	Myeloid differentiation primary response gene (88)
NFκB	Nuclear factor kappa B
NK cell	Natural killer cells
NIMR	National Institute for Medical Research
NO	Nitric oxide
OAS	2'-5'-oligoadenylate synthetase
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
PIM	Phosphatidylinositol mannosides
Poly I :C	Polyriboinosinic polyribocytidylic acid
PPD	Purified protein derivative
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates

SARM	Sterile α and armadillo motifs
SD	Standard deviation
SDS	Sodium dodecyl (lauryl) sulfate
SE	Standard error mean
SLE	Systemic lupus erythematosus
SOCS	Suppressors of cytokine signalling
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TACO	Tryptophan-Aspartate containing coat
TB	Tuberculosis
TBK	TANK binding kinase 1
TELiS	Transcription element listening system
Th-1	T helper cells
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor-6
TRAM	TRIF-related adapter molecule
TRIF	TIR domain containing adaptor inducing interferon-beta
TYK-2	Tyrosine kinase 2
WHO	World health organisation
XDR-TB	Extensively drug resistant tuberculosis

Chapter 1

Introduction

1.1– Tuberculosis- a global disease

Tuberculosis (TB) is an ancient disease known for over 4000 years. One of the earliest records comments on identifying decay in the spinal cord fragments of Egyptian mummies from around 2400BC (Zink, Sola et al. 2003). The disease also called "consumption" or "white plague" is still responsible for approximately 2 million deaths every year. Overall, one third of the world's population is infected with *Mycobacterium tuberculosis (M. tuberculosis)*, generally with no recognisable symptoms. The World Health Organisation (WHO) estimates that in 2004, approximately 14.6 million people were living with TB and about 1.7 million people died from the disease. About 80% of the world burden of TB is borne by 22 countries with one-third of the burden in India and China (WHO 2006). In recent years co-infection with human immunodeficiency virus (HIV) and appearance of multi-drug resistant (MDR) strains have led to the increased number of clinical cases. TB is the most common cause of death in HIV patients. Thus there is a lethal synergy between HIV and *M. tuberculosis*, which causes problems in the administration of TB control programmes. The rise in drug resistant strains, amounting to 300,000 new cases every year, alongside the recent cases of extensively drug-resistant tuberculosis (XDR-TB) has also contributed to increased problems in control of TB (WHO 2006). Chemotherapy for TB patients was established with the discovery of streptomycin in the 1940s. Standard tuberculosis chemotherapy involves (WHO 2006) taking isoniazid, rifampicin, pyrazinamide and ethambutol for two months and subsequently followed by isoniazid and rifampicin for four months. Strains categorised as MDR are resistant to at least the two front line drugs (rifampicin and isoniazid) and XDR-TB strains are resistant to the front-line drugs and in addition, are also resistant to fluoroquinolones and either capreomycin, kanamycin or amikacin (WHO 2006).

Conventional methods of diagnosis of TB include microscopy and bacterial culture. Microscopy is commonly used for identifying bacteria in sputum samples either using Ziehl-Neelsen or auramine-rhodamine staining, the latter being the current preferred option as it is quicker and more sensitive than Ziehl-Neelsen staining (Steingart, Henry et al. 2006). Bacteria from clinical specimens can be cultured conventionally in Löwenstein-Jensen, Kirchner, or Middlebrook media (7H10 or 7H11). In recent years, new automated systems such as

MB/BacT, BACTEC 9000, and the mycobacterial growth indicator tube offer faster methods of diagnosis. Chest X-rays are generally non-specific and expensive thus making them inaccessible to large proportion of patients in developing countries. Abnormalities in radiographs are suggestive but do not provide a conclusive diagnosis of TB infection. The other main method of detecting TB infection is by using the Mantoux test but this method lacks specificity and is highly variable in populations of immunocompromised individuals. Purified protein derivative (PPD), the major component of the tuberculin reagent used for the Mantoux test, cross-reacts with antigens from environmental mycobacteria thus increasing the risk of false positives (Andersen, Munk et al. 2000).

New assays based on detecting IFN-γ produced by T cells in response to early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) are more specific and thus provide more accurate testing than the Mantoux skin test. T-SPOT TB (developed by Oxford Immunotech, UK) and QuantiFERON-TB Gold (developed by Cellestis, Australia) have based their assays on these two antigens, ESAT-6 and CFP-10, encoded within the region of difference-1 locus of *M. tuberculosis*, which is absent from Bacille Calmette Guérin (BCG) and most other strains of mycobacteria; thus these tests offer increased specificity and reduced risk of false positives (Liu, Dosanjh et al. 2004). Despite advances in detection of TB infection in recent years, it remains difficult to distinguish between active vs latent TB.

In a recent report, messenger RNA (mRNA) transcripts for IL-8 (a chemoattractant for neutrophils, monocytes and T cells), forkhead box P3 (Foxp3, a regulatory T cell specific transcription factor) and IL-12p40 were consistently shown to have different expression levels between active and latent TB cases and thus were able to distinguish between these two disease states quite accurately (Wu, Huang et al. 2007). It was further suggested that measuring the expression of these three genes in response to TB antigens would offer an accurate method of distinguishing between latent and active TB cases but the feasibility of such a test would have to be assessed by including individuals co-infected with HIV, which were excluded from the present study (Wu, Huang et al. 2007).

TB control can be achieved using a suitable pre-exposure (prophylactic vaccine) or post-exposure vaccine. The current vaccine BCG is the only approved vaccine for the prevention of TB. BCG has had varying degrees of efficacy against adult pulmonary TB, ranging from providing no protection in South India to 80% protection in trials conducted by the UK Medical Research Council (Hart and Sutherland 1977). BCG is consistently effective against severe childhood forms of TB meningitis (Colditz, Brewer et al. 1994) although in 2005, routine BCG vaccination of school children was ended in the UK and now vaccination is only offered to children in high risk groups (TB incidence of more than 40 cases per 1000,000 individuals) in the UK. The low efficacy of BCG has been attributed to a number of factors including differences in the BCG strain that is being used for vaccination (Behr 2002) and exposure to environmental mycobacteria. The exposure to environmental mycobacteria primes the immune system against mycobacterial antigens shared with BCG and subsequently vaccination with BCG results in the rapid clearance of this strain, due to rapid recall responses (Demangel, Garnier et al. 2005). Another reason for the failure of BCG has been attributed to the increased levels of the cytokine IL-4, which can dampen T-helper 1 (Th-1) type immune response induced by BCG, thus leading to the reduced efficacy of the vaccine (Rook, Dheda et al. 2006). Due to the varying efficacy of BCG and the inability of the vaccine to protect against adult pulmonary TB, it is vital that a new vaccine is available soon. A number of new vaccine candidates are in the pipeline being currently evaluated in clinical trials and the hope is at least one vaccine would complete phase III trials and be licensed by 2015 (Bioventures 2006).

1.2 - *M. tuberculosis*

1.2.1- *M. tuberculosis* - aetiological agent of TB

M. tuberculosis, the causative agent of TB, is a non-motile and acid-fast actinomycete (Russell 2001). The bacterium was discovered by Robert Koch in 1882. This bacterium is part of the *M. tuberculosis* complex which encompasses *M. tuberculosis*, *M. bovis*, BCG, *M. africanum*, *M. canettii* and *M. microti*, all of which share >99% identity at the nucleotide level. *M. tuberculosis* is an aerobic, facultative intracellular pathogen with a generation time of approximately 20

hours. This lengthy generation time contributes to the chronic nature of the disease, thus contributing to the extensive duration of treatment regime. These bacteria possess a unique cell wall rich in mycolic acids, lipoarabinomannan (LAM), arabinogalactan and phosphatidylinositol mannosides (PIM). The mycobacterial genome of CDC1551 (Fleischmann, Alland et al. 2002), *M. bovis* (Garnier, Eiglmeier et al. 2003) and H37Rv (Cole, Brosch et al. 1998), a commonly used laboratory strain of *M. tuberculosis* has been sequenced. The genome of H37Rv consists of approximately 4000 genes. Of these 4000 genes, 40% have a clearly defined function, 40% have been shown to belong to previously identified classes of genes and 20% are completely unknown. Comparative genomic analysis of *M. bovis* BCG with H37Rv, has revealed 11 deletions from the genome of *M. bovis* BCG and the variation in physiology between these two strains may be attributed to the differences in gene expression.

1.22- Pathogenesis of tuberculosis

Approximately one-third of the world's population is infected with *M. tuberculosis.* Individuals who are infected have a 5-10% risk of developing the active form of the disease. Smoking and HIV infection can predispose an individual to acquiring infection, with overcrowding and poverty increasing the risk of exposure to *M. tuberculosis* (Cantwell, Mckenna et al. 1998; Lienhardt, Fielding et al. 2003).

The first stage of interaction between the host and bacteria is initiated after the inhalation of the bacilli via aerosol droplets, measuring 1-2μm, from infectious individuals. In the lung, bacteria are phagocytosed by alveolar macrophages and the generation of a localised inflammatory immune response results in the recruitment of immune cells to the sites of infection. Accumulation of immune cells at the foci of infection, results in the development of a granuloma, which limits the dissemination of the bacteria and localises the inflammation and damage to the lung tissue. Granulomas are composed of infected macrophages, surrounded by foamy giant cells with lymphocytes on the periphery of the structure (Co, Hogan et al. 2004).

The bacteria are contained within these granulomas and the host no longer transmits the infection. Latent, viable bacteria can survive in granulomas for very long periods of time. An alternative possibility is that the bacteria can multiply leading to the development of active TB shortly after infection and this is most likely to happen in with a compromised immune system.

Latency is a hallmark of *M. tuberculosis* infection and re-activation can occur due to a number of reasons such as alcohol abuse and corticosteroid therapy (Flynn and Chan 2001). Under reactivation circumstances, the granuloma becomes caseous and these reactivating granulomas are sites of enhanced bacterial replication. This reactivation leads to clinical symptoms and thus the formation of secondary TB. The different outcomes of the infection reflect the fine balance between the pathogen and host immune responses and any shift in the balance in favour of bacterial replication can lead to reactivation of the disease (Kaufmann 2001; Russell 2001).

1.2.3 - Interplay between host and pathogen

M. tuberculosis is an old foe of man and as a pathogen is highly adapted for survival within an immunocompetent host. Following infection with *M. tuberculosis* there are four potential outcomes:

- Initial response is completely effective and thus bacilli are eliminated
- Mycobacteria can grow and multiply thus causing primary clinical disease (active TB)
- Bacteria become dormant in granulomas leading to latent TB infection
- Latent bacilli reactivate as a failure of immune surveillance thus leading to reactivation TB

Only 10% of infected individuals progress to develop active TB, with a significant proportion of the remaining infected individuals harbouring latent bacilli which pose a reactivation threat in the future. The immune response to *M. tuberculosis* is composed of the early innate immune response, during which bacteria are taken up by phagocytic cells resulting in the production of inflammatory cytokines and induction of antimycobacterial mechanisms by

macrophages. This is followed by a late response in which *M. tuberculosis* specific T cells are activated leading to the priming of a cell mediated immune response accompanied by the production of effector cytokines including IFN-γ. In the final stages of infection, mycobacteria are contained within granulomas thus preventing dissemination of bacteria (latent phase).

1.2.4- Interaction between macrophages and *M. tuberculosis*

Macrophages are considered the primary host cells for mycobacteria. Inhaled mycobacteria within small droplets are phagocytosed by alveolar macrophages where they are confronted by an array of anti-mycobacterial mechanisms. Failure to kill mycobacteria, results in survival of bacteria within macrophages, leading to the establishment of a latent infection. A number of macrophage receptors are involved in the uptake of mycobacteria. Complement receptors CR1, CR3 and CR4 take up opsonised bacteria; monoclonal antibodies against CR3, markedly reduced phagocytosis of *M. tuberculosis* by monocytes thus, showing the relative importance of this phagocytic receptor in taking up mycobacteria (Schlesinger, Bellinger-Kawahara et al. 1990).

The macrophage mannose receptor aids in the uptake of non-opsonised bacteria. This receptor recognises the mannose-capped LAM (manLAM) present on virulent strains of mycobacteria. Other receptors including CD14, macrophage receptor with collagenous structure and fragment crystallisable gamma receptor (Fcγ) receptors, have all been shown to have roles in the phagocytosis of mycobacteria (Ernst 1998). The selective use of receptors by different strains of mycobacteria may hold clues to the different signalling pathways induced, thus influencing the survival of the bacteria within this host cell.

1.2.5- Antimycobacterial mechanisms of the macrophage

Macrophages are considered to be the main effector cells involved in the killing of mycobacteria. Macrophages when activated by interferon-γ (IFN-γ) and tumor necrosis factor-α **(**TNF-α) can exert various microbicidal mechanisms. Of importance, are the induction of reactive nitrogen intermediates (RNI) and

formation of reactive oxygen intermediates (ROI). Hydrogen peroxide is one of the key effector molecules generated as part of the ROI pathway alongside superoxide anions. ROI effectors cause lipid peroxidation, cell membrane, DNA and protein damage. But the role of ROI in killing mycobacteria is controversial, when the data is restricted to human studies, which show that patients with chronic granulomatous disease, who have a defective ROI mechanism, show no increased susceptibility to TB (Winkelstein, Marino et al. 2000). In contrast, a role for ROI in killing mycobacteria is demonstrated by the increased susceptibility of mice to mycobacterial infections, when deficient in the p47 subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase needed for superoxide production (Cooper, Segal et al. 2000) .

The production of increased nitric oxide (NO) is another anti-mycobacterial mechanism employed by an activated macrophage. Activation of macrophages by IFN-γ and TNF-α generates NO and related RNIs which are generated during the conversion of L-arginine to citrullene by inducible nitric-oxide synthase (iNOS). Murine macrophages have been previously shown to produce RNI in response to *M. tuberculosis* infection, which subsequently induced killing of mycobacteria (Chan, Xing et al. 1992). RNI has been shown to contribute to the protective immune responses during acute and chronic stages of infection (MacMicking, North et al. 1997). The role of iNOS in murine infections is fairly well documented but its role in human host defence remains unresolved. *In vitro*, human alveolar macrophages infected with *M. bovis* BCG up regulate iNOS mRNA and the use of iNOS inhibitors resulted in increased bacterial outgrowth (Nozaki, Hasegawa et al. 1997). In contrast, studies have also shown that in murine but not human macrophages the anti-mycobacterial activity is mediated principally by NO (Liu, Stenger et al. 2006). Thus, further work needs to be carried out to elucidate the importance of iNOS in human TB.

Non-oxidative mechanisms are also employed by activated macrophages in order to kill mycobacteria; for example, the induction of autophagy in activated macrophages resulted in the delivery of the bacteria into lysosomes and thus led to the killing of the bacteria by acid hydrolases (Gutierrez, Master et al. 2004; Alonso, Pethe et al. 2007).

1.2.6- Immune evasion mechanisms employed by *M. tuberculosis*

M. tuberculosis is able to survive within macrophages even though a range of effector mechanisms exist in these host cells to control the growth of invading bacteria. Normally, during the process of phagocytosis, microorganisms are taken up into phagocytic vacuoles which eventually fuse with lysosomes resulting in the formation of a phagolysosome. The phagolysosome environment is highly acidic and phagocytosed microbes are degraded by acid hydrolases. This event represents an integral part of the antimicrobial mechanism of phagocytes. However, *M. tuberculosis* employs various mechanisms to survive in the phagosome such as the modification of the phagosome it resides in by exclusion of vesicular H^+ ATPases (Crowle, Dahl et al. 1991; Koszycki-Sturgill, Schlesinger et al. 1994). The exclusion of H^+ ATPases from phagosomes containing live mycobacteria leads to the incomplete acidification of the phagosome, thus allowing the bacteria to survive. It has been additionally shown that, mycobacteria inhibit the formation of the phagolysosome, thus avoiding enzymatic degradation (Armstrong and Hart 1971). The block in maturation of mycobacterial phagosome occurs between the small GTP-binding proteins, Rab5 (early endocytic) and Rab7 (late endocytic), which directs trafficking in eukaryotic cells.

However, during *M. tuberculosis* infection, following the phagocytosis of the bacteria by macrophages, the bacilli are retained within phagocytic vacuoles which tend to be at a pH of 6.2-6.3 (Sturgill-Koszycki, Schlesinger et al. 1994). These vacuoles are not completely isolated from the rest of the host cell as they have been shown to contain lysosomal-associated membrane protein-1, major histocompatibility 2, class II antigen (MHC-II) and transferrin receptor, and are thus able to access transferrin internalised at the cell surface (Russell 2001). Consequently, these vacuoles fail to fuse with lysosomes but they remain fusion competent (Clemens and Horwitz 1996). Thus, mycobacteria-containing vesicles are highly dynamic and fuse with early endosomes although these vesicles do not fuse with lysosomes.

The retention of the host cell protein tryptophan aspartate rich coat proteins (TACO), on phagosomes, has been proposed as a possible reason for the failure of mycobacterial phagosomes to fuse with lysosomes. The association of

TACO to phagosomal membrane is by means of cholesterol rich domains which may provide a docking site for the mycobacteria to gain entry into phagosomes (Ferrari, Langen et al. 1999).

Mycobacterial components LAM and PIM have been shown to intercalate into endosome membranes and traffic within mycobacteria-infected cells (Beatty, Rhoades et al. 2000). LAM has been demonstrated to inhibit phagosomal maturation and PIM stimulates fusion of early endosomes thus maintaining the early endosomal nature of the phagosome (Fratti, Chua et al. 2003). Additionally, it has been reported that the serine/threonine protein kinase G from pathogenic mycobacteria secreted into the macrophage phagosomes prevents phagosome-lysosome fusion (Walburger, Koul et al. 2004). Hence, the bacteria exploit their lipids to modulate host cell membrane and phagolysosome biogenesis processes.

Inhibiting antigen presentation is also known to contribute to the survival of *M. tuberculosis* within macrophages. Recent evidence suggests, that the 19kDa lipoprotein of *M. tuberculosis* inhibits IFN-γ mediated up-regulation of MHC-II molecules and this response seems to be dependent on toll-like receptor 2 (TLR 2) (Noss, Pai et al. 2001; Fulton, Reba et al. 2004). *M. tuberculosis* and the 19kDa lipoprotein have been shown to inhibit IFN-γ induced genes, which are involved in MHC-II antigen processing and presentation such as the class-II transactivator, which is involved in transcriptional regulation of MHC-II genes (Harton and Ting 2000; Pai, Pennini et al. 2004). It has also been reported that post-Golgi transit of nascent MHC-II molecules into the endocytic pathway for antigen loading was impaired in *M. tuberculosis* infected macrophages, thus resulting in intracellular sequestration of MHC-II molecules and a reduced expression of MHC-II on the surface (Hmama, Gabathuler et al.1998).

Targeting specific receptors is another mechanism used by mycobacteria to evade immune surveillance mechanisms. Opsonised mycobacteria taken up by Fcγ receptors are targeted to vacuoles which readily fuse with lysosomes and thus entry of mycobacteria through Fcγ receptors into the host cell can result in a distinct pattern of trafficking but this was found to have no adverse effect on the

growth rate of the bacteria (Armstrong and Hart 1975). The selective use of complement receptors CR1 and CR3 has been shown not to trigger the oxidative burst and additionally, it has been reported that binding of ManLAM on human macrophage mannose receptor leads to the inhibition phagosome-lysosome fusion, thus resulting in survival (Kang, Azad et al. 2005).

Mycobacteria are able to evade the toxic effects of ROI via the use of LAM and phenolic glycolipids which act as ROI scavengers (Chan, Fan et al. 1991) and mycobacterial sulphatides interfere with ROI dependent anti-mycobacterial mechanism of macrophages. *M. tuberculosis* additionally produces superoxide dismutase which catalyses the conversion of superoxide free radical to hydrogen peroxide and oxygen, thus enabling the bacteria to evade the oxidative burst in activated macrophages (Harth and Horwitz 1999).

Employing these immune evasion mechanisms enables *M. tuberculosis* to survive, replicate and avoid immunological detection in the host.

1.3- Dendritic cells- sentinels of the immune system

Macrophages are known to be the primary host cells for mycobacteria but dendritic cells have also been reported to be infected with mycobacteria (Henderson, Watkins et al. 1997; Bodnar, Serbina et al. 2001). Dendritic cells are bone marrow immune cells which populate lymphoid and non-lymphoid tissues of the body (Lipscomb and Masten 2002). They are referred to as “professional antigen presenting cells” and possess the unique capability of activating naïve T-cells. These cells are capable of integrating innate immune responses to initiate adaptive immunity in the host. Dendritic cells are a heterogeneous family of cells, which exist at different developmental and functional stages within the host:

- Proliferating dendritic cell progenitors arise from bone marrow stem cells and these precursor cells migrate to the tissues via the blood stream where they give rise to immature dendritic cells

- Immature dendritic cells serve as sentinels of the immune system where they take up antigens in the peripheral tissues that triggers their maturation and migration to secondary lymphoid tissues
- Mature dendritic cells function as antigen presenting cells in secondary lymphoid tissues

1.3.1 - Dendritic cell subsets

Multiple subsets of dendritic cells exist, all of which exhibit considerable plasticity and trigger diverse immune responses. Precursor dendritic cells from mouse bone marrow, cultured in the presence of granulocyte macrophage colony stimulating factor (GM-CSF), give rise to the myeloid dendritic cell subset ($CD11c^+$, $CD11b^+$ $B220^-$) but when cultured with Flt-3 ligand yields $CD11c^+$, $CD11b^+$, $B220^+$ dendritic cells also commonly known as plasmacytoid dendritic cells (Shortman and Liu 2002). Plasmacytoid dendritic cells have the unique ability to produce large amounts of type I interferons (IFNs) and thus they are primarily involved in anti-viral immunity.

Dendritic cell subsets isolated from murine spleens can influence the type of T cell response generated (Table 1.1). The $CD8\alpha^+$, $CD11c^+$, $CD11b^-$ subset, prime principally naïve $CD4^+$ T cells to generate a Th-1 type response but the $CD8\alpha^-$, $CD11c^+$, $CD11b^+$ subset prime naïve CD4 T cells to produce principally a Th-2 type response (Shortman and Liu 2002; Naik, Proietto et al. 2005). The different subsets can also regulate the amount of cytokines produced by T cells; the $CD4^-CD8^+$ dendritic cell subset induces less interleukin-2 (IL-2) and IFN-γ from $CD8^+$ and $CD4^+$ T cells than the $CD4^-CD8^-$ DC subset (Shortman and Liu 2002; Naik, Proietto et al. 2005). In a similar manner, plasmacytoid dendritic cells can induce a strong Th-1 type immune response in the presence of CpG but not lipopolysaccharide (LPS), whereas myeloid dendritic cells initiate a Th-1 type immune response when stimulated with LPS (Boonstra, Asselin-Paturel et al. 2003). It was additionally demonstrated that both subsets of dendritic cells initiated a Th-1 type immune response when exposed to high doses of antigen (ovalbumin) and in contrast, low doses of antigen induced a Th-2 type immune response (Boonstra, Asselin-Paturel et al. 2003). This set of experiments

demonstrated that the resulting T cell response is influenced by a combination of factors including subset of dendritic cell, type and dose of antigen and the nature of the stimulus.

Features	**Lymphoid-organ-resident DC subsets**			**Migratory DC subsets**		**Monocyte derived**
	CD4$^+$ DCs	**CD8$^+$ DCs**	**DN DCs**	**Interstitial DCs**	**Langerhans cells**	
Location						
Spleen	Yes	Yes	Yes	No	No	Sites of inflammation
Surface markers						
CD11c	+++	+++	+++	+++	+++	+++
CD4	+	-	-	-	-	-
CD8	-	++	-	-	-/+	-
CD205	-	++	-/+	+	+++	-/+
CD11b	++	-	++	++[‡]	++	++
Functional features in the steady state						
Maturity	Immature	Immature	Immature	Mature	Mature	N/A
Co-stimulatory	+	+	+	++	++	N/A
Antigen processing and presentation	+++	+++	+++	+/-	+/-	N/A
MHC class II	++	++	++	+++	+++	N/A
In vitro equivalent						
	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus FLT3L	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow precursors plus GM-CSF, TNF and TGFβ	Bone-marrow, spleen or blood precursors plus GM-CSF

Table 1.1- Murine dendritic cell subsets

Dendritic cells are a heterogeneous population of cells that are capable of processing and presenting antigens to T cells. The different subsets of dendritic cells differ in their location and immunological properties. Table modified from (Villadangos and Schnorrer 2007)

Several human dendritic cell subtypes have also been characterised. In the presence of GM-CSF and IL-4, monocytes differentiate into immature dendritic cells; characterised by low levels of CD86 and MHC-II (Sallusto and Lanzavecchia 1994) and these dendritic cells when stimulated with inflammatory products can mature, expressing increased levels of CD86 and MHC-II. A second sub-type of dendritic cell found in blood is the type I IFN producing plasmacytoid subset, which are characterised as being $CD11c^{-}CD45RA^{+}CD123^{+}$ (Kadowaki and Liu 2002).

1.3.2—Immature dendritic cells

Immature dendritic cells are present in the periphery, where they can phagocytose microbes using an array of receptors, such as FcγRs, complement receptors and toll-like receptors (TLRs). Immature dendritic cells have the ability to take up antigens via different mechanisms such as macropinocytosis, receptor mediated endocytosis (via mannose receptor and DEC 205) and phagocytosis (uptake of apoptotic/necrotic material through CD36 and αvβ5). Immature dendritic cells express low levels of surface MHC-II and co-stimulatory molecules CD80/CD86, thus they are not capable of functioning as efficient antigen presenting cells at this developmental stage (Banchereau, Briere et al. 2000).

1.3.3 – Dendritic cell maturation and migration

Following interaction with antigens, immature dendritic cells undergo a series of functional and phenotypic changes, which result in the transition from antigen capturing cell to antigen presenting cell. Activation of dendritic cells is linked with their migration to the lymphoid organs. Dendritic cell activation is initiated in the peripheral tissues and continues to completion during interaction with T-cells. In response to pathogen derived products like LPS from gram negative bacteria, dsRNA, inflammatory cytokines such as TNFα and IL-1, and T cell derived signals in the form of CD40-CD40L interaction, dendritic cells mature and migrate via the lymphatics to secondary lymphoid tissues (Banchereau and Steinman 1998; Banchereau, Briere et al. 2000). Extravasation of these cells from peripheral tissues to secondary lymphoid organs is mediated by chemokines

and their receptors. One of the key chemokine receptors, chemokine (C-C motif) receptor 7 (CCR7) is expressed on dendritic cells and plays an important role in promoting the migration of these cells to lymph nodes; CCR7 deficient mouse studies revealed a marked defect in migration of dendritic cells to the lymph nodes (Forster, Schubel et al. 1999). Migration of dendritic cells to the lymph nodes occurs via a chemotactic gradient of CCR7 ligands- CCL19 and CCL21. Mature dendritic cells down regulate expression of chemokine receptors such as CCR1, CCR5 and up-regulate the expression of CCR4 and CCR7, which aids the migration of dendritic cells to lymphoid organs (Banchereau, Briere et al. 2000).

1.3.4 Phagocytic receptors on dendritic cells

Phagocytosis can trigger an inflammatory response, which depends on the stimulus in question. Interaction between certain ligands and their receptors, during the process of phagocytosis, initiates the maturation of dendritic cells. Engagement of Fc receptors and mannose receptors, trigger an inflammatory immune cytokine response, resulting in the production of IL-1β, TNF-α and IL-12 (van Crevel, Ottenhoff et al. 2002).C-type lectin receptors on dendritic cells are either type II transmembrane proteins with a single, carboxy terminal lectin domain or type I receptors with multiple lectin domains. Type I transmembrane receptors include the mannose receptor and DEC-205, and type II transmembrane receptors include DC-SIGN (dendritic cell-specific ICAM3-grabbing non-integrin) and dectin-2. The expression of these receptors varies with the developmental stage of the dendritic cell. Immature dendritic cells express abundant mannose receptors and DC-SIGN on the surface, the expression of which decreases on maturation (Kato, Neil et al. 2000). DC-SIGN first gained attention as a receptor that was capable of binding to the HIV-1 glycoprotein gp120. DC-SIGN interacts with intercellular adhesion molecule-2 (ICAM-2) on vascular endothelial cells mediating dendritic cell migration and interacts with ICAM-3 aiding in dendritic cell-T cell interaction. Antigens internalised by DC-SIGN are targeted to lysosomal compartments where antigens are processed and loaded via the MHC-II pathway (Engering, Geijtenbeek et al. 2002).

DC-SIGN as a c-type lectin shows specificity for the ligands it interacts with. DC-SIGN is able to bind dimeric and trimeric mannose residues in ManLAM but it does not recognise ManLAM from *M. avium*, as the ManLAM is capped with a single mannose residues (Maeda, Nigou et al. 2003). ManLAM is a unique component of cell walls from virulent mycobacteria (slow-growing bacteria) as opposed to AraLAM from avirulent mycobacteria (fast-growing bacteria). It has been reported that BCG induced dendritic cell maturation was inhibited by the binding of ManLAM to DC-SIGN on human dendritic cells (Geijtenbeek, van Vliet et al. 2003) and additionally, ManLAM binding to DC-SIGN triggers intracellular signalling pathways that lead to the increased production of anti-inflammatory cytokines such as IL-10. The binding of ManLAM to DC-SIGN results in the inhibition of TLR mediated IL-12 production by LPS matured dendritic cells (Geijtenbeek, van Vliet et al. 2003). Interaction between DC-SIGN and mycobacteria possibly is a route utilised by virulent mycobacteria, to undermine dendritic cell induced immune responses which might enhance survival of the pathogen.

Additional interactions can occur between receptors such as the mannose receptor and pathogen associated mannose structures on mycobacteria. A study in reference to the interaction of LAM and mannose receptor, has demonstrated that LAM is taken up by mannose receptors, which led to its transport to late endosomes for antigen presentation on CD1b molecules (Prigozy, Sieling et al. 1997). Mannose receptor recognises surface polysaccharides of gram positive bacteria, gram negative bacteria and parasites. On the surface of immature dendritic cells, this receptor functions to take up antigens and target them to early endosomes away from MHC-II compartments (Tan, Mommaas et al. 1997; Hawiger, Inaba et al. 2001). The low pH within endosomes results in the dissociation of the receptor and ligand and subsequently, the mannose receptor recycles back to the cell surface. Expression of the mannose receptor is down regulated by inflammatory cytokines and up regulated by anti-inflammatory cytokines such as IL-4 and IL-10 (Martinez-Pomares, Reid et al. 2003).

Dendritic cells also express FCγRs and FCεRs which mediate the presentation of antibody coated and immune complexes on both MHC-I and

MHC-II (Regnault, Lankar et al. 1999). It has been proposed that internalisation of ovalbumin immune complexes via Fc receptors can lead to the delivery of antigens to the cytosol via a pathway unique to dendritic cells. Antigens are trafficked to the cytosol directly from the lumen of the endosome and subsequently degraded by the proteasome and loaded on MHC-I molecules (Rodriguez, Regnault et al. 1999).

1.4- Activation of cellular signalling pathways

In addition, to the above mentioned family of receptors, dendritic cells express a family of pattern recognition receptors: TLRs. Toll was first identified as a gene required for dorsal-ventral patterning during embryonic development and subsequently, was also shown to play a key role in anti-fungal immunity (Lemaitre, Nicolas et al. 1996). A homologous family of genes was then discovered in mammals and was named TLRs. TLRs are type I membrane integral glycoproteins and to-date, there are 10 TLRs identified in humans and 13 in mice. TLRs 1, 2 and 4 are located on the cell surface and are recruited to the phagosome after activation and TLRs 3, 7 and 9 are localised on endosomal and lysosomal compartments.

The structurally conserved motifs recognised by TLRs usually have essential roles in the invading agents and thus are not subjected to high mutation rates. The various ligands recognised by TLRs include peptidoglycan and lipoteichoic acid from gram positive bacteria recognised by TLR 2 (Schwandner, Dziarski et al. 1999) and double stranded RNA from virus which is recognised by TLR 3 (Alexopoulou, Holt et al. 2001). Single stranded RNA is recognised by TLR 7 (Diebold, Kaisho et al. 2004), LPS from gram negative bacteria is the main ligand for TLR 4 (Poltorak, He et al. 1998) and CpG motifs are recognised by TLR9 (Hemmi, Takeuchi et al. 2000).

1.4.1 Adaptors involved in TLR signalling pathways

In total 5 adaptors are known to mediate TLR signalling pathways and the utilisation of different combinations of adaptors lead to the distinct gene

expression patterns observed in response to TLR agonists. The myeloid differentiation primary response gene 88 (MyD88) was originally isolated as a gene, which was shown to play an important role during myeloid cell differentiation and was rapidly induced during the IL-6 stimulated differentiation of M1 myeloleukaemia cells into macrophages (Lord, Hoffman-Liebermann et al. 1990). MyD88 was shown as the adaptor molecule that functions to recruit interleukin-1 receptor-associated kinase (IRAK) to the IL-1 receptor complex following stimulation with IL-1 (Wesche, Henzel et al. 1997). In addition to MyD88 there are 4 other adaptors which are involved in TLR signalling: TIR domain containing adaptor inducing interferon beta (TRIF), TRIF-related adapter molecule (TRAM), MyD88 adapter like (MAL) and sterile α and armadillo motifs (SARM). MAL is essential for TLR 2 and TLR 4 signalling routes, serving as a link to recruit MyD88 (Yamamoto, Sato et al. 2002) and thus plays a role in the MyD88 dependent pathway of TLR4 signalling. TRIF is important in the TLR4 mediated MyD88 independent pathway and is also the adaptor used for initiation of signalling via TLR 3 (Yamamoto, Sato et al. 2003). TRAM is another TLR 4 pathway adaptor whose role includes induction of IL-6, TNF and CD86 (Yamamoto, Sato et al. 2003). Similar to TRIF, TRAM is also required for the late activation of NFκB. SARM, in contrast to the other TLR adaptors, is a negative regulator of TLR signalling and its expression is specifically shown to block TRIF dependent gene activation.

1.4.2- MyD88 dependent and independent TLR signalling pathways

The cytoplasmic portion of TLRs exhibit a high degree of similarity to that of the IL-1 receptor family and is known as the Toll/IL-1 receptor (TIR) domain. Extracellular portions of the TLRs and IL-1 receptor are structurally unrelated, with TLRs possessing leucine rich repeats and the IL-1 receptor having immunoglobulin like domains. Binding of ligands to TLRs leads to its dimerisation and conformational change, which triggers its association with the adaptor MyD88. This in turn recruits IRAK-4, which subsequently associates itself with IRAK-1 (Barton and Medzhitov 2003). Binding of IRAK-4 to MyD88 induces the phosphorylation of IRAK-1. TNF receptor associated factor-6 (TRAF-

6), a signalling mediator adaptor protein is also recruited to the IRAK-1/4-MyD88 complex and associates itself with IRAK-1 (Figure 1.1).

Phosphorylated IRAK-1 and TRAF-6 then dissociate and form a complex with members of the mitogen activated protein kinase family (MAPK): TGF-activated kinase 1 (TAK-1), TAK-1 binding protein 1 (TAB-1) and TAB-2. This subsequently leads to the phosphorylation of TAB-2 and TAK-1. IRAK-1 is degraded and following this TRAF-6 forms a complex with the ubiquitin ligases, ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A). Ubiquitylated TRAF-6 results in the activation of TAK-1 which phosphorylates both the MAPK and inhibitor of nuclear factor kappa-B kinase (IKK) complex (IKK-α, IKK-β and IKK-γ). Phosphorylation of inhibitor of kappa light polypeptide gene enhancer in B-cells (IκB) by the IKK complex results in its degradation, which results in the translocation of nuclear factor kappa B (NFκB) to the nucleus, thus resulting in the transcription of NFκB dependent target genes (e.g. inflammatory cytokines), as illustrated in figure 1.1.

Recognition of microbial components through TLRs can result in signalling via the MyD88 dependent or independent pathways. The MyD88 dependent pathway involves the early phase activation of NFκB and the MyD88 independent pathway leads to the late phase activation of NFκB, with delayed kinetics compared to wild-type mice (Covert, Leung et al. 2005). Engagement of TLR3 and TLR4 is known to activate the MyD88 independent signalling pathway. As part of the MyD88 independent pathway, TANK binding kinase 1 (TBK-1) and IKKε/IKKi induces interferon regulatory-3 (IRF-3) phosphorylation thus leading to the activation of IRF-3. Activated IRF-3 translocates to the nucleus and initiates the cascade of events leading to type I IFN production (Figure 1.1).

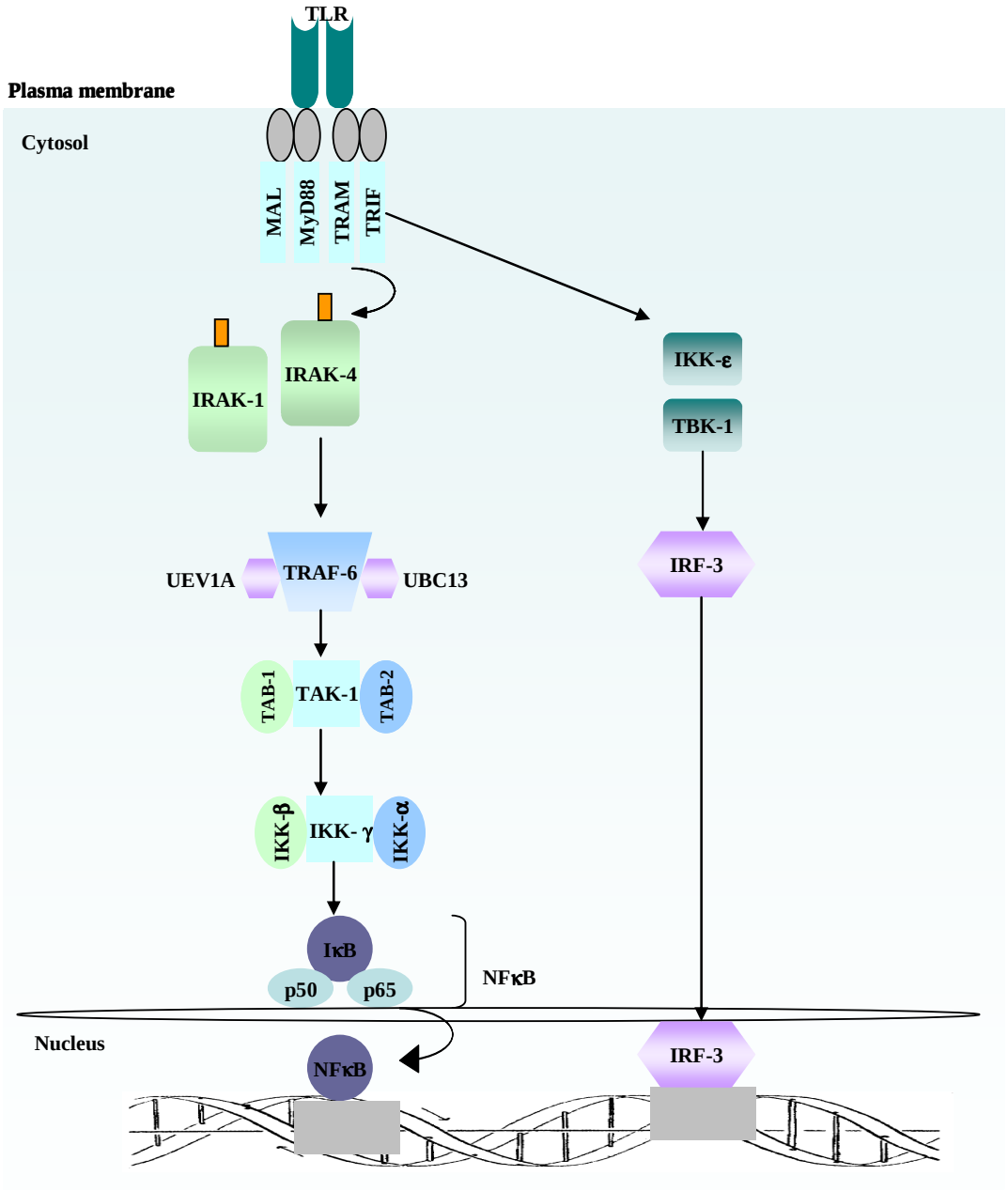


Figure 1.1- Overview of the TLR signalling pathways

TLRs are type I transmembrane receptors involved in innate immunity. Upon engagement of TLRs with microbial products, several signalling pathways are activated; this results in the activation of NFκB or IRF-3. Activation of NFκB via TLR signalling pathways represents an integral part of the host innate immune response.

1.4.3- Negative regulators of the TLR signalling pathway

A balance must be achieved between activation and inhibition of immune and inflammatory responses mediated by TLRs. This is important in preventing the induction of detrimental inflammatory responses in the host. Thus, the TLR signalling pathway is tightly regulated by various negative regulatory components such as, soluble decoy TLRs, which attenuate TLR function by blocking the interaction between the ligand and TLR, even though the finer details of the mechanism by which this is achieved still remains unclear (Iwami, Matsuguchi et al. 2000). Other negative regulators include MyD88s (short form of MyD88), IRAKM, suppressor of cytokine signalling 1 (SOCS 1), phosphoinositide 3-kinase (PI3K), toll interacting protein (TOLLIP) and A20.

MyD88s has been shown to be induced in the THP-1, monocytic cell line in response to LPS stimulation and over expression of this regulator leads to the inhibition of LPS and IL-1 induced NFκB activation (Janssens, Burns et al. 2002). Many immune cell receptors relay intracellular signals via PI3K, which results in a variety of biological effects including chemotaxis, growth, survival and cytokine synthesis (Katso, Okkenhaug et al. 2001). Mice deficient in PI3K (p85) show enhanced TLR signalling, which results in enhanced IL-12 production in dendritic cells in response to LPS, and a dominant Th-1 cell response (Fukao, Tanabe et al. 2002). A20 is a unique negative regulator that regulates both the MyD88 dependent and independent pathways by interacting with TRAF6 (Boone, Turer et al. 2004). Macrophages from A20 deficient mice produce elevated levels of pro-inflammatory cytokines in response to peptidoglycan, CpG and poly I:C (Boone, Turer et al. 2004).

The presence of negative regulators maintains a delicate balance between inflammatory and immune responses generated by the TLR signalling pathway.

1.4.4- Interaction between TLRs and microbes

TLRs maybe expressed as homodimers or heterodimers and function, as mentioned above, in recognising an array of microbial ligands. An overview of the three TLRs (TLR 2, TLR 4 and TLR 9), which are known to be key players in the recognition of mycobacterial components is addressed below.

TLR 2 is able to recognise a diverse range of microbial components which include lipoteichoic acid, lipoproteins from gram positive and gram negative bacteria and peptidoglycan. This unique ability to recognise various ligands can be attributed to its ability to form heterodimeric interactions with TLR 1 and TLR 6 (Ozinsky, Underhill et al. 2000). The different heterodimeric combinations enable recognition of different bacterial lipopoproteins. TLR 2/TLR 6, along with CD36, facilitate the recognition of diacylated lipoproteins, in contrast, triacylated lipoproteins induce the activation of the immune system through interaction with TLR 1/TLR 2 dimers (Hoebe, Georgel et al. 2005). A role for TLR 2 during bacterial infections has been demonstrated in various studies. The enhanced growth of *Legionella pneumophila* in TLR $2^{-/-}$ macrophages, in conjunction with decreased production of IL-12p40 production, led to the proposal of a role for TLR 2 in mediating host resistance to *L. pneumophila* at early stages of the infection (Akamine, Higa et al. 2005)**.** In another bacterial infection model, mice infected with *Streptococcus pneumoniae* had an increased level of TLR 2 mRNA in the brain, with TLR $2^{-/-}$ mice exhibiting an increased disease severity, accompanied by higher bacterial titres in both the cerebellar and blood (Koedel, Angele et al. 2003).

One of the most extensively studied TLRs is TLR 4, whose primary ligand is the hexacetylated form of the lipid A component of LPS. TLR 4 also requires co-receptors including CD14, (a glycosylphosphatidyl inositol anchored glycoprotein) and MD-2, to respond to LPS (Wright, Ramos et al. 1990). Murine models have provided evidence that TLR 4 is the receptor for LPS; C3H/HeJ mice has a point mutation in the TIR domain of TLR 4 and another strain of mice B10.ScCR lack the genomic region consisting of the TLR 4 gene; these two strains of mice have been shown to be hypo-responsive to LPS (Poltorak, He et al. 1998) . An important role for TLR 4 was demonstrated during *Candida albicans*

infection using TLR $4^{-/-}$ mice (Netea, Van Der Graaf et al. 2002). The increased fungal load in TLR $4^{-/-}$ mice was associated with impaired chemokine production (lower levels of CXCL1 and macrophage inhibitory protein-2) and reduced neutrophil recruitment to the sites of infection. Host defence against respiratory syncitial virus is impaired in TLR $4^{-/-}$ mice due to defective IL-12 release and natural killer cells (NK cell) trafficking and function in the lungs of these mice (Haynes, Moore et al. 2001). This observed defect in NK cells was proposed to be due to the reduced NFκB activation in the lungs of TLR $4^{-/-}$ mice (Haeberle, Takizawa et al. 2002).

TLR 9 was initially described as an endosomal receptor recognising unmethylated CpG motifs in bacterial DNA (Krieg, Yi et al. 1995). The fact that vertebrate DNA is likely to be methylated provides the discrimination needed for TLR 9 to identify self from foreign DNA. TLR $9^{-/-}$ macrophages were defective in their ability to produce inflammatory cytokines in response to CpG DNA and furthermore, dendritic cells failed to enhance the expression of co-stimulatory molecules thus indicating that TLR 9 played a crucial role in inducing cellular responses to CpG DNA (Hemmi, Takeuchi et al. 2000).

1.4.5- Role of TLRs in the immune response to mycobacteria

Studies in TLR knock out mice have demonstrated the importance of this family of receptors in mediating host resistance during *M. tuberculosis* infections. Mycobacterial ligands including LAM, lipomannan (LM) and the 19kDa lipoprotein (Brightbill, Libraty et al. 1999) are recognised by TLR 2 and TLR 4, and mycobacterial DNA is recognised by TLR 9 (Bafica, Scanga et al. 2005).

TLR $2^{-/-}$ and TLR $4^{-/-}$ mice displayed no increased susceptibility to low dose aerosol *M. tuberculosis* infections and survived for up to 200 days post-infection similar to wild-type mice (Reiling, Holscher et al. 2002). In response to a high dose aerosol challenge, TLR $2^{-/-}$ mice were found to be significantly more susceptible to *M. tuberculosis* infection than TLR $4^{-/-}$. TLR $9^{-/-}$ mice were more susceptible to a high dose (500CFU/mouse) aerosol challenge succumbing to infection after about 70 days and in contrast, TLR $9^{-/-}$ mice infected with a low

aerosol dose showed no changes in pathology and had similar survival levels to wild-type mice. In addition, antigen presenting cells (splenic dendritic cells, bone marrow dendritic cells and macrophages) from TLR $9^{-/-}$ mice did not display any defects in TNF-α production *in vitro* (Bafica, Scanga et al. 2005). In general, TLR knockout studies have shown that, defects in a single TLR does not have a critical impact on the host resistance to *M. tuberculosis* infection at low dose of infection (Reiling, Holscher et al. 2002; Drennan, Nicolle et al. 2004).

The use of double TLR knockout mice has shown the importance of synergism between TLRs and the critical role of this synergism in mediating immune responses to *M. tuberculosis* (Drennan, Nicolle et al. 2004). TLR 2 and TLR 9 double knock out mice had significantly increased bacterial loads and a mean survival time of 120 days with a low dose of *M. tuberculosis*. In addition, these mice had widespread inflammation, focal necrosis and abundant acid fast bacilli in lung tissues (Bafica, Scanga et al. 2005). All synergistic responses of TLRs may not play an important role during mycobacterial infections. TLR 2/TLR $4^{-/-}$ double knock out mice were resistant to BCG infection, which was similar to wild-type mice (Nicolle, Fremond et al. 2004).

Human case studies have indicated a possible link between a TLR 2 polymorphism Arg753Gln (arginine to glutamine substitution at residue 753) and increased susceptibility to tuberculosis (Ogus, Yoldas et al. 2004) with a more recent study, suggesting a strong association between TLR 2 T597C polymorphism and the development of tuberculosis meningitis (Thuong, Hawn et al. 2007). The identification of TLR 2 polymorphisms highlights the role played by this receptor in tuberculosis disease.

The current literature provides evidence for the role of various TLRs in the host resistance to mycobacterial infections.

1.5- Type I IFNs

Signalling via the TLR mediated, MyD88 independent pathway, can lead to the induction of type I IFNs. IFNs were first described in 1957 by Isaacs and Lindemann and were described as proteins that interfere with viral replication (Isaacs A 1957). Interferons are divided into two main classes: type I interferons (IFN alpha and IFN beta) and type II interferon (IFN-γ). In addition, there are reports of another class of human, type I like interferons termed IFNλ1 (IL-28A), IFNλ2 (IL-28B) and IFNλ3 (IL-29) (Sheppard, Kindsvogel et al. 2003).

Genes encoding type I IFNs are clustered on chromosome 9 in humans and on chromosome 4 in mice. IFN alpha and IFN beta are the main type I interferons that are synthesised during viral and bacterial infections thus making them interesting candidates for further study, from an immunological perspective.

The production of type I interferons is not restricted to a single cell type and it is plausible that a wide range of cells produce these cytokines in response to viral and bacterial infections. Type I IFNs can be produced by B-cells, monocytes, macrophages, myeloid and plasmacytoid dendritic cells. The amount of type I IFNs produced varies between cell types, where plasmacytoid dendritic cells tend to produce higher levels of type I IFNs in response to infection and are thus known as ‘natural interferon producing cells’ (Asselin-Paturel and Trinchieri 2005). Recently, a class of cells which are distinct from conventional dendritic cells and plasmacytoid dendritic cells were described as ‘interferon producing killer dendritic cells’. These hybrid cells have molecular features of both natural killer cells (NK cells) and dendritic cells in particular the ability of these cells to produce high levels of type I IFNs upon activation (Chan, Crafton et al. 2006). Thus, it is well established that a range of cells possess the capacity to produce type I IFNs upon activation.

1.5.1- Type I IFN signalling pathway

Type I IFN signal transduction occurs through a ubiquitously expressed heterodimeric receptor– interferon (alpha and beta) receptor 1 (IFNAR), which is composed of two subunits IFNAR1 and IFNAR2 (Sen 2001). Binding of type I IFNs to their cognate receptor leads to the induction of transcriptional responses through the JAK-STAT signalling pathway. The JAK-STAT signalling pathway is a universal pathway utilised by all cytokines to induce cell type specific cytokine induced gene expression patterns. There are 4 mammalian janus kinases (JAKs) and 7 members of the signal transducers and activators of transcription (STAT) family identified to date (Darnell 1997; Stark, Kerr et al. 1998).

IFN alpha/beta binds to IFNAR, which leads to ligand induced receptor dimerization, resulting in phosphorylation of JAK-1 and tyrosine kinase 2 (Tyk2), two members of the Janus protein tyrosine kinase family. Phosphorylation and activation of JAK-1 and Tyk2, leads to the phosphorylation of IFNAR1, thus creating binding sites for STAT-2, as illustrated in figure 1.2. Subsequently, STAT-2 is phosphorylated and serves as a platform for recruitment and tyrosine phosphorylation of STAT-1. The STAT 1-STAT 2 heterodimer dissociates from the IFNAR and translocates to the nucleus resulting in the association of the heterodimer with IRF-9 to form a heterotrimeric complex known as interferon stimulated gene factor 3 (ISGF3) as shown in figure 1.2. This complex binds to the DNA sequence, interferon-stimulated response element (ISRE) which results in the initiation of transcription of the type I IFNs and IFN alpha/beta inducible genes. ISRE sequence is found in the promoters of several type I IFN inducible genes, the consensus sequence of which is 5'AANNGAAA 3' where N denotes any nucleotide (Tailor, Tamura et al. 2006).

The production of type I interferons is primarily regulated at the transcriptional level. IFN beta promoter consists of four positive regulatory domains [PRD I, II, III and IV] and these regulatory cis elements provide binding sites for specific transcription factors. PRD I and III are binding sites for IRFs, NFκB binds to PRDII and activating protein-1 (AP-1) docks onto PRD IV (Honda, Yanai et al. 2005) . The binding of these transcription factors results in

the recruitment of transcriptional machinery and chromatin modifiers which results in the induction of IFN beta.

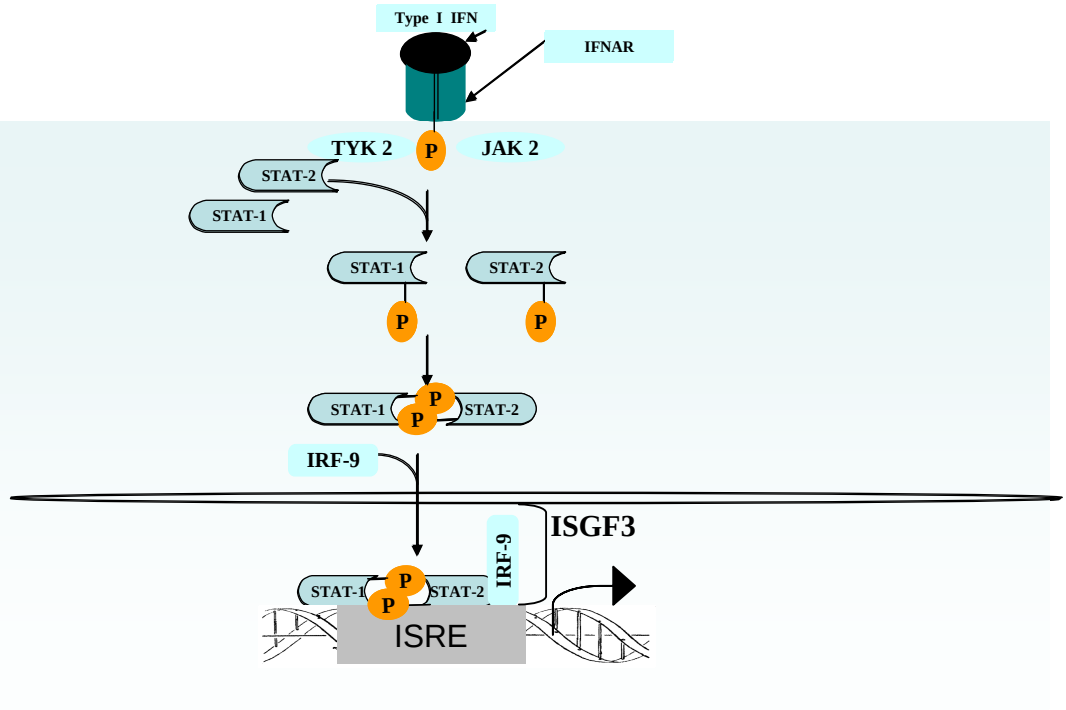


Figure 1.2- Type I IFN signal transduction pathway

The binding of type I IFNs to the IFNAR receptor, triggers the activation of the cardinal JAK-STAT signalling pathway, thus leading to the downstream activation of type I IFN inducible genes.

1.5.2- IRFs - transcriptional regulators of the type I IFN pathway

IRFs play an important role in the transcription of type I IFN inducible genes. The IRF family consists of 9 members: IRF 1-IRF 9. The two highly homologous proteins IRF-3 and IRF-7 are considered to be the key transcriptional proteins that play a role in the induction of the type I IFN pathway (Taniguchi, Ogasawara et al. 2001). IRF-3 is constitutively expressed in the cytoplasm of all cell types and in response to stimulation, is post-translationally modified by phosphorylation at serine residues, which results in the translocation of the protein from the cytosol into the nucleus. Phosphorylation of IRF-3 is by one of the two IκB kinases- TBK1 and IKK-i. Upon entry into the nucleus, IRF-3 associates with CREB binding protein (CBP)/p300, NFκB and AP-1. The resulting complex binds to the promoter of type I IFN genes thus inducing their expression (Taniguchi, Ogasawara et al. 2001) . IRF-7 also plays a pivotal role in the type I IFN gene induction. IRF-7 unlike IRF-3, is absent from most cells or expressed at very low levels constitutively. In response to viral infection, IRF-7 is phosphorylated and activated by the kinases TBK1, IκB or IRAK-1 at serine/threonine residues. Phosphorylated IRF-7 undergoes nuclear translocation and binds to PRD I and PRD III elements in the promoter of the type I genes. Most cells utilise an amplification loop in order to increase the available amount of IRF-7 thus increasing the production of IFN alpha and IFN beta (Tailor, Tamura et al. 2006).

1.5.3- Type I interferons and their role in host defence

The activation stimuli for the production of type I IFNs can be in the form of viral infections and as shown more recently in the last few years, bacterial components such as LPS and bacterial DNA (unmethylated CpG motifs in bacterial DNA). The induction of host antiviral immunity utilises either the TLR signalling pathway or the retinoic acid-inducible gene I - mitochondrial antiviral protein signalling pathway which functions to recognise cytosolic double stranded RNA to induce the production of type I IFNs (Creagh and O'Neill 2006).

Induction of type I IFNs leads to the assembly of antiviral proteins like 2'-5'-oligoadenylate synthetase (OAS), a dsRNA dependent synthetase that activates endoribonuclease RNase L to degrade ssRNA and myxovirus (influenza virus)

resistance (Mx) proteins, which bind to viral proteins thus inhibiting their replication (Stark, Kerr et al. 1998). Mx proteins have been shown to bind to the nucleocapsid proteins of bunyaviruses and causes re-distribution of the viral nucleocapsid, thus inhibiting the replication of these viruses (Kochs, Janzen et al. 2002). Type I IFNs contribute to immune responses against *Leishmania major* by inducing the expression of iNOS during the innate phase of the immune response (Diefenbach, Schindler et al. 1998) and additionally it has been demonstrated, that application of low doses of IFN beta protects mice from progressive cutaneous leishmaniasis (Mattner, Wandersee-Steinhauser et al. 2004). In contrast, during infection with *Listeria monocytogenes,* elevated levels of type I IFN production are accompanied by increased levels of apoptosis in the spleens of infected mice thus leading to enhanced susceptibility to infection (O'Connell, Saha et al. 2004). Other studies with extracellular bacteria which include *Streptococcus pneumoniae*, encapsulated *E. coli* and group B streptococci demonstrated that in the absence of type I IFN signalling there was a defect in the production of TNF-α and IFN-γ, which contributed to the impaired responses to these bacteria (Mancuso, Midiri et al. 2007). Furthermore, it has been reported that interferon (alpha and beta) receptor knock out mice exhibited a delay in controlling BCG growth and this defect in controlling early growth is thought to be attributed to the defect in nitric oxide production (Kuchtey, Fulton et al. 2006) .

Thus, type I IFNs have shown to have important roles in both viral and bacterial infections. Both protective and detrimental effects of type I IFNs have been observed, depending on the microbial pathogen.

1.5.4- Immunomodulatory effects of type I IFNs

Type I IFNs can have both immunostimulatory and immunospuppressive effects which forms the basis for their role during various microbial infections.

Type I IFNs can induce the production of IL-15 which plays a role in the proliferation and maintenance of NK cells and memory $CD8^+$T cells (Nguyen, Salazar-Mather et al. 2002). Similarly, type I IFNs are known to play a role in the differentiation and function of effector $CD8^+$T cells; this was demonstrated in a model of lymphocytic choriomeningitis virus infection, where $CD8^+$T cells from interferon (alpha and beta) receptor knockout mice, displayed impaired ability to differentiate and expand into functional effector $CD8^+$T cells (Kolumam, Thomas et al. 2005). It has also been demonstrated that, IFN alpha/beta can induce the maturation of dendritic cells and furthermore, endogenously expressed type I IFNs by dendritic cells can act in an autocrine manner to activate more type I IFN production (Gallucci, Lolkema et al. 1999; Montoya, Schiavoni et al. 2002).

Low level constitutive expression of IFN alpha/beta has been reported to be critical for IFN-γ mediated signalling, as the IFNAR1 provides a docking site for STAT-1, which is a crucial component of the IFN-γ signalling pathway (Takaoka, Mitani et al. 2000). The production of very high physiologically relevant amounts of type I IFNs can have a negative effect on the production of IL-12; a decrease in IL-12 production occurs due to the specific inhibition of the transcription of the IL-12p40 gene (Adriana A. Byrnes 2001). Thus the amount of type I IFN produced is crucial, with very high levels negatively regulating IL-12 production.

1.5.5- Clinical applications of type I interferons

IFN alpha has been approved for the treatment of viral infections such as chronic hepatitis B and hepatitis C. Additionally, IFN beta has also been approved for treatment of relapsing-remitting multiple sclerosis, the mechanism by which it works is still not clear (Wingerchuk and Noseworthy 2002).

Thus in general, type I interferons represent a family of cytokines which elicit multifaceted responses in the host and have important roles in both innate and adaptive immune responses in the host.

1.6- Principal cytokines modulating the immune response to *M. tuberculosis*

The modulation of the immune response to pathogens is critically dependent on the production of cytokines. Dendritic cell maturation, in addition to the altered expression of chemokine receptors, is also characterised by the production of pro-inflammatory cytokines. Recognition of microbial patterns via TLRs leads to signalling via the NFκB pathway resulting in the production of inflammatory cytokines.

1.6.1-IL-12

The IL-12 family of cytokines is composed of IL-12p40, IL-12p70 and IL-23. IL-12p40 can be secreted as a monomer or as a dimmer. IL-12p40 is always produced in excess, at much higher levels compared to the other IL-12 family members, which is partially attributed to the fact that it forms part of the bioactive complex of IL-12p70 and IL-23.

The role of IL-12 as part of the immune response is wide and varied. It has been reported that IL-12p40 is an important promoter of dendritic cell migration from the lung to the draining lymph nodes during *M. tuberculosis* infection, as this migratory process was impaired in IL-12p40$^{-/-}$ mice (Khader, Partida-Sanchez et al. 2006). The reduced ability of IL-12p40$^{-/-}$ dendritic cells to migrate, may be partly linked to the fact that these dendritic cells also produce increased levels of IL-10 compared to the wild-type mice. IL-10 specifically inhibits migration of dendritic cells by preventing the downregulation of CCR1, CCR2 and CCR5, and up regulation of CCR7 (D'Amico, Frascaroli et al. 2000). IL-12p40 deficiency also results in the reduced ability of IL-12p40 $^{-/-}$ dendritic cells to activate naïve T cells, thus linking the chemokine responsiveness and IL-12p40 production by dendritic cells.

IL-12p70, the bioactive IL-12 molecule, plays a key role in the priming of CD4^{+}T cells (Flynn, Goldstein et al. 1995; Holscher, Atkinson et al. 2001; Cooper, Kipnis et al. 2002). Lack of IL-12p35 has a less critical effect on the host, compared with IL-12p40 deficiency during *M. tuberculosis* infection, possibly

reinforcing the importance of the IL-12p40 subunit and suggesting IL-12p40 activity independent of IL-12p35 (Holscher, Atkinson et al. 2001; Cooper, Kipnis et al. 2002). IL-12p35$^{-/-}$ mice were less susceptible to BCG and H37Rv infection compared to IL-12p35$^{-/-}$p40$^{-/-}$ mice. Furthermore, IL-12p35$^{-/-}$ mice were able to mount antigen specific Th-1 responses and granuloma formation was normal in these mice. In addition, gene polymorphisms in the IL-12B gene have been linked to the increased susceptibility in humans (Tso, Lau et al. 2004).

1.6.2-IL-6

IL-6 is thought to play a role in controlling *M. tuberculosis* infection during the initial stages. In a low dose aerosol model of infection, IL-6$^{-/-}$ mice had increased bacterial burden in the lungs, at early time points post-infection. The bacterial counts stabilised at later time points post-infection, which coincided with the induction of the acquired immune response (Saunders, Frank et al. 2000).

1.6.3-TNF-α

TNF-α contributes to the immunity and the pathology of TB infections. *M. tuberculosis* induces the production of TNF-α by macrophages and dendritic cells and this cytokine plays a key role in the acute stage of *M. tuberculosis* infection (Henderson, Watkins et al. 1997). This role has been highlighted in TNF-α deficient mice, which exhibited increased mortality and extensive necrosis in the lungs, with defects in granuloma formation and overall higher bacterial burdens compared to control mice (Flynn, Goldstein et al. 1995; Roach, Bean et al. 2002).

TNF-α as part of the innate immune response has roles as the mediator of macrophage activator and in synergy with IFN-γ stimulates inducible nitric oxide synthase expression (Chan, Xing et al. 1992). Additional studies in TNF-α$^{-/-}$ mice have demonstrated that this cytokine plays a role in the early induction of chemokines (CCL2, CCL3, CCL4, CCL5 and CCL8) and initial recruitment of leukocytes like macrophages, which are essential for the production of RNI to clear the bacterial infection.

Anti-TNF antibody therapy is commonly used in rheumatoid arthritis and Crohn's disease but the use of such therapy has been presented with problems of reactivation of latent TB infections. It has been proposed that TNF blockers could compromise the integrity of the granuloma, thus contributing to the reactivation of the latent bacilli (Keane 2005). The cytokine TNF-α can be considered as a double edged sword, where on one side it has constructive roles in the formation and maintenance of granulomas but in addition, contributes to increased lung pathology and necrosis (Bekker, Moreira et al. 2000).

1.6.4- IL-10

IL-10 is principally an anti-inflammatory cytokine that is produced by T cells, dendritic cells and macrophages during *M. tuberculosis* infection. The engagement of TLR 2, TLR 7, TLR 9 and DC-SIGN induces the production of IL-10 by dendritic cells (Geijtenbeek, van Vliet et al. 2003; Samarasinghe, Tailor et al. 2006). IL-10 inhibits production of cytokines and chemokines from macrophages *in vitro* (Moore, O'Garra et al. 1993). Importantly, IL-12 production is modulated by IL-10 in response to *M. tuberculosis* infection. Additionally, it has been shown that IL-10 production can interfere with dendritic cell trafficking thus reducing their migration to the draining lymph nodes (Demangel, Bertolino et al. 2002). Most of the inhibitory properties of IL-10 are dependent on STAT-3 pathways and are specific to a defined set of genes, as approximately 40% of LPS induced genes remained unchanged in response to IL-10 in a microarray profiling study (Lang, Patel et al. 2002). The interference of IL-10 in the host defence against mycobacteria was demonstrated in a mouse model of infection, which showed that IL-10 transgenic mice (constitutively expressing IL-10) were unable to clear BCG infection and thus developed higher bacterial burdens. In contrast, the absence of IL-10 had no effect on the course of *M. tuberculosis* infection (Murray, Wang et al. 1997; North 1998). It is postulated that IL-10 plays an important role during the chronic stage of infection, promoting reactivation of the disease (Turner, Gonzalez-Juarrero et al. 2002).

1.6.5-IL-4

IL-4 is a Th-2 cytokine, playing an important role in directing development of Th-2 cells. Contrasting observations have been reported with regard to the role of IL-4 during *M. tuberculosis* infection. It has been demonstrated that IL-4 expression is increased in patients with TB, with a possibility that these higher IL-4 levels are found in patients closer to the equator, as a consequence of simultaneous infection with helminths (Schauf, Rom et al. 1993; Sanchez, Rodriguez et al. 1994; Malhotra, Mungai et al. 1999). In murine models of infection, the detection of IL-4 coincides with the appearance of pneumonia and necrosis, leading to increased mortality. But, in the absence of IL-4 (disrupted IL-4 gene) in mice, there is no effect on the course of TB infection (North 1998). It has also being shown that IL-4 can down regulate the expression of iNOS, thus down regulating macrophage mediated anti-mycobacterial immunity (Bogdan, Vodovotz et al. 1994). Hence the precise roles of IL-4 during *M. tuberculosis* infection still remain to be elucidated.

1.6.6-IFN-γ

The type II interferon- IFN-γ has a critical role in mediating the host resistance to *M. tuberculosis* infection. The binding of IFN-γ triggers the rearrangement and dimerisation of the interferon gamma receptor (IFNGR) subunits and autophosphorylation of the associated JAKs. JAK1 and JAK2 regulate the phosphorylation of STAT-1, thus resulting in the formation of STAT 1-STAT 1 homodimers (Stark, Kerr et al. 1998). The homodimer of STAT-1 translocates to the nucleus and binds to the IFN-γ activated sequence (GAS) elements, to initiate transcription as illustrated in figure 1.3 (Stark, Kerr et al. 1998).

In the absence of IFN-γ, there is defective macrophage activation and thus reduced expression of inducible nitric oxide synthase. A role for IFN-γ in the expression of MHC-II has also been suggested, with peritoneal macrophages from IFN-γ receptor$^{-/-}$ mice having lower levels of MHC-II in response to BCG

infection (Kamijo, Shapiro et al. 1993). IFN-γ is produced mainly by T-cells and NK cells (Orme, Roberts et al. 1993; Lalvani, Brookes et al. 1998).

IFN-γ receptor knock out mice are highly susceptible to *M. tuberculosis* infection. These mice display increased bacterial burdens, with a mean survival time of approximately 20 days and although granulomas form, they tend to be necrotic. Furthermore, IFN-γ receptor knock out mice fail to produce RNIs in response to *M. tuberculosis* infection, contributing to the failure to control *M. tuberculosis* infection (Flynn, Chan et al. 1993). IFN-γ receptor knock out mice are also susceptible to infection with *Legionella pneumophila* and *Toxoplasma gondii*, where IFN-γ was shown to contribute to the activation of macrophages, with a reduction in TNF-α, iNOS and IL-1β in the livers of these knock out mice (Deckert-Schluter, Rang et al. 1996). The importance of this cytokine has also been demonstrated in humans with defective genes for IFN-γ, as these individuals are prone to suffer from mycobacterial infections (Ottenhoff, Kumararatne et al. 1998). Patients possessing a mutation in the IFNGR1 gene at nucleotide position 395, introduced a stop codon, leading to the formation of a truncated protein and these individuals were found to be more susceptible to mycobacterial infections (Newport, Huxley et al. 1996). In addition null, recessive IFNGR2 mutation has also been postulated to contribute to severe mycobacterial infection (Altare, Jouanguy et al. 1998). Thus IFN-γ is a critical cytokine is mediating macrophage based anti-mycobacterial responses.

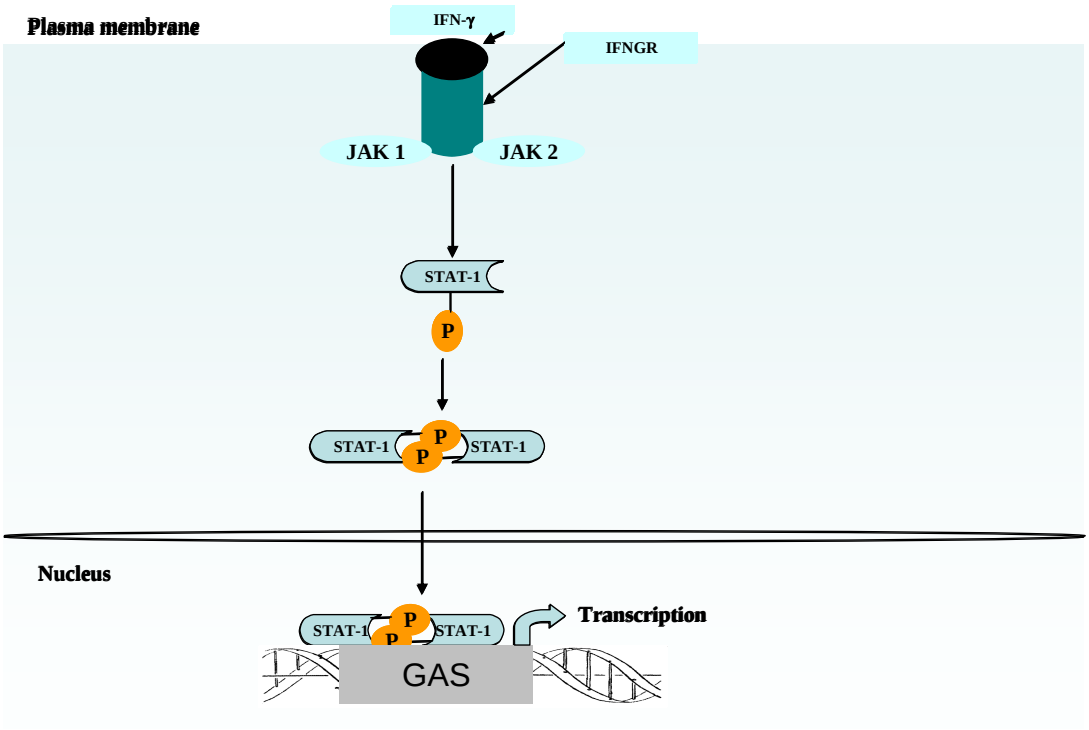


Figure 1.3 – The Type II IFN signalling pathway

The binding of IFN-γ to the IFNGR receptor triggers the activation of the STAT phosphorylation and formation of STAT homodimers, which translocate to the nucleus and initiates transcription of type II IFN inducible genes.

1.6.7- IL-18 and IL-15

Other cytokines such as IL-18 and IL-15 have also been implicated in the host response to *M. tuberculosis*. IL-18 is an IFN-γ inducing factor and augments Th-1 type immune responses induced by IL-12. In response to *M. tuberculosis* infection, IL-$18^{-/-}$ mice exhibited a decrease in IFN-γ synthesis, which was partially attributed to the defect in IL-12 production in these mice. Thus this cytokine is thought to contribute to the induction of Th-1 type immune response against *M. tuberculosis* infection (Kinjo, Kawakami et al. 2002).

IL-15 has many biological roles including stimulating proliferation of memory $CD8^+$ T cells (Zhang, Sun et al. 1998) and promoting optimal dendritic cell activation thus influencing antigen presentation functions (Mattei, Schiavoni et al. 2001). In addition, IFN alpha/beta is capable of inducing IL-15 via a STAT-1 mediated mechanism which contributes to the accumulation of NK cells. IL-15 transgenic mice (overexpressing IL-15 under the control of a MHC-I promoter), increased the efficacy of BCG vaccination with memory $CD8^+$ T cells being maintained for a prolonged period of time after BCG vaccination. Hence a role for IL-15 in the maintenance of memory $CD8^+$T cells was proposed (Umemura, Nishimura et al. 2003). In addition, it has been reported that IL-$15^{-/-}$ mice in response to *M. tuberculosis* infection recruit $CD4^+$ and $CD8^+$ T cells to the lungs but the recruitment of these cells is delayed, although the induction of cytotoxic $CD8^+$ T cell responses was normal after the primary infection with *M. tuberculosis*. It was thus proposed that IL-15 plays a role in the recall T cell responses during *M. tuberculosis* infection (Lazarevic, Yankura et al. 2005).

1.7- Antigen presentation and T cell stimulation

Analysis of entire kinetic sets of data has revealed that dendritic cells undergo extensive re-organisation of gene expression with dendritic cell maturation being almost complete 24 hours after activation (Granucci, Vizzardelli et al. 2001). In addition to cytokine production, mature dendritic cells down-regulate mechanisms of antigen capture and express high levels of long-lived surface MHC-II and co-stimulatory molecules making them potent T cell

stimulators (Banchereau, Briere et al. 2000). Dendritic cells can process and present antigens in the context of both MHC-I and MHC-II molecules to $CD8^+$ and $CD4^+$ T cells respectively. T cell activation requires two signals: signal one involves the recognition of peptide–MHC complexes on the antigen presenting cell by antigen specific T-cell receptor, and signal two, involves the interaction of co-stimulatory molecules on the antigen presenting cell with their ligands on the T cell (Banchereau and Steinman 1998). Since dendritic cells express high levels of co-stimulatory and MHC molecules, they are equipped with the ability to provide both signals required for stimulation and amplification of T cell responses.

Other factors which can influence the T cell stimulatory capacity of dendritic cells include, the release of cytokines into the local environment and expression of high levels of adhesion molecules, which may facilitate clustering and TCR engagement (Cella, Sallusto et al. 1997). During dendritic cell-T cell interactions, dendritic cells enter their final maturation stage by receiving additional signals from T cells in the form of CD40-CD40L interactions (Cella, Scheidegger et al. 1996). Thus, mature dendritic cells possess increased T-cell stimulatory capacity and produce inflammatory cytokines which further modulate the outcome of the immune response.

1.7.1-T-cell subsets

M. tuberculosis infected antigen presenting cells present mycobacterial antigens on MHC-I, MHC-II and CD1 molecules to $CD8^+$, $CD4^+$, and CD1 restricted T cells respectively. However, immunity against *M. tuberculosis* is primarily mediated via a Th-1 response. $CD4^+$ T cells are known to be important in generating protective immune responses in the host primarily by producing IFN-γ, a protective cytokine known to activate macrophages, thus leading to the control of the pathogen. Antibody mediated depletion of $CD4^+$ T cells has demonstrated that these cells may be important in preventing reactivation of the infection (Scanga, Mohan et al. 2000). $CD8^+$ T cells probably have two effector mechanisms which are important during a *M. tuberculosis* infection; the first is the production of IFN-γ similar to the $CD4^+$T cell population and the second is the

lysing of target cells via granzyme/perforin and Fas/Fas L pathway (Flynn and Chan 2001; Kaufmann 2001). The CD1 restricted T cells are specific for mycobacterial lipids and can produce IFN-γ and exhibit cytolytic activity. In the mouse model of *M. tuberculosis* infection, there is an increase in the number of $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes, within the first week. Activated $CD4^+$ and $CD8^+$ T cells migrate to the lungs, between 2-4 weeks post-infection and interact with antigen presenting cells at the site of infection. The granuloma also consists of $CD4^+$ and $CD8^+$ T cells which, as part of the granuloma composition, encloses the bacteria and prevents reactivation (Feng, Bean et al. 1999; Serbina, Liu et al. 2000). An overview of the T cell responses during *M. tuberculosis* infection is illustrated in figure 1.4.

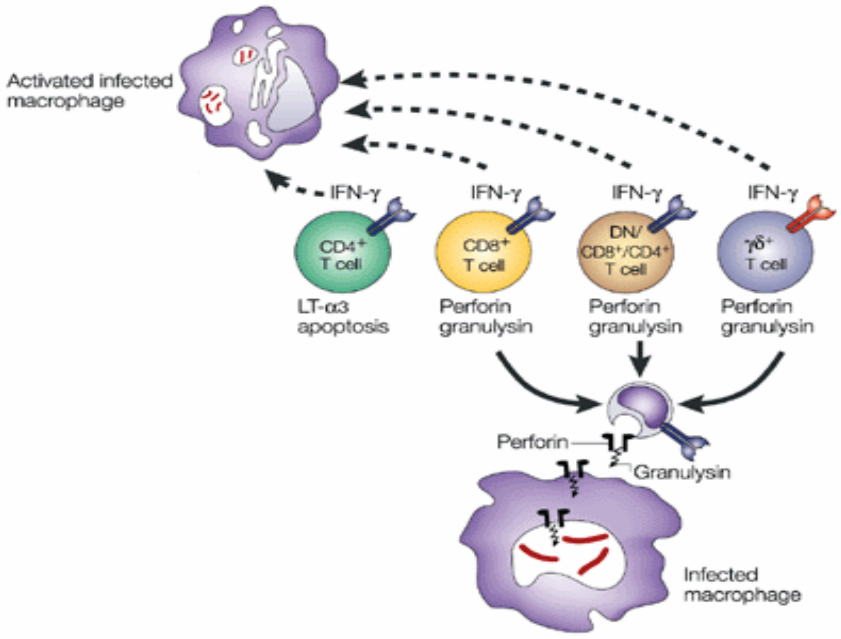


Figure 1.4- An overview of the T cell responses during *M. tuberculosis* infection

The immune response to *M. tuberculosis* is for the most part dependent on T cell responses which mainly activate macrophages by secreting cytokines or exhibit direct cytolytic activity. Figure modified from (Kaufmann 2001).

1.7.1.1-$CD4^+$ T cells

Mycobacteria are phagocytosed and reside within phagosomes. Bacterial derived proteins from these intracellular vesicles in macrophages are not accessible to the proteasomes in the cytosol and MHC-I loading complex. But mycobacterial antigens are readily accessible to the MHC-II antigen processing and presentation complex, thus resulting in the presentation of antigens to $CD4^+$ T cells. The importance of this subset of cells has been demonstrated by antibody depletion, adoptive transfer and the use of mouse strains deficient in $CD4^+$ T cells or MHC-II (Caruso, Serbina et al. 1999; Scanga, Mohan et al. 2000). The increased susceptibility of patients to TB, during the course of HIV infection, further highlights the importance of this subset of T cells. IFN-γ production by these cells is proposed to play a major role during *M. tuberculosis* infection, which is supported by the work, which showed that $CD4^+$ T cell–depleted and MHC-$II^{-/-}$ mice exhibited approximately 50% reduced IFN-γ levels at 2 weeks post-infection but at later time points the level of IFN-γ was similar between the wild-type and knockout strains of mice (Scanga, Mohan et al. 2000). Thus it was demonstrated that $CD4^+$ T cells are required early in infection for the production of IFN-γ.

In a model of latent TB, antibody mediated depletion of $CD4^+$ T cells led to reactivation of a persistent infection although, the levels of IFN-γ and iNOS remained unaffected (Scanga, Mohan et al. 2000). This work showed an additional role for $CD4^+$ T cells in the prevention of TB reactivation. This role has been further supported by work, which demonstrated that in the absence of this subset of T cells mice formed abnormal lesions, which were unable to control bacterial burden and prevent dissemination of the bacteria (Mogues, Goodrich et al. 2001; Saunders, Frank et al. 2002). One of the mechanisms utilised by *M. tuberculosis* to survive in the macrophages is by down regulating MHC-II expression on the surface (discussed in section 1.2.6). Hence, infected macrophages are defective in their ability to present antigens to $CD4^+$ T cells and thus contribute to the inability of the host to eliminate persistent infection. Experimental evidence from human studies has also shown that $CD4^+$ T cells can perform cytolytic functions in the lung. One possible mechanism is via the Fas-ligand-induced apoptosis of infected macrophages, thus reducing the viability of

intracellular bacteria (Tan, Canaday et al. 1997; Oddo, Renno et al. 1998). However, the primary function of $CD4^+$ T cells during mycobacterial infection appears to be the production of IFN-γ which activates macrophages resulting in the induction of anti-mycobacterial killing mechanisms within the host.

1.7.1.2-$CD8^+$ T cells

Cytosolic pathogen derived peptides are presented on MHC-I and recognised by $CD8^+$ T cells. Although *M. tuberculosis* is not a cytosolic pathogen, it is widely recognised that $CD8^+$ T cells are important in the host mediated immune response against this bacteria. A number of reports have provided evidence to support the importance of this subset of T cell in mediating the host immune response. Mice deficient in β2-microglobulin (aids in the folding and assembly of MHC-I molecule), TAP (transports peptides from the cytosol into the lumen of endoplasmic reticulum for loading onto MHC-I) and perforin, display enhanced susceptibility to *M. tuberculosis* infection in comparison to wild-type mice (Flynn, Goldstein et al. 1992; Cooper, D'Souza et al. 1997; Behar, Dascher et al. 1999).

Previous work has also shown that $CD8^+$ T cells are important in generating protective immune responses in the host against *M. tuberculosis* infection by producing IFN-γ (Tascon, Stavropoulos et al. 1998). Mice lacking MHC-Ia were less susceptible over the course of the infection compared to β2-microglobulin knockout mice, suggesting that other cells apart from classically MHC-I restricted $CD8^+$ T cells were playing a role in the resistance mechanism to TB infection (Perarnau, Saron et al. 1999). The effector mechanisms of this subset of T cells include cytokine production in the form of IFN-γ and TNF which have important roles in mediating anti-mycobacterial immunity (roles of TNF and IFN-γ discussed in sections 1.6.3 and 1.6.6) and in TB patients, IFN-γ secreting MHC-I restricted $CD8^+$ T cells could be detected by enzyme-linked immunosorbent spot (ELISPOT) from the peripheral blood (Pathan, Wilkinson et al. 2000). The killing of mycobacteria was dependent on perforin and granulysin and this direct killing of *M. tuberculosis* could also be demonstrated by incubating *M. tuberculosis* with granulysin alone (Stenger, Hanson et al. 1998).

1.7.1.3-Unconventional T cells

Mycobacterial glycolipids such as LAM, PIM and mycolic acids are presented on CD1 molecules. There exists two main groups of CD1 molecules, with group 1 comprising of CD1a, CD1b and CD1c, found in primates and guinea pigs and group 2 – CD1d found in mice (Porcelli and Modlin 1999). CD1 molecules are present in distinct intracellular compartments with CD1a being expressed in the early recycling endosomes, CD1b in late endosomes/lysosomes and CD1c being localised in endosomes. CD1a and CD1c have ready access to mycobacterial lipids from these compartments. A correlation between CD1 T cells in the peripheral blood and prior *M. tuberculosis* infection, with CD1 responses being reduced during active TB infection, has been reported (Ulrichs, Moody et al. 2003).

Double negative ($CD4^-$ $CD8^-$) CD1 restricted T cells which recognise mycobacterial antigens are capable of producing IFN-γ and expressing cytotoxic activity (Stenger, Mazzaccaro et al. 1997). In contrast, to $CD8^+$ T cells, these cells exert their cytotoxic activity via the Fas-FasL pathway.

In addition, to the above mentioned subsets of T cells, a minor T cell population expressing the γδ T cell receptor has been implicated in response to mycobacterial infections and shown to accumulate during early infection of mice. δ T cell receptor gene-disrupted mice infected with *M. tuberculosis* did not exhibit increased susceptibility to infection compared with wild-type animals, although at the site of granuloma formation, there was an increased neutrophil infiltration, suggesting a potential role for these cells in directing cellular trafficking and granuloma formation (D'Souza, Cooper et al. 1997).

Another sub-population of T cells, which represent approximately 5-10% of the circulating $CD4^+$T cells in healthy individuals are the regulatory T cells (Baecher-Allan, Brown et al. 2001). These cells are characterised as being $Foxp3^+CD4^+CD25^+$ or $CD8^+$. Foxp3 is a T cell lineage specification factor, with an essential role in the development of $CD4^+CD25^+$ regulatory T cells. A recent study reported an increased frequency of $CD4^+CD25^+$ T cells in patients with active TB and depletion of this subset of T cells improved IFN-γ production from

$CD4^+$T cells (Ribeiro-Rodrigues, Resende Co et al. 2006). Another set of experiments demonstrated an increase in the frequency of $Foxp3^+CD4^+$ in the spleen and lungs of mice infected with *M. tuberculosis.* Hence, it was suggested that, regulatory T cells limited the function of $CD4^+$ T cells during the course of infection (Kursar, Koch et al. 2007). The role of regulatory T cells during *M. tuberculosis* infection remains unclear but a common consensus seems to be, the depression of protection afforded by $CD4^+$ T cells, during the course of TB infection, by regulatory T cells.

1.8-Involvement of other immune cells in the host mediated response to *M. tuberculosis*

Additional subsets of immune cells are also thought to contribute to the host immune response during *M. tuberculosis* infection. In the inflammatory response during the course of infection, neutrophils are the first phagocytes to arrive at the site. Neutrophils are present early at sites of TB infections, as well as in granulomas (Peters and Ernst 2003). They also appear to be recruited to the lung, where they have a role during acute TB infection (Fulton, Reba et al. 2002). Additionally, neutrophils are thought to have an immunomodulatory role, as they produce cytokines and chemokines thus contributing to the global responses to *M. tuberculosis* infection (Petrofsky and Bermudez 1999).

B-cells were thought not to play a role during mycobacterial infection as B-cell mice deficient displayed similar bacterial loads to the wild-type mice. But a recent report demonstrated that B $cell^{-/-}$ mice displayed profound immunopathology and increased IL-10 production in the lung in response to *M. tuberculosis* infection. Furthermore B $cell^{-/-}$ mice were susceptible to a high dose of *M. tuberculosis* which was reflected in the elevated bacterial counts in the lungs (Maglione, Xu et al. 2007). The contribution of antibodies to the host response during mycobacterial infection is still not clear. Recent reports have demonstrated a use for high dose intravenous immunoglobulin in reducing bacterial loads at early and late phase of infection and could be used as an adjunct to therapy of TB (Roy, Stavropoulos et al. 2005; Roy, De Silva et al. 2006).

NK cells have been associated with early resistance to intracellular pathogens and are thought to be an early source of IFN-γ prior to the emergence of antigen specific T cells. In the aerosol infection model, it was found that NK cells increase initially over a 3 week period in the lung and then subsequent depletion of NK cells had no effect on the bacterial load in the lungs (Junqueira-Kipnis, Kipnis et al. 2003). NK cells also exhibit enhanced lytic capability against *M. tuberculosis* infected macrophages following stimulation with IL-12 (Bermudez, Wu et al. 1995).

The protective role against *M. tuberculosis* is generally accepted to be cell mediated with dendritic cells among the key subsets of innate immune cells bridging the gap between innate and adaptive immune responses.

1.9-Dendritic cells and mycobacteria

M. tuberculosis can readily infect dendritic cells (Bodnar, Serbina et al. 2001; Jiao, Lo-Man et al. 2002). Dendritic cells are not considered the natural host cells for mycobacteria. Thus, less is know about the interface between dendritic cells and mycobacteria, when compared to the wealth of information available for the interaction between macrophages (primary host cells for the bacilli) and mycobacteria.

Key aspects of dendritic cells underlie the importance of studying this subset of immune cells during mycobacterial infections. These include:

1. The ability of these cells to secrete IL-12, which plays a key role in the development of a protective immune response
2. The capability to transport antigens from the periphery into the draining lymph nodes and activate naïve T cells, as these cells are equipped with an array of antigen presenting molecules (MHC-I, MHC-II and CD1 molecules) and co-stimulatory molecules
3. The possibility that these cells act as “Trojan horses”, harbouring mycobacteria, thus providing a safe environment for the bacilli by preventing uptake and destruction by activated macrophages.

A study comparing the ability of activated murine macrophages and dendritic cells demonstrated that, dendritic cells were able to restrict the growth of the bacteria but in comparison activated macrophages were highly efficient in killing *M. tuberculosis* (Bodnar, Serbina et al. 2001). Dendritic cells utilise TLRs to recognise mycobacteria and it has been reported that stimulation of TLR 2 and TLR 4 on human dendritic cells, with BCG cell wall skeleton, (consists of mycolic acids, arabinogalactan and peptidoglycan) induces the secretion of TNF-α which leads to the maturation of dendritic cells. Other receptors like DC-SIGN aid in the uptake of mycobacteria and engagement of this receptor has been reported to suppress dendritic cell maturation by inducing production of IL-10. The interaction between DC-SIGN and mycobacteria has been discussed in section 1.3.4).

Maturation of dendritic cells via engaging the TLRs, results in the induction of signalling via NF-κB. It has been previously reported that dendritic cells in response to *M. tuberculosis* infection exhibited a mature phenotype. In contrast to these studies, a study has reported that dendritic cells derived from monocytes exhibit a limited maturation capacity in response to *M. tuberculosis* infection which was reversible upon removal of IL-4 and GM-CSF from dendritic cell cultures (Hanekom, Mendillo et al. 2003).

It has been postulated that dendritic cells function to transport the bacilli from the lungs which is usually the primary site of infection to the draining lymph nodes. A recent report provides evidence to this statement. This study reported that a significant proportion of BCG was detected in dendritic cells in the draining lymph nodes. In the draining lymph nodes dendritic cells would present mycobacterial antigens to T cells thus evoking a protective T cell response or act as bystander cells to present antigens from other mycobacterial infected cells (Humphreys, Stewart et al. 2006).

M. tuberculosis infected dendritic cells can prime naïve T cells *in vitro* and induce an IFN-γ producing Th-1 type response (Giacomini, Iona et al. 2001; Hickman, Chan et al. 2002). In addition, mycobacteria infected dendritic cells

prime protective T cell responses when administered into mice with priming of both $CD4^+$ and $CD8^+$ T cells (Roy, De Silva et al. 2006). T cell responses primed in this manner are protective against a subsequent challenge with virulent *M. tuberculosis*, achieving levels of protection similar to, or improved upon that conferred by BCG (Roy, De Silva et al. 2006).

The process of cross-priming has been shown to be an essential component of priming T cells thus an integral part of the immune response against pathogens (Sigal, Crotty et al. 1999; den Haan and Bevan 2001). Apoptotic blebs derived from mycobacteria infected cells are phagocytosed by uninfected dendritic cells and presented to MHC-I and CD1 restricted T cells. Dendritic cells were found to be indispensable for this new detour pathway of priming T cells, as dendritic cells possess a unique endosome to cytosol pathway, and they are superior to macrophages in priming T cell responses (den Haan and Bevan 2001; Aaronson and Horvath 2002; Schaible, Winau et al. 2003). Hence, uninfected dendritic cells are also important in cross-priming *in vivo* during *M. tuberculosis* infection thus, contributing to the T cell mediated immune responses generated by the host.

1.10-Microarrays

Global transcriptional responses of immune cells to a pathogen like *M. tuberculosis* can be deciphered using microarrays. Microarrays represent a high throughput screening method to monitor the whole genome on a single chip. Affymetrix arrays provide high reproducibility and specific detection of gene expression but the downside being that they cannot be produced in-house. They have to be purchased from commercial sources hence making them very expensive thus limiting the number of analysis that can be carried out.

Affymetrix GeneChips are manufactured using semiconductor fabrication technology, combining this with photolithography to enable a vast amount of information to be accommodated on a single chip. Mouse genome 430 2.0 array is a 11μm sized chip representing approximately 39,000 transcripts thus, providing coverage of the entire transcribed mouse genome on a single chip (source -

Affymetrix data sheet). Expression chips consist of 11 probe pairs; each probe is about 25 basepairs long, weighted towards the 3' end of the transcript and spans the entire length of each gene measured. One probe from the pair perfectly matches the target gene, while the other has a single mismatch located in the middle of the sequence, thus serving as an internal control for correcting non-specific binding. The mismatch probe differs from the perfect match probe in the 13^{th} base. The expression level for each gene is a summary of the entire probe set hybridisation data.

Microarray analysis is useful in uncovering pathways and identifying biomarkers of gene expression patterns under a given experimental condition or in human diseases. Using Affymetrix arrays, it has been possible to identify transcriptional responses for infections with influenza A and systemic lupus erythematosus (SLE). The analysis of peripheral blood mononuclear cell profiles from patients with influenza A or SLE, allowed for the identification of genes common to both these dieases and also unique gene patterns which were then further translated into functional modules, to obtain a distinct gene expression signature for each disease (Chaussabel, Allman et al. 2005). Profiling gene expression of human dendritic cells and macrophages, in response to a group of diverse microbes including *Toxoplasma gondii, Leishmania major, Leishmania donovani, Brugia malayi* and *M. tuberculosis*, identified clusters of genes which showed cell type specificity. A greater change in gene expression was observed in dendritic cells (1519 genes) in comparison to macrophages (1060 genes) (Chaussabel, Semnani et al. 2003).

Additionally, microarrays can be used to assign probable functions to genes with no known biological role by comparing expression patterns to genes with known function, to discover vaccine targets and to elucidate genetic differences between pathogens. It has been possible to use microarrays to identify genes at the various stages of infection with *Neisseria meningitides* (Kurz, Hubner et al. 2003) and *Plasmodium falciparum* (Le Roch, Zhou et al. 2003), which served as the basis of identification of a number of vaccine candidates.

Microarray technology had made it possible to have a genome-wide view of complex interactions rather than focussing on a single aspect of the host-pathogen relationship. The biggest limiting step in microarray based experiments is the post-analytical step. With the advent of microarrays, the interest in the use of various bioinformatics tools to identify transcription factors regulating gene expression changes also increased exponentially. Identifying *cis*-regulatory sequences is crucial to understanding gene expression. Transcription factor binding sites are usually short sequences (5-15 bases) and hence potentially these sites can occur frequently by chance thus leading to a large number of false positives. A approach that could be used is to identify over-represented transcription factor binding motifs in the promoters of differentially regulated genes by using position weight matrices which are available as databases such as JASPAR (Sandelin, Alkema et al. 2004) and TRANSFAC (Wingender, Chen et al. 2000).

1.11- Aims of the project

The main objective of the project was to study the global interactions between bone marrow derived dendritic cells and *M. tuberculosis*. Particular emphasis was placed on characterising the immune signalling pathways that are evoked by dendritic cells in response to *M. tuberculosis* infection.

The specific objectives of the project were:

1 To use Affymetrix gene expression profiling to identify differentially expressed genes in response to infection on a global scale

2 To utilize a bioinformatic approach to identify potential transcription factor binding domains within genes that are differentially regulated by dendritic cells infected with *M. tuberculosis*

3 To validate selected gene expression data from microarray analysis using real-time PCR, ELISA , FACS and biochemical approaches

4 To investigate the role of MyD88 independent signalling pathway in the priming of anti-mycobacterial immunity, in an intravenous model of *M. tuberculosis* infection.

Chapter 2

Materials and Methods

2.1- Mice

Adult 6-8 week old male C57BL/6 and MyD88$^{-/-}$ mice were obtained from breeding colonies maintained under specific pathogen-free conditions in the animal facilities at the National Institute for Medical Research (NIMR). MyD88$^{-/-}$ mice (Adachi, Kawai et al. 1998) were kindly provided by Dr.Jean Langhorne, NIMR.

2.2-Bacteria

The *M. tuberculosis* strain H37Rv was grown in Dubos media supplemented with 0.2% (v/v) glycerol (Merck, Germany) and Dubos medium albumin (Difco, New Jersey) in roller bottles at 37^{0}C. Aliquots of H37Rv (optical density 0.55-0.60) were stored at –80^{0}C.

2.3- Dendritic cells

2.3.1-Generation of bone marrow derived dendritic cells (BMDCs)

Dendritic cells were cultured using the protocol adapted from Inaba et al (Inaba, Inaba et al. 1992). Briefly, bone marrow cells were derived from murine femurs and cultured for 6 days in Iscove's modified Dulbecco's medium (Invitrogen, UK) supplemented with 5% fetal calf serum, 200mM glutamine, 2mM mercaptoethanol and 10ng/ml of recombinant GM-CSF (R&D systems, UK). Media and non-adherent cells were removed on day 3 and then replaced with fresh media containing GM-CSF. Non-adherent cells were harvested on day 5 and these clusters of cells were sub-cultured in fresh medium with recombinant GM-CSF for a further 24 hours and on day 6 these cells were used as a source of dendritic cells. Using the above method, approximately 8x10^6 dendritic cells were generated per mouse. The cells were cultured in media free of antibiotics.

2.3.2-Isolation of CD11c$^+$ dendritic cells from bone marrow

CD11c antigen is commonly used as a marker of dendritic cells. Purification of CD11c$^+$ dendritic cells was performed by the method adapted from the protocol supplied by Miltenyi Biotec. Day 6 dendritic cells were pooled and

washed extensively with sorting buffer (PBS pH7.2, supplemented with 0.5% fetal calf serum and 0.5mM EDTA). After washing the supernatant was removed and the pellet re-suspended in 400µl of sorting buffer per 10^8 total cells. Fc receptors were blocked with anti-mouse CD16/CD32 (Clone 2.4G2, Pharmingen, San Diego) for 20 minutes at 4^0C. The cells were then further incubated with 100µl MACS CD11C beads (Miltenyibiotec, clone N418) per 10^8 cells at 4^0C for 15- 20minutes. The cells underwent a final wash using sorting buffer and were then re-suspended in 500µl per 10^8 cells. The cells were then separated using magnetic LS columns (Miltenyi Biotec, UK) on a SuperMACS™(Miltenyi Biotec, UK) magnetic separator . As shown in figure 2.1, dendritic cells after sorting, were consistently 95 ±3% $CD11c^+$.

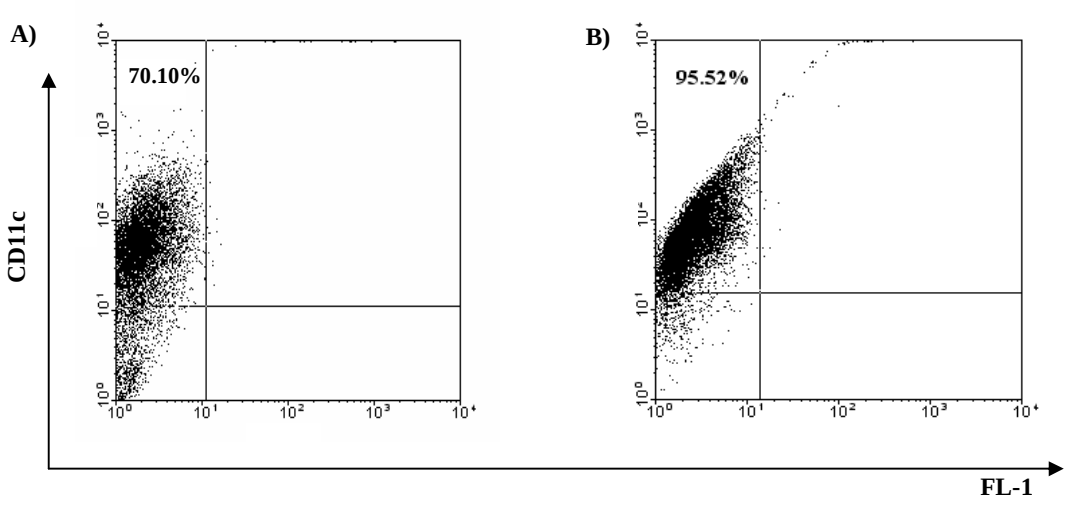


Figure 2.1- Flow cytometric analysis of bone marrow cells
A) before magnetic sorting B) after magnetic sorting using CD11c beads. Results shown percentage of total cells and represent a typical experiment. Consistently, the purity of the cells was 95± 3%.

2.3.3-Flow cytometry

Cells were pooled and washed with PBS supplemented with 1% fetal calf serum (Gibco, Paisley, UK) and after extensive washing the pellet was re-suspended in 0.1ml of PBS supplemented with 1% fetal calf serum. The cell suspension was incubated with anti-CD16/CD32 (clone 2.4G2) for 20 minutes at 4^0C to prevent non-specific antibody binding and then stained with directly conjugated antibodies for 1 hour at 4^0C. The following directly conjugated antibodies were used for the experiments: PE CD11c (Clone HL3), PE CD80 (Clone 16-10A1), PE CD86 (Clone GL1), FITC I-A^b (Clone 25-9-17), FITC F4/80 (Clone BM8), FITC IgG2a (Clone R19-15), PE IgG1 (Clone A85-1). After the staining process the cells were washed with PBS supplemented with 1% fetal calf serum and subsequently the cells were fixed in 4% paraformaldehyde and analysed using a FACScan flow cytometer (Becton Dickinson, United Kingdom). Data analysis was performed using WinMDI (Scripps Research Institute, California). All the antibodies used for flow cyometry were purchased from B.D Pharmingen, San Diego.

2.3.4-Cytokine measurement

BMDCs at a concentration of $1x10^6$/ml were stimulated with 1µg/ml LPS from *E.coli* 026:B6 (Sigma-Aldrich, USA), 25µg/ml poly I:C – synthetic double stranded RNA (Autogen Bioclear, UK), infected with H37Rv 10:1 (10 bacteria per cell) or left uninfected (negative control) and supernatants were collected usually between 6-72 hours post-stimulation, unless specified. All cytokine measurements were carried out by enzyme-linked immunosorbent assay (ELISA) using commercial kits according to the manufacturer's protocol. IL-12p40 (sensitivity 8 pg/ml), IL-12p70 (sensitivity 15pg/ml), IL-6 (sensitivity 4pg/ml), TNF-α (sensitivity 8pg/ml) and IL-10 (sensitivity 30pg/ml) were purchased from eBioscience (San Diego). CXCL10 (sensitivity 30pg/ml) ELISA kit was purchased from R&D systems (Oxford, UK) and IFN alpha (sensitivity 12.5pg/ml), IFN beta (sensitivity 15.6 pg/ml) kits were acquired from PBL Biomedical Laboratories ,New Jersey.

2.4- RNA and DNA microarray hybridisation and analysis

2.4.1- RNA isolation

Total RNA was extracted using TRIzol reagent (Invitrogen Ltd, UK) according to the manufacturer's protocol. Briefly, TRIzol is a mono-phasic solution of phenol and guanidine isothiocyanate used for the isolation of total RNA from cells. $5x10^6$ cells were lysed in 1ml of TRIzol at room temperature for 5 minutes. Subsequently, 200µl of cholorform per 1ml of TRIzol was added and then further incubated at room temperature for 3 minutes. This was followed by a centrifugation step at 12,000g for 15 minutes at 4^0C which separates the mixture into three phases and the RNA remains in the upper aqueous phase. The aqueous phase was carefully pipetted into a fresh tube and RNA was precipitated out of the aqueous phase with the addition of 0.5ml of isopropanol. The sample was incubated at room temperature for 10 minutes and then centrifuged at 12,000g for 10 minutes at 4^0C. The RNA pellet which is visible after the centrifugation step was washed with 1ml of 75% ethanol per 1ml of TRIzol and then further centrifuged at 7,500g at 4^0C for 5 minutes. The RNA pellet was air-dried and re-suspended in 100µl of RNase free water and stored at -70^0C . RNA was cleaned up to remove any contaminants such as phenol or proteins using the RNeasy Mini Kit (Qiagen Ltd, UK) according to the manufacturer's protocol. The total amount of RNA in each sample was determined using the Nanodrop spectrophotometer (Nanodrop technologies, USA).

2.4.2- Determining the quality of RNA

The quality of RNA was determined using the Agilent 2100 Bioanalyzer (Agilent technologies Inc, Santa Clara, CA). The bioanalyzer generates an electropherogram for each RNA sample and key characteristics of the graph aids in the confirmation of the quality of RNA like the presence of well-defined 28S/18S peaks, low noise between 18S/28S peaks, minimal low molecular weight contamination. A 28S:18S, ribosomal RNA (rRNA) ratio of 1.8-2.1, generally indicates a good quality of RNA. An example of an electropherogram is given in figure 2.2.

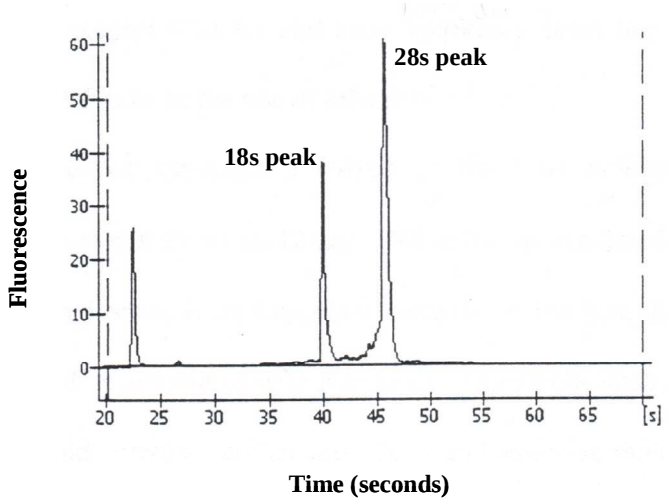


Figure 2.2- Electropherogram of a RNA sample generated by the Agilent bioanalyzer.

Above is a typical plot generated by the bioanalyzer which depicts sharp peaks for 18S and 28S and additionally the lack of noise between the peaks indicates a good quality RNA sample which can be used for processing and hybridisation to the Affymetrix GeneChip.

2.4.3 - Microarray hybridisation and analysis

Three independent experiments were conducted using 4-6 mice per experiment on different days, to generate triplicate biological RNA samples for microarrays.

2.4.4- MyD88$^{-/-}$ BMDCs -two-cycle target labelling and hybridisation

Triplicate biological samples were prepared from MyD88$^{-/-}$ BMDCs and from each biological sample, 50ng of total RNA from MyD88 $^{-/-}$ BMDCs was used for two cycle target labelling and hybridisation to the Affymetrix Mouse

genome 430 2.0 array as per the manufacturer's protocol (Affymetrix Inc, Santa Clara, CA). Two cycle target labelling was used due to the limited availability of $MyD88^{-/-}$ mice which was required to carry out the experiments. Briefly, 50 ng of RNA was reverse transcribed into cDNA using the Affymetrix two-cycle synthesis kit (Affymetrix Inc, CA) and T7-Oligo(dT) primer (Affymetrix Inc, Santa Clara, CA).cRNA was subsequently generated from the cDNA sample by an in vitro transcription reaction using MEGAscript T7 kit (Ambion, Austin, USA). A further step of cDNA synthesis was needed in the 2-cycle cDNA synthesis cycle to generate sufficient amounts of cRNA for target labelling and hybridisation. Subsequently, the unlabelled cRNA was reverse transcribed into first strand cDNA using random primers (Invitrogen Ltd, UK followed by the 2^{nd} strand cDNA synthesis using T7-Oligo(dT) primer (Affymetrix Inc, Santa Clara, CA). The amplified cDNA was labelled with biotin during an in-vitro transcription reaction to produce biotin labelled cRNA which was then fragmented and hybridised to the Affymetrix Mouse genome 430 2.0 array. The fluorescence was measured using the Affymetrix GeneChip scanner 3000.

2.4.5- C57BL/6 BMDCs-one-cycle target labelling and hybridisation

Triplicate biological samples were prepared from C57BL/6 (wild-type) BMDCs and from each sample 5-8μg of total RNA was used for one cycle target labelling and hybridisation to the GeneChip (Affymetrix, UK).The further step for the cDNA synthesis was not needed in the one cycle labelling and hybridisation reactions. The rest of the protocol is the same as described above for the two-cycle target labelling and hybridisation.

2.4.6- Analysis of microarray data

Microarray data was analysed using GeneSpring version 7.3 expression analysis platform (Agilent technologies Inc, Santa Clara, CA). The algorithm GC-Robust Multiarray Analysis (GCRMA) was computed from GeneSpring 7.3 (Agilent technologies, Santa Clara) to generate the absolute values from the .cel files (contains fluorescence intensities for each probe on the microarray) for

further normalisation. The GCRMA algorithm was chosen as it provides a good signal-noise ratio thus giving the best signal specificity and also achieves a more accurate coverage of the low expressing genes (Wu and Irizarry 2004). The MAS 5.0 algorithm was used to obtain the flagged data for each gene (genes are flagged as present, marginal or absent). The following normalisation parameters were applied to each experimental sample:

1. Data transformation- cut-off value set at 10 (Enables transformation of data into the log scale by eliminating any zero or negative values)
2. Per chip- Normalise to 50^{th} percentile (Removes non-biological variation across GeneChips)
3. Per gene- Normalise to median (Enables all transcript values to be centred around 1, thus making comparisons of gene expression patterns straightforward)

Individual GeneChips and each gene were normalised to 50% median intensity. The normalised data was transformed to the log scale. All the GeneChip data was normalised and log transformed before averaging and comparing each gene's expression value.

After normalisation of the data, the following filtering parameters were used to identify differentially regulated genes.

1. Flag filter- selects for gene flags which are either 'Present' or 'Marginal' in 3 out of the 6 samples
2. Expression level filter- removes genes which are non-changing across the GeneChips
3. AFFX control genes filter- enables removal of the Affymetrix control genes

Following filtering, differentially regulated genes were identified by analysing a change in transcript expression level between the uninfected (control) and *M. tuberculosis* infected group by a factor of 1.4 fold (minimum cut-off value suggested by Affymetrix consultant, Dr. Euan Hunter, personal communication) with a p value cut-off set at ≤0.05. The level of significance (p value-≤0.05) for each gene was tested using the one-way analysis of variance; Welch t-test (parametric t-test; assuming variances are unequal).

Quality control of all the samples was assessed using RNA digestion plots and differences in the distribution of intensities across arrays were evaluated using box plots for samples from C57BL/6 BMDCs (Appendix I) and MyD88$^{-/-}$ BMDCs (Appendix II).

2.4.6.1- Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways

KEGG pathways are a collection of biological pathways depicting the molecular interactions for various biological processes within an organism. These biological pathways are available for use as plug-in feature of GeneSpring 7.3. Differentially regulated genes can be superimposed onto these biological pathways, thus providing an overview of the various pathways that exhibit a change in response to *M. tuberculosis* infection of BMDCs.

2.5- Real-time PCR

2.5.1- Real-time PCR using SYBR green based method

Selected genes of interest were obtained from microarray data analysis and their expression levels were then examined using quantitative real-time PCR. Real-time PCR was performed on cDNA generated from RNA samples that were prepared independently of the RNA used for microarray analysis. Total RNA was reverse transcribed using random primers (Invitrogen, Paisley, UK) and superscript II reverse transcriptase (Invitrogen, Paisley, UK). Quantitect SYBR green PCR kit (Qiagen, UK) was used to produce fluorescently labelled PCR products. The quantification of PCR products was done using the ABI PRISM 7000 sequence detection system (Applied Bisosystems, U.S.A). Commercially available primers (SuperArray Biosciences, U.S.A) were used for the real-time PCR reactions. The following temperature cycling profile was used for all the primers: 95^0C, 15 minutes and 40 cycles of (95^0C, 30 seconds; 55^0C, 30 seconds and 72^0C, 30 seconds).

Real-time PCR reactions were performed with reverse transcriptase negative samples to exclude any DNA contamination. All amplifications were done in optical reaction plates (Applied Biosystems, U.S.A) in duplicates. The expression values of individual genes were normalised to the 18S rRNA, house-keeping gene and the transcript level of each gene was expressed as a fold change for the *M. tuberculosis* infected sample relative to the uninfected sample.

Expression value of the gene of interest (GOI) = $10^{[(C_t - b)/m]}$

(C_t- threshold cycle)

2.5.2- RT profiler PCR array system (IFN array)

Total RNA was reverse transcribed using random primers (Invitrogen, Paisley, UK) and superscript II reverse transcriptase (Invitrogen, Paisley, UK) to generate first strand cDNA. Diluted cDNA with RT (Real-time) SYBR green /ROX (SuperArray biosciences, Maryland) and water was used to generate the experimental cocktail which was added to the 96 well reaction PCR plate pre-coated with IFN genes and controls (SuperArray Biosciences, Maryland). The thermal cycling was performed using the following temperature profile: Cycle 1- 10 minutes at 95^0C and 40 cycles of 15 seconds at 95^0C followed by 1 minute at 60^0C. The expression value for each gene was normalised to a housekeeping gene- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the fold change was expressed relative to the *M. tuberculosis* infected sample.

The following formula was used to calculate the relative expression level for each gene- $2^{\wedge (-\Delta\Delta Ct)}$.

$\Delta C_t = C_t$ (GOI) - average (C_t (HKG)), where HKG- housekeeping gene

2.6- Transcription Element Listening system (TELiS)

TELiS (www.telis.ucla.edu) identifies transcription factor binding motifs in the promoters of genes differentially regulated under a given experimental condition which allows inferences about transcription factors mediating the observed changes in gene expression (Cole, Yan et al. 2005). The database computes a statistical list of transcription factor binding motifs in the promoters of differentially regulated genes. Representation of transcription factors is calculated relative to the basal level of prevalence of transcription factor binding motifs represented on the Affymetrix microarray platform. The following parameters were supplied to the bioinformatics database:

Sampling frame: Affymetrix Mouse Genome 430.2 array
Gene list: Genes with a fold change of 1.4 or more with a p value of less than 0.05
Promoter size: -1000 to +200 nucleotides
Stringency of analysis: 0.90 (90% confidence limit)

The above parameters were chosen to maximise detection sensitivity.
A z-test (used to determine if changes detected between the population and sample mean are statistically significant) is used to identify the significance of the representation of each transcription factor binding motif in the gene list compared to the sampling frame. Additionally the risk of false positives of the gene list that is considered to be significant (p value <0.01) is calculated using False discovery rate (Benjamini and Hochberg test). The data retrieval function in TELiS is useful for the identification of genes and their respective transcription factor binding sites.

2.7- Western Blot

2.7.1- Extraction of nuclear lysates

Nuclear lysates were prepared using the Transfactor Extraction Kit (Clontech, California) according to the manufacturer's protocol. Briefly, $1x10^7$ cells were lysed for 15 minutes on ice in 1ml of lysis buffer (100mM HEPES pH7.9, 15mM $MgCl_2$, 100mM KCl) supplemented with 0.1M dithiothreitol and

15µl of protease inhibitor cocktails (Aprotinin, Pepstatin A, Bestatin, trans-Epoxysuccinyl-L- leucylamido (4-guanidino) butane, and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride in dimethyl sulfoxide) for 15 minutes and then centrifuged at 11,000g for 20 minutes. The resulting supernatant is the cytosolic fraction which was removed and the crude nuclear pellet was then further re-suspended in pre-extraction buffer (20mM HEPES pH 7.9, 1.5mM MgCl2, 0.42M NaCl, 0.2mM EDTA, 25% [v/v] glycerol) supplemented with 0.1M dithiothreitol and 1.5µl of protease inhibitor cocktails for 30 minutes on ice. The nuclear suspension was centrifuged at 21,000g for 5 minutes and the supernatant was frozen in aliquots at -80^{0}C.

2.7.2- Determining protein concentration in nuclear lysates

Concentration of protein was determined using Coomassie Plus Bradford assay reagent (Pierce, Illinois) according to the manufacturer's protocol. Briefly, the protein sample was mixed with Coomassie plus reagent and then the absorbance at 595nm was determined using a Benchmark plus microplate spectrophotometer (Bio-Rad, UK). Protein concentrations were estimated by reference to absorbance measurements obtained for a series of Albumin (Pierce, Illinois) protein dilutions which served as the standard.

2.7.3- Gel electrophoresis

Nuclear lysates were mixed with NuPage LDS sample buffer (Invitrogen, Paisley, UK) and then incubated at 70 ^{0}C for 10 minutes. A protein concentration of 15µg/well was resolved using Tris-HCl Gels 10% (Bio-Rad, UK) in Tris-glycine SDS buffer. Separated proteins were transferred onto nitrocellulose membranes in transfer buffer (GE Healthcare, Buckinghamshire). Following transfer, the membrane was blocked overnight at 4^{0}C with 5% skimmed milk + 0.05% Tween 20 in PBS. After blocking the membrane was washed and incubated with anti-IRF-3 (Santa Cruz biotechnology, California) or anti-IRF-7 (Santa Cruz biotechnology, California) followed by incubation with HRP conjugated anti-rabbit IgG (Santa Cruz biotechnology, California) or anti-goat IgG (Santa Cruz biotechnology, California). The membranes were then developed with enhanced chemiluminescent western blot detecting reagents (GE Healthcare,

Buckinghamshire) and exposed to Lumi-Film chemiluminescent detection film (Roche, USA). Detailed compositions of western blotting reagents are listed in Appendix-III.

2.8- *In vivo* experiments

2.8.1- In vivo infection procedure

Six to eight week old female C57BL/6 and MyD88$^{-/-}$ mice were obtained from breeding colonies maintained under specific pathogen-free conditions in the Division of Biological Services, NIMR. Mice were injected intravenously with 10^6 viable H37Rv and spleens and lungs were harvested at 14 and 30 days post-infection.

2.8.2- Determining colony forming units from M. tuberculosis infected murine tissues

Tissues were homogenised by shaking with 2mm glass beads in ice cold saline using a ribolyser (Hybaid) for 30 seconds and 10 fold serial dilutions of the homogenate in saline was plated on 7H11 Middlebrook medium supplemented with Dubos oleic albumin complex supplement . The number of colony forming units was determined after the plates had been incubated at 37^0C for 14-21 days.

2.8.3- Lung histology

Mice were administered with a lethal injection of anaesthetic (Sagatal, RMB Animal health, United Kingdom) via the intra-peritoneal route and the lungs were perfused with 1ml of 10% neutral buffered formalin solution (Sigma-Aldrich, UK) via the trachea in situ and with an additional 1ml on the removal of the lungs. The lungs were then placed in 10ml of neutral buffered formalin for 3 days prior to histology. Following dehydration in a graded series of ethanol and clearing in xylene, the lungs were embedded in fibrowax (VWR International, UK). Sections of the embedded lungs were stained with hematoxylin and eosin.

2.8.4- Ex-vivo whole spleen cell assay

Spleens were harvested from three mice at 14 and 30 days post-infection, homogenised via a 40μm strainer (Falcon, San Jose), washed and re-suspended in red blood cell lysing buffer (Sigma, UK) for 2 minutes. Cells were washed and re-suspended in AIM-v serum free media (Gibco life technologies, Paisley). In 24 well plates, whole spleen cells were treated with saline (negative control), PPD at various concentrations (0.1μg/ml, 1μg/ml and 10μg/ml) or 1μg/ml of anti-CD3/CD28 (Pharmingen, San Diego) which served as the positive control. PPD was a kind gift from Dr.Martin Vordermier (VLA laboratories, Weybridge). Supernatants were collected at 24 and 72 hours post-infection. Culture supernatants were run on commercially available ELISA's from e-Bioscience for quantification of IL-2 (sensitivity 2pg/ml) and IFN-γ (sensitivity 15pg/ml).

2.9- Statistics

Statistical analysis was carried out using a Student's t-test (Sigma plot version 10, San Jose) with a p value < 0.05 being considered significant.

Chapter 3

Activation of dendritic cells in response to *M. tuberculosis* infection

3.1 -Introduction

Dendritic cells are potent antigen presenting cells that play a key role in priming immune responses to various pathogens. Dendritic cells in their immature state function as effective phagocytic cells ingesting microbes or their components. It has been previously reported that dendritic cells can efficiently take up microbes such as *Borrelia burdorferria* (Filgueira, Nestle et al. 1996), *Salmonella Dublin* (Ian Marriott 1999) and *M. tuberculosis* (Bodnar, Serbina et al. 2001). By using green fluorescent protein tagged BCG, it has also been demonstrated that lung dendritic cells can phagocytose mycobacteria, *in vivo* (Reljic, Di Sano et al. 2005).

Pathogen derived products including LPS, bacterial DNA and viral dsRNA can promote dendritic cell activation (Brunner, Seiderer et al. 2000). Hallmarks of dendritic cell activation include: increased surface expression of antigen presenting molecules like MHC-II and co-stimulatory molecules such as CD80 and CD86 on the surface and the production of inflammatory cytokines such as IL-12. It has been demonstrated that following phagocytosis of bacteria such as *Salmonella typhimurium* (Ian Marriott 1999) and *M. tuberculosis* (Henderson, Watkins et al. 1997), dendritic cells can produce inflammatory cytokines. *M. tuberculosis* infection of an immortalised dendritic cell line also resulted in increased inflammatory cytokine production, similar to the observation made with primary dendritic cells (Tascon, Soares et al. 2000).

The main aim of the work described in this chapter, was to study the activation of primary murine BMDCs in response to *M. tuberculosis* infection, by determining the expression of co-stimulatory and MHC-II molecules and assaying levels of inflammatory cytokine production both of which function as indicators of dendritic cell activation. The experiments described in this thesis were done using BMDCs which were grown in recombinant GM-CSF, as described in the materials and methods section (chapter 2, section 2.3.1). BMDCs are commonly used as a model to study murine dendritic cells, *in vitro*. The major advantage of using bone marrow as the source of dendritic cells is the ease of obtaining large numbers of dendritic cells from relatively low numbers of mice.

3.2-Characterisation of BMDCs using flow cytometry

BMDCs were generated by culturing bone marrow cells in the presence of GM-CSF for 6 days. To characterise day 6 BMDCs, non-adherent cells were stained with directly conjugated fluorescent antibodies and analysed using flow cytometry. BMDCs were determined by primarily gating on forward scatter (FSC) vs side scatter (SSC), as shown in figure 3.1, A. Visible on the FSC vs SSC plots in figure 3.1, A, are also two populations of cells outside the gate. These cells have a low FSC and thus most likely indicative of the granulocyte population (Inaba, Inaba et al. 1992) and hence were not included within the gated population. CD11c antigen, also known an integrin alpha X was used to initially identify the dendritic cell population. Day 6 BMDCs were 70± 5% $CD11c^{+}$ (Figure 3.1, B). In addition, these cells were also 30.29 ±10% $CD80^{+}$, 28.95 ±8% $CD86^{+}$, 24.57% ±5% MHC-II^{+} and 5.62 ±2% $CD11c^{+}F4/80^{+}$ (Figure 3.2).

Thus, day 6 BMDCs exhibit an immature phenotype as indicated by the low expression of CD86 and MHC-II. Although a small portion of cells stained positive for the macrophage specific marker, F4/80 (Figure 3.2, D), the majority of the cells were characteristic of dendritic cells.

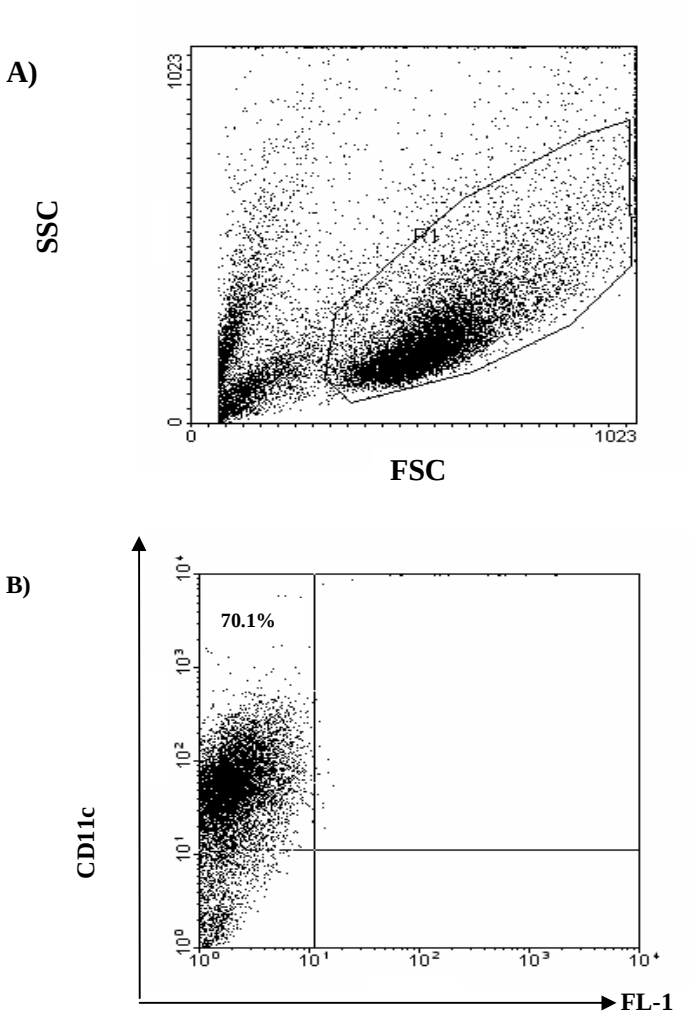


Figure 3.1- Expression of CD11c on BMDCs.

Day 6 BMDCs were gated on **A)** FSC vs SSC and **B)** the expression of CD11c was quantified using flow cytometry. Data shown are representative of 3 independent experiments.

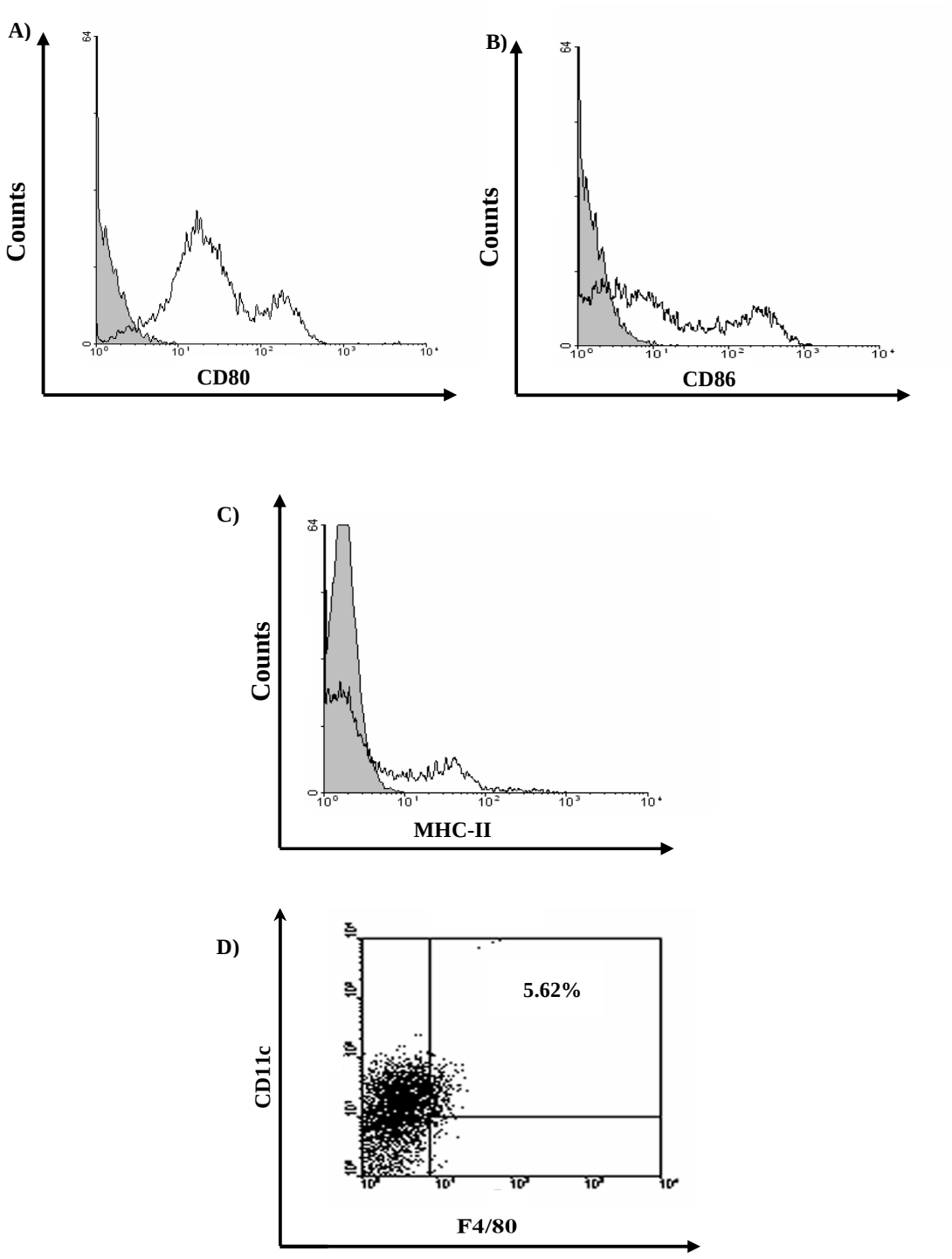


Figure 3.2-Analysis of day 6 BMDCs using flow cytometry.

Non-adherent cells, on day 6 were stained with fluorescent antibodies and the surface expression of **A)** CD80 **B)** CD86 **C)** MHC-II and **D)** CD11c F4/80 were analysed using flow cytometry. Data shown are representative of 3 independent experiments.

3.3-Phenotypic analysis of *M. tuberculosis* infected BMDCs using flow cytometry

In the previous section it was shown that BMDCs expressed low levels of co-stimulatory and antigen presenting molecules thus identifying them as immature dendritic cells. In order to investigate whether BMDCs can be activated in response to *M. tuberculosis* infection, day 6 BMDCs were either left uninfected, stimulated with LPS (1µg/ml) or infected with H37Rv at a multiplicity of infection (M.O.I) of 10:1 and the expression of activation markers were assessed by flow cytometry 24 hours later. LPS has shown to be an effective activator of dendritic cells and was thus used as a positive control for the experiments (Francesca Granucci 2001). The time-point, 24 hours and the ratio of infection (M.O.I 10:1) was chosen as an early time point to assess dendritic cell responses to *M. tuberculosis* infection, as a result of previous work in our lab, which identified 24 hours and 10:1 as an optimal time point for measuring the activation status of dendritic cells in response to *M. tuberculosis* infection (Dr. Ricardo Tascon, personal communication).

M. tuberculosis infection of BMDCs, up regulated the expression of CD80 to 83% , CD86 to 82% and MHC-II to 80%, thus indicating that these immature cells are activated in response to infection. These results allows us to conclude that *M. tuberculosis* infection provides the microbial stimuli needed for the activation of dendritic cells thus providing transition to a mature phenotype.

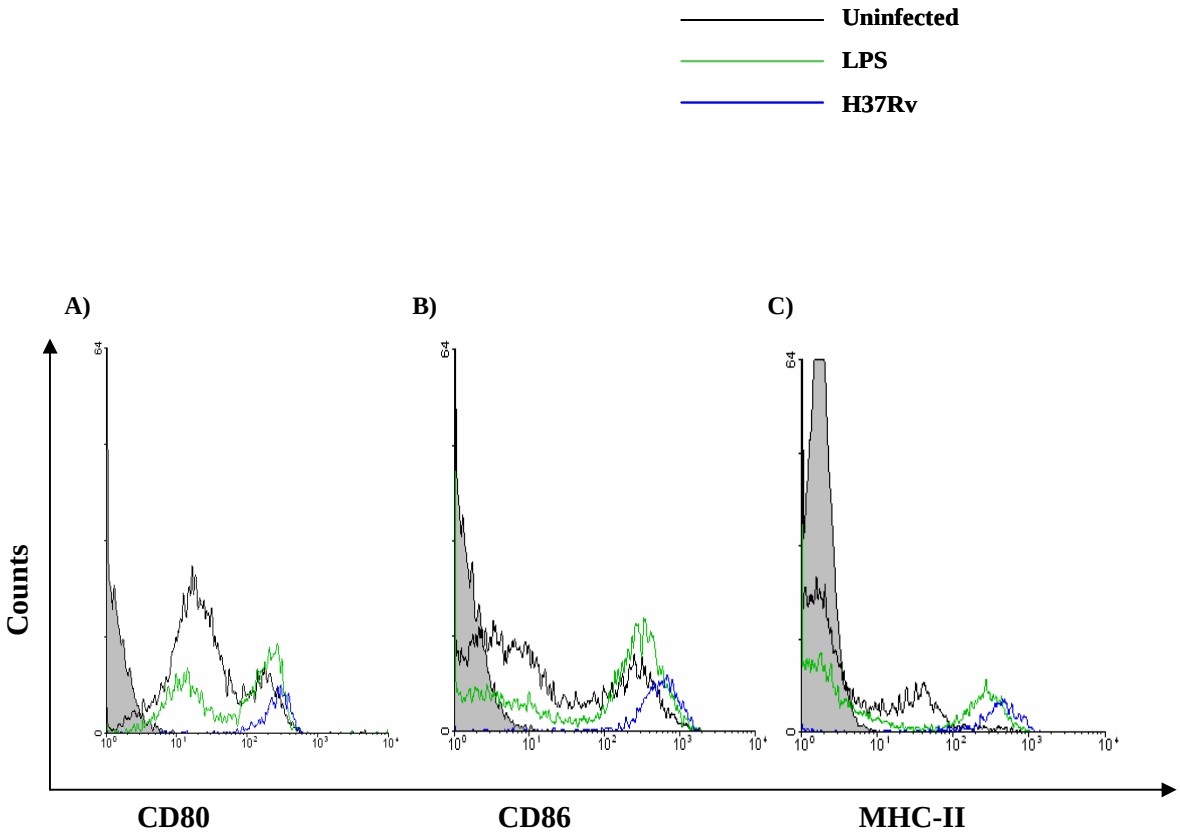


Figure 3.3- Cell surface phenotype of BMDCs infected with H37Rv.

Day 6 BMDCs were either left uninfected, stimulated with LPS (1µg/ml) or infected with H37Rv at a M.O.I of 10:1. 24 hours post-infection the expression of co-stimulatory molecules and MHC-II were analysed using flow cytometry. Histograms compare the expression of **A)** CD80 **B)** CD86 and **C)** MHC-II on uninfected cells (black line), LPS stimulated cells (green line) and H37Rv infected BMDCs (blue line). Gray filled histogram represents the isotype IgG control. Data are representative of 3 independent experiments.

3.4- A kinetic analysis of cytokines induced by *M. tuberculosis* infection of BMDCs

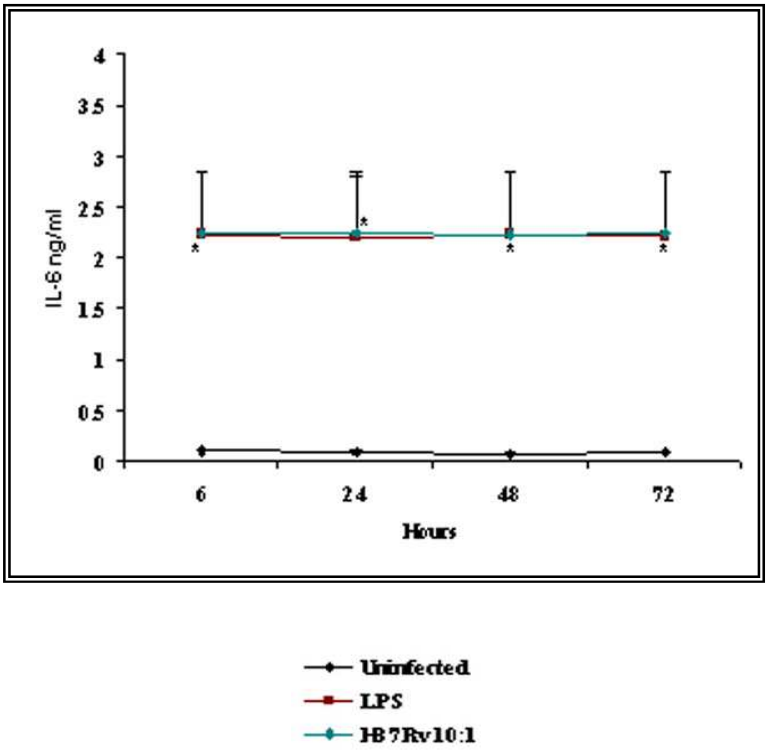
Dendritic cell activation triggers the production of inflammatory cytokines, which play a role in the polarisation of T cell responses either towards a Th-1 or Th-2 type. In the previous section it was shown that immature dendritic cells when exposed to microbial stimuli during *M. tuberculosis* infection were activated hence acquiring increased antigen presenting and co-stimulatory capacity thus moulding these cells to become effective stimulators of T cells. We also studied the repertoire of cytokines that are produced in response to *M. tuberculosis* infection as another indicator of dendritic cell activation.

Day 6 unsorted BMDCs (70± 5% $CD11c^{+}$) were infected with H37Rv at a M.O.I of 10:1 and uninfected and LPS stimulated BMDCs were included as control groups. At various time points post-infection (6-72 hours), supernatants were collected and the production of cytokines was assayed using ELISA.

3.4.1- IL-6 and TNF-α production by *M. tuberculosis* infected BMDCs

In response to *M. tuberculosis* infection, BMDCs consistently produced significantly high levels of IL-6 and TNF-α, in comparison to uninfected BMDCs over the time points assayed (6-72 hours), as shown in figure 3.4. Uninfected BMDCs produced negligible amounts of IL-6 and TNF-α at all the time points examined. LPS stimulated BMDCs also produced similar levels of IL-6 to *M. tuberculosis* infected BMDCs over the course of infection (6-72 hours). Although, the production of TNF-α is comparable between LPS stimulated and *M. tuberculosis* infected BMDCs between 6 and 24 hours, there is a decrease in the level of TNF-α by 48 hours (3.9 ±1.9 ng/ml) only in LPS stimulated BMDCs (Figure 3.4, B). Similar levels of TNF-α were secreted by *M. tuberculosis* infected BMDCs between 6-72 hours of infection, as shown in figure 3.4, B.

A)



B)

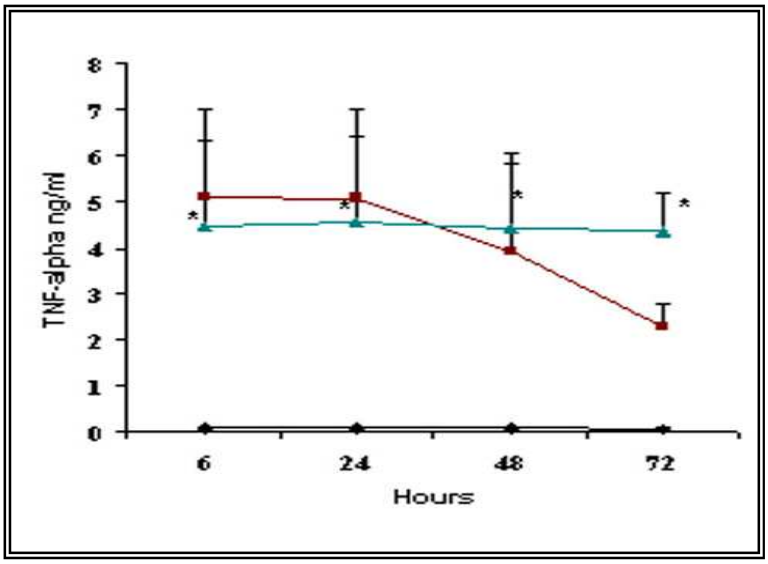


Figure 3.4- IL-6 and TNF-α secretion by BMDCs in response to *M. tuberculosis* infection.

Day 6 BMDCs were either left uninfected, stimulated with LPS or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at various time points and quantified by ELISA for the amount of A) IL-6 and B) TNF-α produced. Results are the mean of ±SE of 3 independent experiments. Statistical analysis was performed between H37Rv infected group and uninfected group with the Student's t-test, *- p value <0.05.

3.4.2- Induction of IL-10 secretion by *M. tuberculosis* infected BMDCs

The production of IL-10 by *M. tuberculosis* infected BMDCs reached a peak at 24 hours post-infection (0.19 ±0.05 ng/ml) and decreased over later time points, as shown in figure 3.5. IL-10 was produced by LPS stimulated BMDCs by 24 hours (0.2 ±0.05 ng/ml), with decreased production at later time points. The levels of IL-10 produced by *M. tuberculosis* infected BMDCs were not significantly different statistically, to the uninfected group.

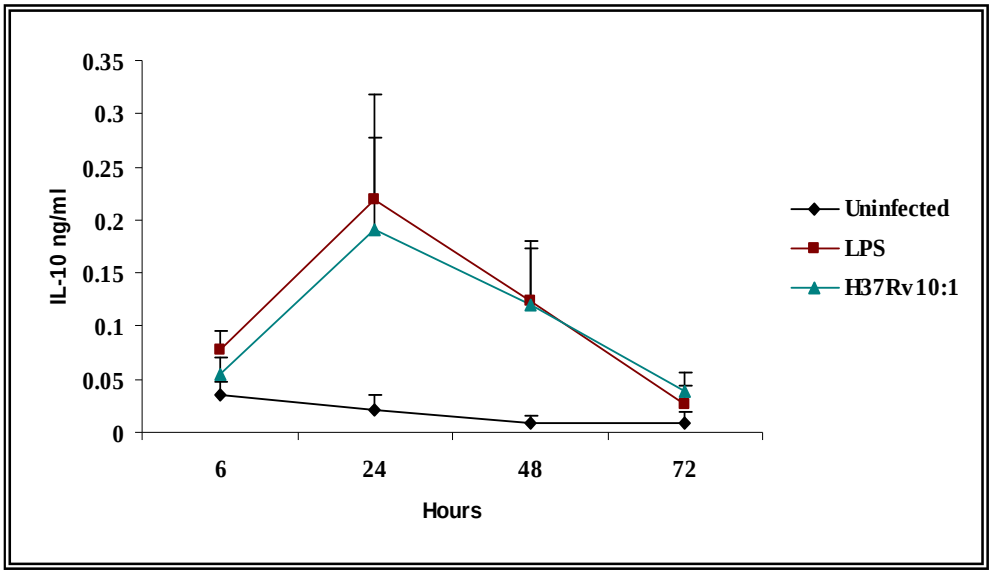


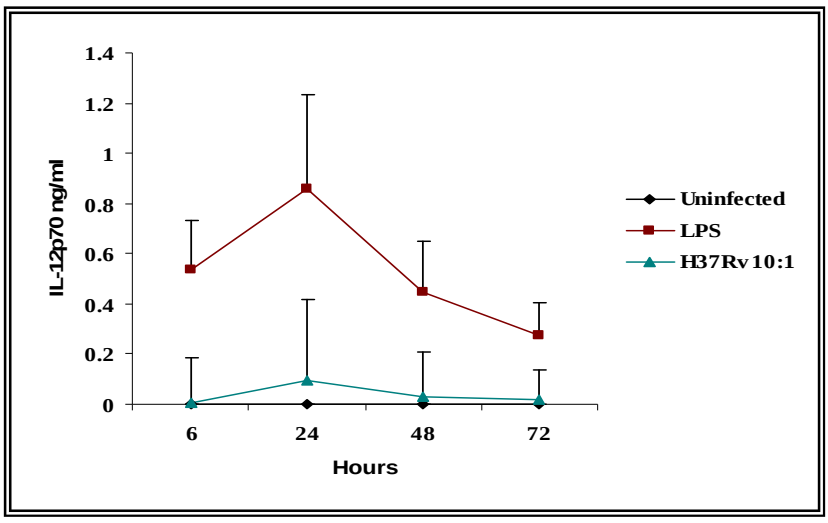
Figure 3.5- IL-10 secretion by unsorted BMDCs in response to *M. tuberculosis* infection.

Day 6 BMDCs were either left uninfected, stimulated with LPS or infected with H37Rv (M.O.I 10:1). IL-10 levels were quantified using ELISA. Results are the mean of ±SE of 3 independent experiments.

3.4.3- The production of IL-12 in response to *M. tuberculosis* infection of BMDCs

M. tuberculosis infected unsorted BMDCs, consistently produced, lower levels of IL-12p70 in comparison to LPS stimulated cells, as shown in figure 3.6, A. The maximum IL-12p70 production was detected at 24 hours in both LPS stimulated and *M. tuberculosis* infected BMDCs. High levels of IL-12p40 secretion were detected as early as 6 hours in LPS stimulated BMDCs (5.7±1.4 ng/ml) and only by 24 hours in *M. tuberculosis* infected BMDCs (5.1±1.4 ng/ml), as shown in figure 3.6, B. At later time points (48-72 hours), the production of IL-12p40 was comparable in both LPS stimulated and *M. tuberculosis* infected BMDCs.

A)



B

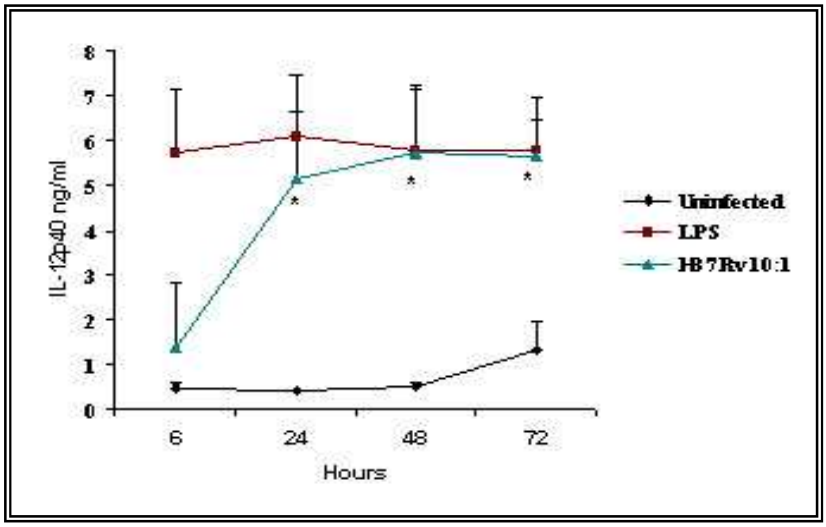


Figure 3.6- Production of IL-12 by BMDCs in response to *M. tuberculosis* infection.

Day 6 BMDCs were either left uninfected, stimulated with LPS or infected with H37Rv and the amount of IL-12 were determined using ELISA. The production of A) IL-12p70 and B) IL-12p40 was analysed using ELISA. Results are the mean of ±SE of 3 independent experiments. Statistical analysis was performed between H37Rv infected group and uninfected group with the Student's t-test, *- p value <0.05.

3.5-Discussion

The experiments described in chapter 3 were carried out to study the activation of murine BMDCs in response to *M. tuberculosis* infection. Analysis of BMDCs using flow cytometry, demonstrated that these cells displayed an immature phenotype (low expression of CD86 and MHC-II) before microbial stimulation. Following *M. tuberculosis* infection, BMDCs up regulated the co-stimulatory molecules and MHC-II. This is in agreement with published data which also demonstrated similar up regulation of co-stimulatory and MHC-II molecules on murine dendritic cells (Bodnar, Serbina et al. 2001).

In addition to the acquisition of increased surface expression of activation markers, BMDCs also produced increased inflammatory cytokines in response to *M. tuberculosis* infection. IL-6 and TNF-α were induced at significantly high levels as early as 6 hours post-infection. The early production of IL-6 and TNF-α represents rapid induction of the synthesis followed an accumulation of these cytokines even at 72 hours post-infection. In contrast, IL-10 and IL-12p70 production peaked at 24 hours, with decreased production at later time points (Figure 3.5 and Figure 3.6, A) . The amount of IL-12p70 produced in response to infection is very low in comparison to IL-6, TNF-α and IL-12p40. This could be attributed to the increased IL-10 production observed at 24 hours post-infection (Figure 3.5). It has been previously shown that IL-12 production by dendritic cells could be augmented by the addition of neutralising anti-IL-10 antibodies in the presence of LPS (Corinti, Albanesi et al. 2001). Thus, it is likely that the increased levels of IL-10 between 6- 24 hours post-infection could be responsible for the low IL-12p70 production between 6-48 hours by *M. tuberculosis* infected BMDCs.

In conclusion, BMDCs are activated in response to *M. tuberculosis* infection as denoted by the up regulation of co-stimulatory molecules, MHC-II and increased inflammatory cytokine production.

Chapter 4

Profiling transcriptional responses of *M. tuberculosis* infected dendritic cells

4.1 Introduction

Using a global approach to study transcriptional responses, it was possible to have an overview of the gene expression profiles of *M. tuberculosis* infected dendritic cells. In recent years, a number of studies have been performed using microarrays which have allowed the profiling of genes, that are differentially regulated under experimental conditions and genes that are tightly regulated across different experimental conditions simultaneously (Chaussabel, Semnani et al. 2003; Chaussabel, Allman et al. 2005). The possibility of acquiring gene expression data across various parameters (e.g. different stimuli and time course analysis) for a vast number of genes in a single experiment is probably the major advantage of using microarrays.

Experiments described in the previous chapter demonstrated that BMDCs were activated in response to *M. tuberculosis* infection as, depicted by the increased level of co-stimulatory molecules (CD80, CD86), antigen presenting molecule (MHC-II) and augmented levels of cytokine production. The process of dendritic cell activation in response to a pathogen like *M. tuberculosis*, results in an array of co-ordinated gene expression patterns. In order to have a global understanding of these transcriptional events, a microarray based approach was used to profile genes that are differentially regulated in dendritic cells in response to *M. tuberculosis* infection.

The Affymetrix Mouse 430.2 GeneChip was used to profile gene expression of BMDCs in response to *M. tuberculosis* infection at a single time point (24 hours). The Mouse GeneChip 430.2 has over 39,000 transcripts on a single chip, so comprehending such vast amounts of data is complex. Thus a single time point analysis was chosen to act as a starting point for future work, in understanding the cohort of signalling pathways and gene expression patterns, which are evoked in response to *M. tuberculosis* infection of BMDCs. After evaluating the cytokine and dendritic cell activation profiles using a kinetic approach, 24 hours was chosen as a time point of optimum dendritic cell activation in response to *M. tuberculosis* infection (Figures 3.3, 3.4 and 3.6).

The microarray analysis method is described in detail in the Materials and Methods (section 2.4.6). Briefly, day 6 unsorted BMDCs (70±5% $CD11c^{+}$) were infected with H37Rv at a M.O.I of 10:1 and 24 hours post-infection, total RNA was extracted. Triplicate RNA samples from biological replicates were used for Affymetrix GeneChip labelling and hybridisation. The microarray data was processed with the GCRMA algorithm and differentially regulated genes were considered as those, which had a change in expression of at least 1.4 fold in *M. tuberculosis* infected samples, when compared to the control group (uninfected sample). Differentially regulated genes were statistically analysed using the 1-way ANOVA t-test, with a p value cut-off set at 0.05, in order to select genes with a high level of statistical significance. Differentially regulated genes were grouped into KEGG biological pathways using BioScript library 2.2, a plug-in feature of the GeneSpring software. The use of KEGG pathways allows for visualisation of gene clusters that are differentially regulated as part of various biological pathways.

4.2- An overview of the KEGG biological pathways for differentially expressed genes in response to *M. tuberculosis* infection of BMDCs

Differentially regulated genes were organised into KEGG biological pathways. There were 29 pathways which contained genes being differentially regulated in response to *M. tuberculosis* infection of BMDCs. Each pathway was designated a p value, which indicated the level of confidence that the pathway is part of the cellular response generated by BMDCs in response to infection. A list of the top 10 significant KEGG pathways is displayed in table 4.1 and a more complete list of all the KEGG pathways is in Appendix-IV. In response to infection, pathways which are components of the immune response (toll-like receptor, hematopoietic cell lineage and antigen processing and presentation pathways), signal transduction (JAK-STAT signalling and cytokine-cytokine receptor interaction pathways), cell adhesion and membrane transport (cell adhesion molecules, ABC transporters) and others (type I diabetes mellitus, type

II diabetes mellitus and adipocytokine signalling pathway) were among the most significant biological pathways, which contained genes displaying a change in expression in response to *M. tuberculosis* infection of BMDCs.

Genes overlapping with Pathways	p-value
Toll-like receptor signaling pathway	8.68E-12
Jak-STAT signaling pathway	1.17E-10
Cytokine-cytokine receptor interaction	2.06E-09
Type I diabetes mellitus	3.44E-06
Hematopoietic cell lineage	1.14E-04
Antigen processing and presentation	4.89E-04
Cell adhesion molecules (CAMs)	7.68E-04
Adipocytokine signaling pathway	1.01E-03
Type II diabetes mellitus	9.52E-03
ABC transporters	1.17E-02

Table 4.1 – List of the top 10 significant biological pathways identified from microarray analysis of *M. tuberculosis* infected BMDCs.

Genes which displayed differential regulation in response to *M. tuberculosis* infection were integrated within KEGG pathways. The p value indicates the statistical significance; the likelihood of the pathway being part of the cellular response generated by BMDCs in response to *M. tuberculosis* infection

4.3- Transcription patterns of *M. tuberculosis* infected BMDCs

In the previous section, an overview of the 10 most significantly represented biological pathways in response to *M. tuberculosis* infection was highlighted. The following sections examine in detail, patterns of toll-like receptor signalling pathway, JAK-STAT and cytokine-cytokine receptor pathway genes as these pathways are the 3 most significantly represented pathways identified from microarray analysis (Table 4.1). In addition, expression of phagocytosis and pattern recognition receptors in response to infection was also explored.

4.3.1- Gene expression patterns of phagocytosis and pattern recognition receptors

Dendritic cells as sentinels of the immune system circulate in the periphery and use an array of receptors (e.g TLRs) to recognise foreign antigens and utilise receptors like the c-type lectin receptors and complement receptors for phagocytosis of pathogens. GeneChip profiles of receptor expression involved in microbial pattern recognition and phagocytosis are shown in figure 4.1. In response to infection, BMDCs up regulated complement component 1, s subcomponent (C1s) and TLR 2 but interestingly phagocytic receptors like mannose receptor, C type 1 (Mrc1), C1qr1 (CD93) and CD209a (DC-SIGN) were down regulated. C1qr1 is a surface glycoprotein which acts as a phagocytic receptor on monocytes. The expression of C1qr1 is down regulated on mature human dendritic cells (Steinberger, Szekeres et al. 2002) and furthermore, the murine orthologue has been shown to play a role in the clearance of apoptotic cells in vivo (Norsworthy, Fossati-Jimack et al. 2004). The binding of c1q to antigen: antibody complex results in the activation of the serine protease, C1s (up-regulated by a factor of 3 fold) which aids in the initiation of the classical complement pathway.

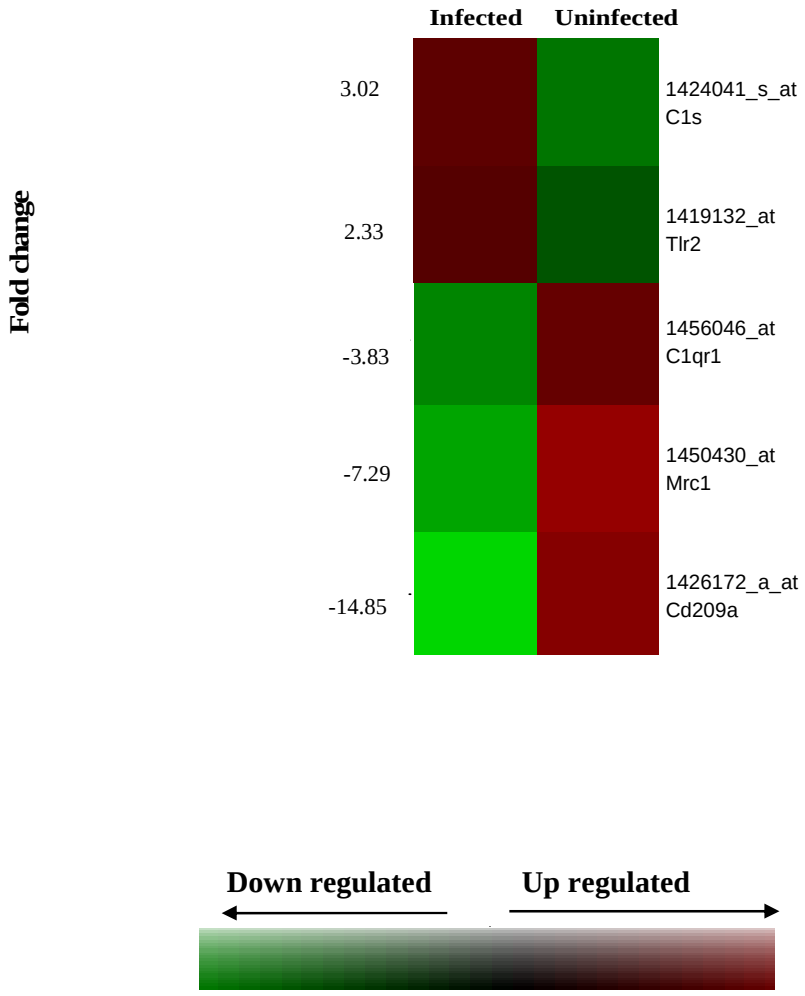


Figure 4.1 – Clustering of phagocytic and pattern recognition receptor genes differentially regulated in *M. tuberculosis* infected BMDCs.

Differentially regulated phagocytic and pattern recognition receptor genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection are displayed in the clustering diagram. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

4.3.2- Gene expression patterns of TLR signalling pathway genes in response to *M. tuberculosis* infection

Engagement of TLRs results in the induction of signalling pathways, which leads to the production of a strong inflammatory response. A number of genes differentially regulated in response to *M. tuberculosis* infection which are part of the TLR signalling pathway, also converge with other pathways like the JAK-STAT signalling pathway and cytokine receptor interaction pathway. This overlap of genes highlights the cross talk that takes place between signalling pathways, thus adding to the complexity of the analysis of data. In this section, only genes which are primarily part of the TLR signalling pathway have been clustered and displayed in figure 4.2. Components of the MyD88 independent signalling pathway, Tbk1 and Ikbkε are significantly up regulated in response to infection (up regulated by a factor >1.9 fold). phosphatidylinositol 3-kinase-p85 alpha (Pik3r1), a negative regulator of the TLR signalling pathway is up regulated in response to infection (up regulated by a factor of 2.5 fold), as shown in figure 4.2. TLR 4 is reported to recognise lipomannan and TLR 2 recognises LAM from mycobacterial cell walls (Quesniaux, Nicolle et al. 2004). Although TLR 4 was up regulated in response to infection, the fold change was not statistically significant and thus was excluded from the final gene list. However, TLR 2 was significantly up regulated by 2.3 fold in response to infection as shown in figure 4.2.

The up regulation of CD86 (downstream response of the TLR signalling pathway) at the mRNA level, (3.5 fold up regulation) is in good agreement with increased CD86 protein expression on the surface of *M. tuberculosis* infected BMDCs, (Figure 3.3, B) as measured using flow cytometry.

Figure 4.2– Clustering of differentially regulated TLR pathway genes.
GeneChip profiles of TLR pathway genes were identified using the GCRMA algorithm and normalised values for fold change in response to *M. tuberculosis* infection of unsorted BMDCs are displayed for each gene. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

4.3.3- JAK-STAT signalling pathway genes differentially regulated in *M. tuberculosis* infected BMDCs

The JAK-STAT signalling pathway has an important role in the control of immune responses, as it is a common signalling pathway used by an array of cytokines (e.g. IL-4, IFN-γ). As shown figure 4.3, differentially regulated genes that are part of the JAK-STAT pathway show high induction levels in response to infection. STAT-1, STAT-2, STAT-3 and SOCS 2 all display a fold change greater than 2. Mutations in the STAT 1 gene can enhance patient susceptibility to mycobacterial infections and this was attributed to inability to produce or respond to IFN-γ *in vitro* (Dupuis, Jouanguy et al. 2003). SOCS are negative regulators of the JAK-STAT signalling pathway and in response to mycobacterial infection; SOCS 2 was up regulated by a factor of 2 fold. Isgf3g (IRF-9), up regulated by a factor of 2.5 fold, is part of the complex comprised of Isgf3g –STAT 1-STAT 2. This complex binds to the interferon stimulated response element and initiates transcription of type I IFN genes.

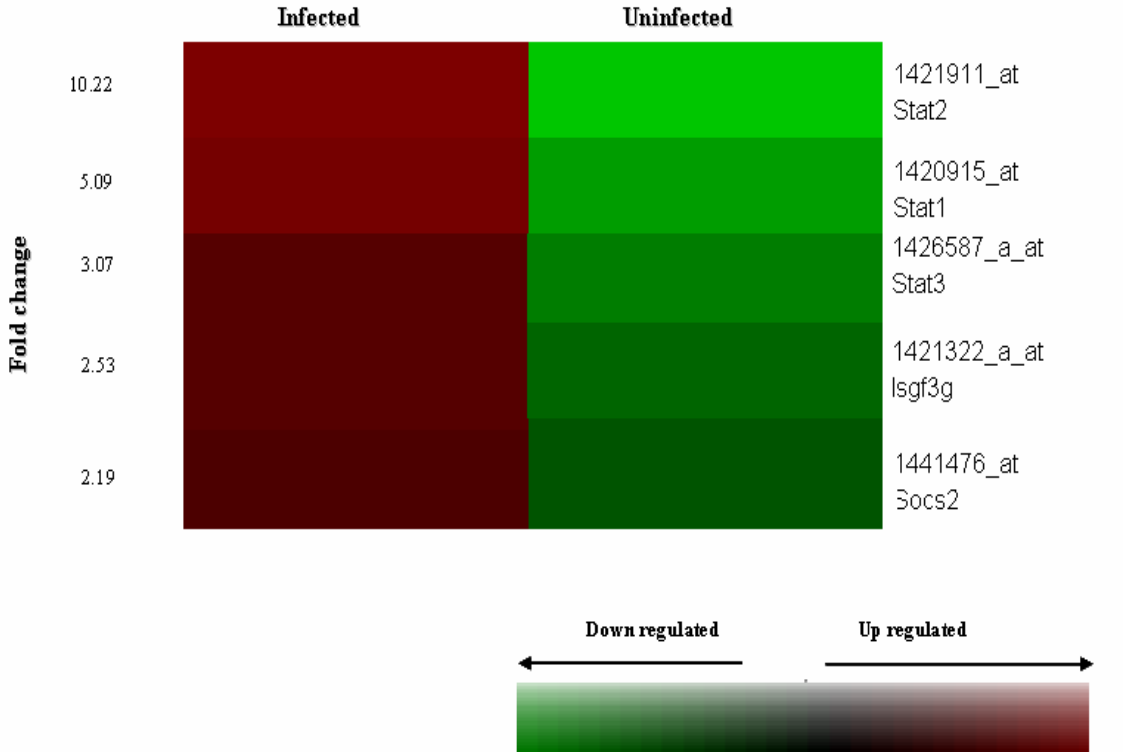


Figure 4.3 - Clustering of JAK-STAT signalling pathway genes regulated in *M. tuberculosis* infected BMDCs.

GeneChip profiles for JAK-STAT signalling pathway genes were identified using the GCRMA algorithm and normalised values for fold change in response to *M. tuberculosis* infection are displayed for each gene. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

4.3.4 Clustering of cytokine and chemokine genes differentially regulated in *M. tuberculosis* infected BMDCs

The induction of inflammatory cytokines is a key part of an efficient immune response and up regulation of chemokines, aids in the migration of dendritic cells out of the periphery into secondary lymphoid organs, and in the recruitment of immune cells to the sites of infection. The microarray results demonstrate that in response to *M. tuberculosis* infection, numerous cytokine and chemokine genes were differentially regulated.

As shown in the clustering diagram (Figure 4.4), inflammatory cytokines like IL-6, IL-12b (IL12p40) and TNF-α were up regulated by >6 fold in response to *M. tuberculosis* infection. TNF-α has been reported to play an important role in granuloma formation during mycobacterial infections (Roach, Bean et al. 2002) and IL-12p40 has been suggested to be involved in dendritic cell migration from the lungs to the draining lymph nodes (Khader, Partida-Sanchez et al. 2006). Two sub-families of chemokines including the C-C sub family of cytokines (CCL12, CCL4) up regulated >5 fold and the CXC family of cytokines (CXCL1, CXCL2, CXCL5, CXCL9, CXCL10) were up regulated >2 fold in response to infection. The chemokines CXCL9 and CXCL10 are ligands for the receptors CXCR3 and are involved in preferentially attracting $CXCR3^{+}$ T cells to the site of infection (Lande, Giacomini et al. 2003). Tgfbr2 (transforming growth factorβ, receptor II), the receptor for the cytokine transforming growth factor β was down regulated by 1.9 fold in response to infection.

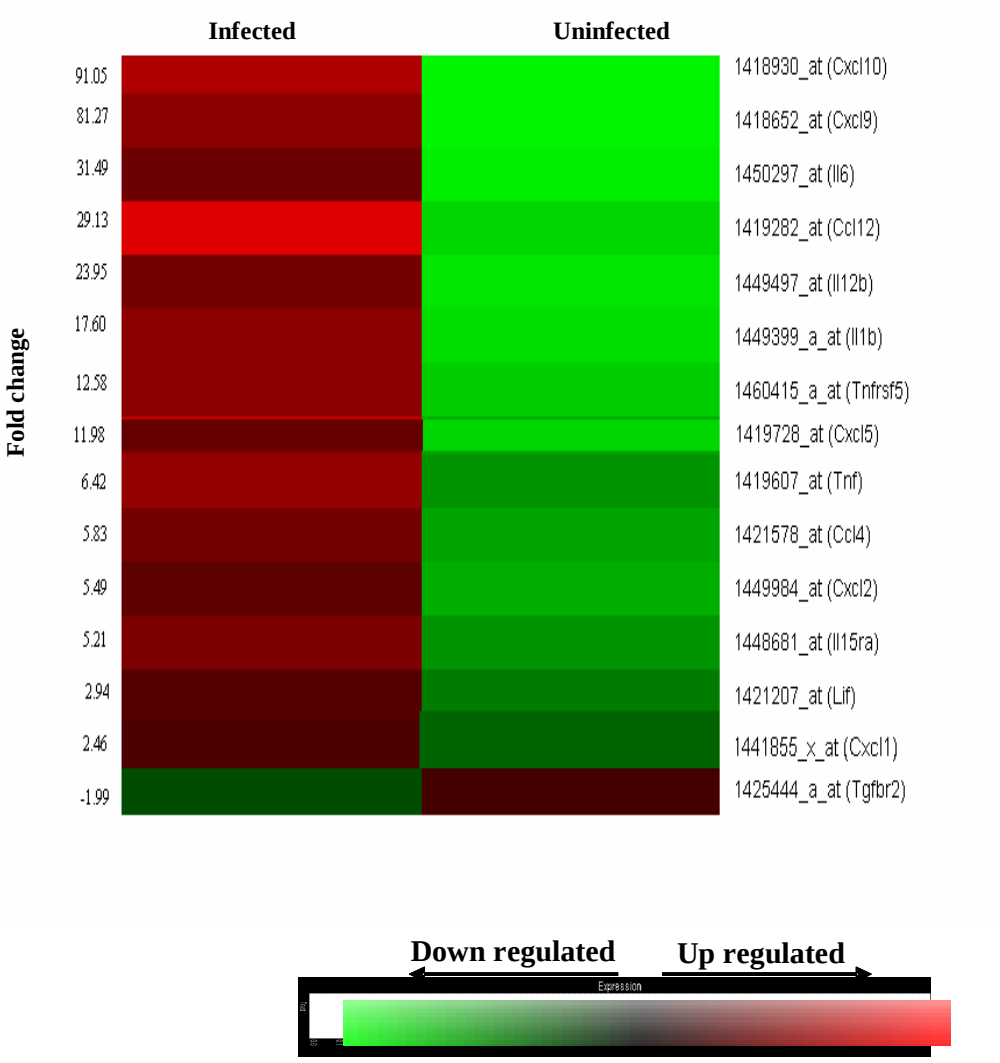


Figure 4.4 - Clustering of cytokine and chemokine genes regulated in *M. tuberculosis* infected BMDCs.

Differentially regulated cytokine and chemokine genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection are displayed in the clustering diagram. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

In conclusion, a range of immune response and signal transduction pathways are significantly represented in *M. tuberculosis* infected BMDCs. Expression of TLR 2 and cytokines (TNF, IL12b) with know roles in mycobacterial infections were significantly up regulated. In contrast, phagocytic receptors (Mrc1, CD209a) were down regulated in response to infection as shown in figure 4.1. An array of chemokines with roles in attracting immune cells to sites of infection e.g. CXCL2, CXCL5, CXCL9 and CXCL10 were highly up regulated (>5 fold) in response to infection, as shown in figure 4.4. Additionally, the co-stimulatory molecule CD86 was up regulated by 3.5 fold in response to infection. The differential expression of these genes highlights the roles played by dendritic cells as microbial sensors, immune modulators and T cell stimulators.

4.4-Validation of microarray results using real time PCR

Confirmation of microarray data was carried out using real time PCR. Day 6, unsorted (70±5% $CD11c^{+}$) BMDCs were infected with H37Rv (M.O.I 10:1) or left uninfected (control sample) and 24 hours post-infection, total RNA was extracted as described in the materials and methods (Chapter 2, section 2.4.1). cDNA was generated from the RNA samples for real time PCR and expression of selected genes were measured using the SYBR Green method (Chapter 2, section 2.5.1). All gene expression values were normalised to an endogenous control gene 18S rRNA. The relative fold change was calculated as the degree of up regulation or down regulation in the *M. tuberculosis* infected sample relative, to the uninfected sample.

CXCL9 and CXCL10 were the two most highly up regulated genes (up regulated by 81 and 91 fold respectively) obtained from the microarray analysis and thus their expression was confirmed using real-time PCR. IL-6, IL-10, IL-12p35, IL-12p40, TNF, TLR 2, TLR 4 and STAT-1 were chosen on the basis of their importance during mycobacterial infections (Reiling, Holscher et al. 2002; Sugawara, Yamada et al. 2004).

Microarray and real time PCR data are displayed for each of the genes as shown in figure 4.5. The gene expression patterns obtained using both microarrays and real time PCR displayed the same trend. Although microarray represents a useful tool for analysing global gene expression patters, some genes fail to be included in the final gene list due to the lack of statistical significance (TLR 4, IL-10) or they are detectable only in one experiment (IL12p35). But TLR 4 and IL-12p35, a sub-component of IL-12p70 (IL-12p35/IL-12p40) involved in the $CD4^+$ T cell response (Flynn, Goldstein et al. 1995) play important roles during mycobacterial infections. TLR4, IL-12p35 and IL-10 were induced at 24 hours post-infection, as shown in figure 4.5. Additionally, the induction of IL-6, TNF, IL-10, IL-12p70 and CXCL10 was quantified using ELISA (Figures 3.4, 3.5, 3.6 and 4.6). At 24 hours post-infection, increased levels of the cytokines and CXCL10 were detected at both the mRNA and protein level *M. tuberculosis* infected BMDCs compared to uninfected BMDCs.

Using real-time PCR, expression of key cytokines, chemokines and TLR genes were confirmed. Additionally, gene expression of IL-10, IL-12p35 and TLR 4 were quantified using real-time PCR, even though the expression of these genes was not detected using microarrays.

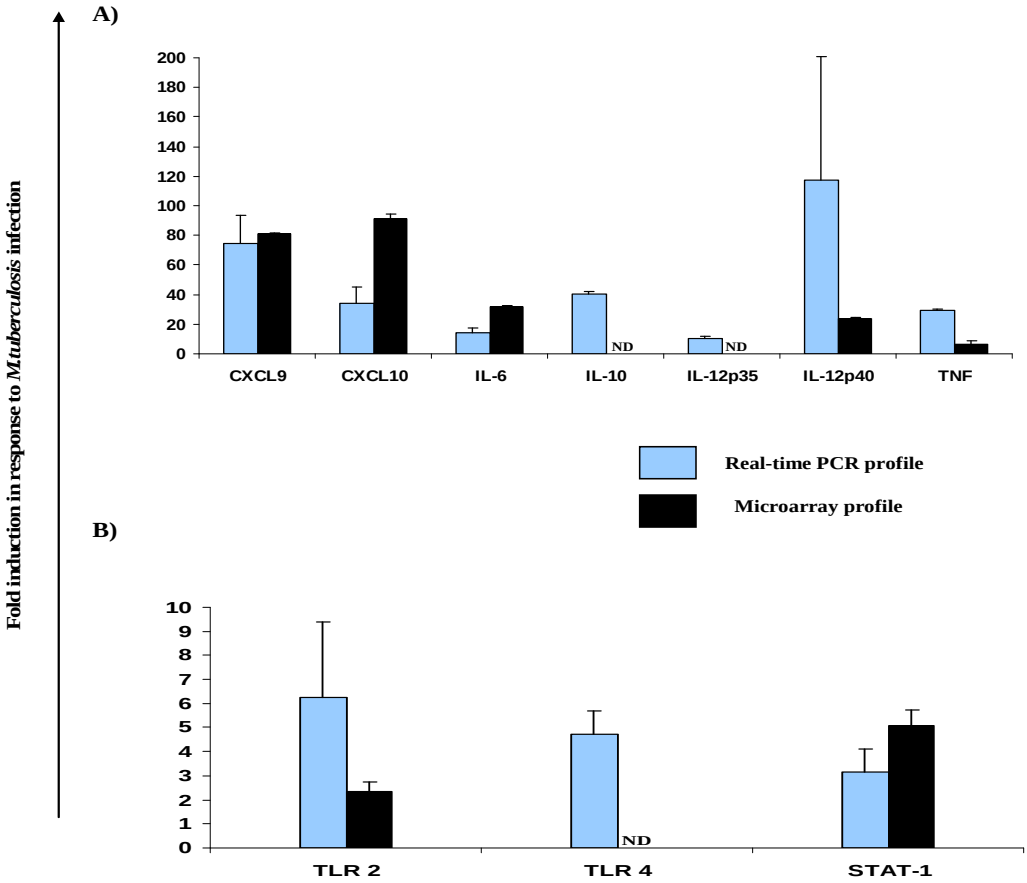


Figure 4.5 – Quantitative real time PCR and microarray gene expression profiles A) cytokines and chemokines B) Toll-like receptors and STAT-1 in *M. tuberculosis* infected BMDCs.

RNA was extracted from BMDCs 24 hour's post- infection, converted to cDNA and gene expression values as indicated were measured, by SYBR Green incorporation during PCR. For real time PCR, the expression of each gene was normalized to the internal control, 18S rRNA. Fold change for each gene in response to infection was calculated relative to the uninfected sample. Results are representative of 2 independent experiments. Fold change obtained using real-time PCR for each gene was compared with the fold induction values obtained using microarray anlaysis. ND- genes showed no statistically significant change in the microarray analysis.

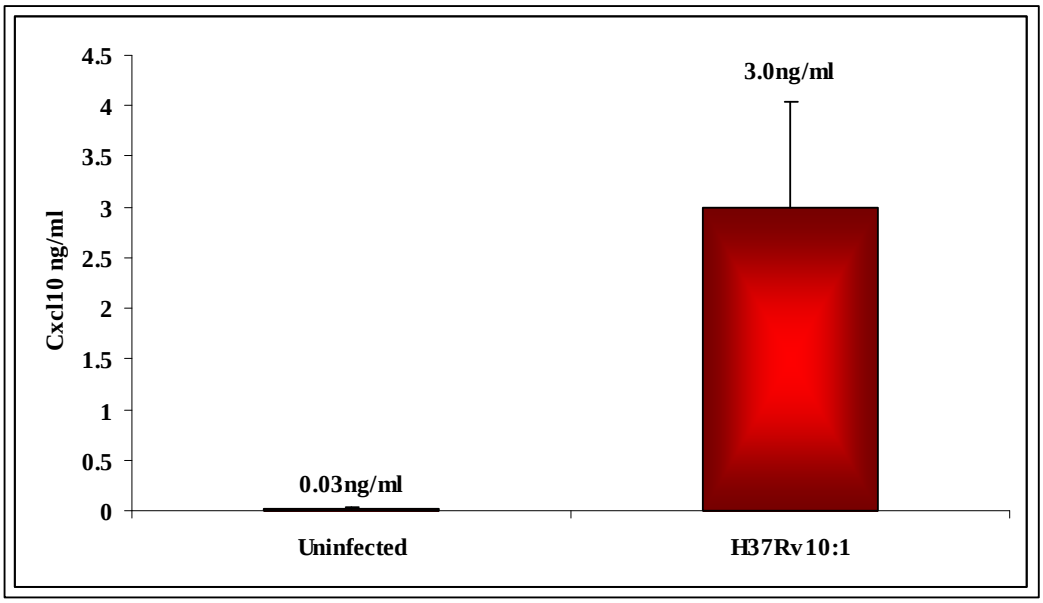


Figure 4.6- CXCL10 production by *M. tuberculosis* infected BMDCs

Day 6 unsorted BMDCs were either left uninfected or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at 24 hours and quantified by ELISA for the amount of CXCL10 produced. Data shown are the mean ±SD of duplicate determinations in one experiment, representative of three independent experiments.

4.5- *In silico* promoter analysis of differentially expressed genes

Transcriptional regulation of genes is mediated by transcription factor proteins that form protein-DNA or protein-protein interactions. Each transcription factor binds to its associated transcription factor binding sites, which are located near the gene usually in defined clusters. Using differential expression analysis, it was possible to identify transcription factor binding motifs, represented in the promoter of differentially regulated genes identified from the microarray data. The identification of transcription factors, contributed to the identification of signalling pathways that might be playing important roles, in modulating the variation in gene expression observed from the microarray data.

Genes differentially regulated in response to infection (fold change 1.4 and a p value ≤0.05) were analysed using the TELiS database in order to identify the transcription factor binding motifs that are represented in the promoters of differentially regulated genes, as described in materials and methods (chapter 2, section 2.6). Briefly, the following parameters were supplied to the TELiS database for the identification of transcription factor binding motifs:

- Sampling frame- Affymetrix Mouse Genome 430-2 array
- Gene list- Genes with a fold change ≥ 1.4 and a p value ≤ 0.05
- Promoter size- -1000 to +200 nucleotides
- Stringency of analysis- 0.90

The NFκB transcription factor binding motif was one of the most significantly represented motifs as shown in table 4.2. The NFκB family is comprised of multiple subunits and binding motifs for NFκB p65 and reticuloendotheliosis oncogene (CREL) were significantly represented in the promoters of genes that were differentially regulated in response to infection. This is not surprising, as the NFκB family of transcription factors are considered to be central regulators of the both the innate and adaptive immune response. NFκB proteins play key roles in dendritic cell development and survival (Ouaaz, Arron et al. 2002) and T cell proliferation (Gerondakis, Grumont et al. 1998).

Interestingly, in addition to NFκB, ISRE binding motif was also significantly represented in the promoters of genes differentially regulated in response to infection (Table 4.2). ISRE sequences are DNA binding motifs for IRFs which are known to be important regulators of the type I IFN response (Taniguchi, Ogasawara et al. 2001).

The other significant binding motif that was represented in the promoters was AP-1, which is a heterodimer formed by jun oncogene (c-jun) and FBJ osteosarcoma oncogene (c-fos) that plays a key role in the binding to the IFN beta promoter along with NFκB and IRF-3/7 and thus plays a role in IFN beta (type I IFN) induction (Wathelet, Lin et al. 1998). Other indentified motifs include CEBP binding sites, which are specific for CEBP (CCAAT/enhancer binding protein) family of transcription factors, with known functions in cell proliferation, cell cycle arrest and response to inflammatory stimuli (Lekstrom-Himes and Xanthopoulos 1998), ecotropic viral integration site 1 (EVI1), a transcription factor which has a role in cell cycle progression and myeloid differentiation and heat shock factor-1 (Hsf-1) binding motifs which bind the transcription factor Hsf-1 which is known to play a role in modulating NFκB responses .

In conclusion, IRFs and NFκB appear to be the principal transcription factors mediating gene changes in response to *M. tuberculosis* infection of BMDCs.

Transcription factors binding motifs	P- value	Z-test score
ISRE (Interferon stimulated response element)	$1.00x10^{-10}$	11.86
NFKB (Nuclear factor kappa B)	$1.00x10^{-10}$	5.42
NFKAPPAB65 (Nuclear factor kappa B- p65)	$1.48x10^{-5}$	4.33
CEBP_C (C/EBP binding site)	$3.86x10^{-5}$	4.12
CREL	$2.00x10^{-4}$	3.76
EVI1(Ectopic viral integration site 1 encoded factor)	$1.4x10^{-3}$	3.19
AP1_C (AP-1 binding site)	$3.70x10^{-3}$	2.91
HSF-1 (Heat shock factor 1)	$5.80x10^{-3}$	2.76
IRF-2 (Interferon regulatory factor-2)	$7.60x10^{-3}$	2.67
IRF-1 (Interferon regulatory factor-1)	$4.72x10^{-2}$	1.98

Table 4.2- Transcription factor binding motifs represented in *M. tuberculosis* infected BMDCs.

Significant transcription factor binding motifs prevalent in the promoters of differentially regulated genes in response to *M. tuberculosis* infection was identified using the bioinformatic database TELiS. The positive values obtained with the Z-test indicate an increased prevalence of the individual transcription factor binding motifs and the p value represents the level of significance.

4.6- Data mining of type I IFN inducible cluster of genes from microarray data

ISRE sequences were found to be significantly represented in the promoters of genes differentially regulated in response to infection, as shown by TELiS analysis. The microarray data was used to mine type I IFN inducible genes. Genes were identified as being type I IFN inducible using literature based evidence or if the genes have ISRE sequences in their promoter. The ISRE sequences were detected using the data retrieval function in TELiS which makes it possible to identify the prevalence of ISRE motifs in the promoters of the type I IFN cluster of genes.

There were 39 genes mined from the microarray data which could be classified as type I IFN inducible genes (Table 4.3). Identification of this cluster of type I IFN inducible genes was unanticipated as type I IFNs are associated with mediating anti-viral immunity and only recently, been documented in relation to intracellular bacterial infections (Smith, Lombardi et al. 2005).

Type I IFN inducible genes identified from the microarrays was clustered into 6 groups (Table 4.3). Genes involved in anti-viral immunity (e.g. Isg20,OAS1c,OAS2, Mx2), MHC-I antigen processing and presentation (H2-Q10, H2-Q8, H2-T10, H2-T24, Psme2, Tap1) and regulators of transcription (Adar, Ell2, Sp100) were all up regulated in response to *M. tuberculosis* infection. H2-T24, H2-T10, H2-Q8, H2-Q10 genes were identified by TELiS as having ISRE sequences in their promoters. These genes are predicted to have roles in antigen processing and presentation of peptides via MHC-I proteins, thus promoting the development of $CD8^+$ T cell responses. MHC-I synthesis has been previously reported in response to type I IFNs (Kuchtey, Chefalo et al. 2005).

Affymetrix ID	Fold change	Common name	Description	
1426278_at	6.14	2310061N23Rik	interferon, alpha-inducible protein 27	
1423555_a_at	90.56	A430056A10Rik	interferon-induced protein 44	
1418240_at	3.97	Gbp2	guanylate nucleotide binding protein 2	
1450783_at	84.62	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	
1418293_at	62.53	Ifit2	interferon-induced protein with tetratricopeptide repeats 2	
1419569_a_at	32.59	Isg20	interferon-stimulated protein 20	
1419676_at	11.78	Mx2	myxovirus (influenza virus) resistance 2	**Antiviral immunity**
1418686_at	5.48	Oas1c	2'-5' oligoadenylate synthetase 1C	
1425065_at	8.00	Oas2	2'-5' oligoadenylate synthetase 2	
1424339_at	56.58	Oasl1	2'-5' oligoadenylate synthetase-like 1	
1422005_at	5.34	Prkr	eukaryotic translation initiation factor 2-alpha kinase 2	
1418587_at	1.97	Traf3	Tnf receptor-associated factor 3	
1427736_a_at	5.84	Ccrl2	chemokine (C-C motif) receptor-like 2	
1418930_at	91.05	Cxcl10	chemokine (C-X-C motif) ligand 10	**Cytokines and chemokines**
1449497_at	23.95	Il12b	interleukin 12b	
1417244_a_at	65.58	Irf7	interferon regulatory factor 7	
1421322_a_at	2.53	Isgf3g	interferon dependent positive acting transcription factor 3 gamma	
1420915_at	5.01	Stat1	signal transducer and activator of transcription 1	**IRFs and Type I IFN pathway**
1421911_at	10.22	Stat2	signal transducer and activator of transcription 2	
1426324_at	1.79	H2-Q10	histocompatibility 2, D region locus 1	
1430802_at	5.85	H2-Q8	histocompatibility 2, Q region locus 8	
1449875_s_at	3.05	H2-T10	histocompatibility 2, T region locus 10	
1422160_at	6.47	H2-T24	histocompatibility 2, T region locus 24	**Antigen processing and presentation via MHC-I**
1417189_at	2.03	Psme2	proteasome (prosome, macropain) 28 subunit, beta	
1416016_at	2.90	Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	
1434268_at	2.58	Adar	adenosine deaminase, RNA-specific	
1450744_at	2.64	Ell2	elongation factor RNA polymerase II 2	
1451821_a_at	3.48	Sp100	nuclear antigen Sp100	**Regulation of transcription**
1425053_at	2.12	2610034N03Rik	isochorismatase domain containing 1	
1435446_a_at	2.67	Chpt1	choline phosphotransferase 1	
1451426_at	11.86	D11Lgp2e	DNA segment, Chr 11, Lothar Hennighausen 2, expressed	
1417314_at	50.13	H2-Bf	histocompatibility 2, complement component factor B	
1422433_s_at	-1.78	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	**Miscellenaeous**
1421207_at	2.94	Lif	leukemia inhibitory factor	
1452178_at	6.04	Plec1	plectin 1	
1434015_at	3.30	Slc2a6	solute carrier family 2 (facilitated glucose transporter), member 6	
1417961_a_at	8.49	Trim30	tripartite motif protein 30	
1421550_a_at	5.98	Trim34	tripartite motif protein 34	
1418191_at	7.15	Usp18	ubiquitin specific peptidase 18	

Table 4.3 – Profiling IFN inducible gene expression levels in *M. tuberculosis* infected BMDCs.

GeneChip profiles for type I IFN genes were calculated using the GCRMA algorithm and all genes displayed have a p value$\leq$ 0.05. Fold change indicates the degree of differential regulation in response to *M. tuberculosis* infection (positive value indicates up-regulation and negative value indicates down-regulation). Displayed values for each gene is an average of three biological replicates.

Another cluster includes the cytokines and chemokines (CXCL10, IL12b) and the type I IFN pathway genes (IRF-7, STAT-1, STAT-2 and Isgf3g). As shown in table 4.3, these genes are significantly up regulated in *M. tuberculosis* infected BMDCs. The expression of STAT-1, CXCL10 and IL-12b (IL12p40) was verified using real time PCR, as shown previously in figure 4.5.

From the list of genes, it is evident that an array of type I IFN pathway genes are highly up regulated (Table 4.3). A cartoon representation of the type I IFN pathway and the genes exhibiting differential regulation are illustrated in figure 4.7. The expression of IFN beta, IFN alpha, IRF-3 and type I IFN receptors (IFNAR1 and IFNAR2) were not significantly represented in the microarray data and thus not represented in figure 4.7.

Thus, the significant up regulation of type I IFN inducible genes in *M. tuberculosis* infected BMDCs, strongly indicates induction of the type I IFN pathway as part of the cellular response of BMDCs in response to infection.

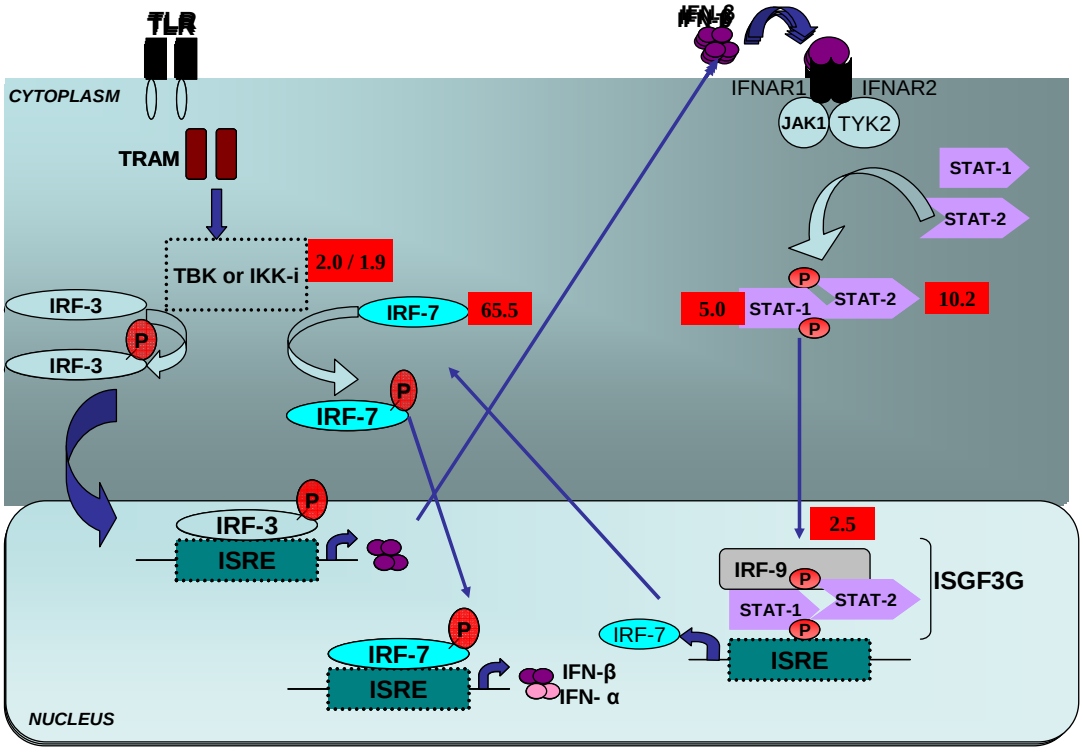


Figure 4.7 – Schematic representation of the type I IFN pathway depicting expression of type I IFN pathway genes induced in *M. tuberculosis* infected BMDCs

Expression of type I IFN pathway genes were extracted from microarray data. All the genes displayed have a fold change of ≥ 1.4 and a p value of ≤0.05.

- Up regulated genes

4.7-Validation of selected type I IFN pathway genes using real time PCR

Real time PCR was used for the validation of type I IFN pathway gene expression, in response to *M. tuberculosis* infection of BMDCs. IFNAR1, IFNAR2, IFN beta and IRF-3 were not significantly represented in the microarray data and hence the expression of these genes was quantified using real time PCR. The expression of IRF-7 was confirmed using real time PCR because IRF-7 is considered to be a "master regulator" of the type I IFN response induced during viral infections (Honda, Yanai et al. 2005) and IRF-7 was additionally, up regulated during *M. tuberculosis* infection of BMDCs (Table 4.3).

To extend the gene expression analysis, day 6 unsorted BMDCs were either left uninfected or infected with H37Rv at a M.O.I of 10:1 and RNA was isolated at 4 and 24 hours post infection. Using cDNA generated from 4 hours and 24 hours post-infection, it was possible to quantitatively assess the gene expression across two different time points. The time point 4 hours was chosen to represent an early time point of interferon beta induction (Remoli, Giacomini et al. 2002) and 24 hours correlated with the time point of microarray analysis.

The expression of IFNAR1, IFN beta, IRF-3 and IRF-7 was detected at 24 hours, in response to *M. tuberculosis* infection and in addition, the expression of IFNAR1, IFNAR2 and IRF-3 was down regulated at 4 hours in response to *M. tuberculosis* infection (Figure 4.8). Nevertheless, the expression IFN beta and IRF-7 increased over a period of time as shown in figure 4.8.

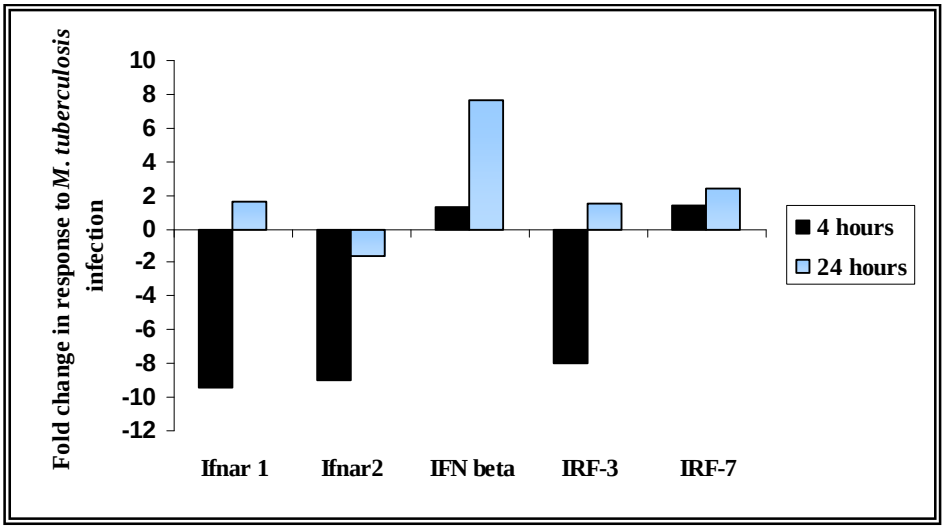


Figure 4.8 – Time course analysis of selected type I IFN pathway genes

RNA was extracted from unsorted BMDCs at 4 hours and 24 hours post-infection, converted to cDNA and real-time PCR was performed using the SYBR green detection method. Each gene was normalised to the internal control 18S rRNA. Fold induction of each gene, in response to infection, was calculated relative to its normalised value in the uninfected sample. Data is representative of 2 independent experiments.

4.8- Quantifying IFN beta levels in *M. tuberculosis* infected BMDCs

IFN beta is one of the type I IFNs and is a by-product of the type I IFN pathway. This cytokine also plays a key role in the induction of the feedback loop mechanism, leading to the amplification of type I IFN responses (Stark, Kerr et al. 1998). Day 6 unsorted BMDCs, were either stimulated with LPS (1μg/ml) or infected with H37Rv at a M.O.I of 10:1 and at various time points post-infection supernatants were collected and the amount of IFN beta was measured using ELISA.

As shown in figure 4.9, IFN beta is secreted from BMDCs in response to *M. tuberculosis* infection, albeit at lower levels compared to LPS stimulation, between 6-72 hours. The amount of IFN beta produced in response to infection peaks at 24 hours (60 ±7.3 pg/ml) and then slightly decreases at later time points post-infection, whereas the production of IFN beta was detected as early as 6 hours (545 ±1.3 pg/ml) in response to LPS stimulation. In contrast, significant production of IFN beta was not detected until 24 hours, in response to *M. tuberculosis* infection.

At 24 hours post-infection, there is a clear correlation between the secretion of IFN beta (Figure 4.9) and up regulation of its transcript (Figure 4.8).

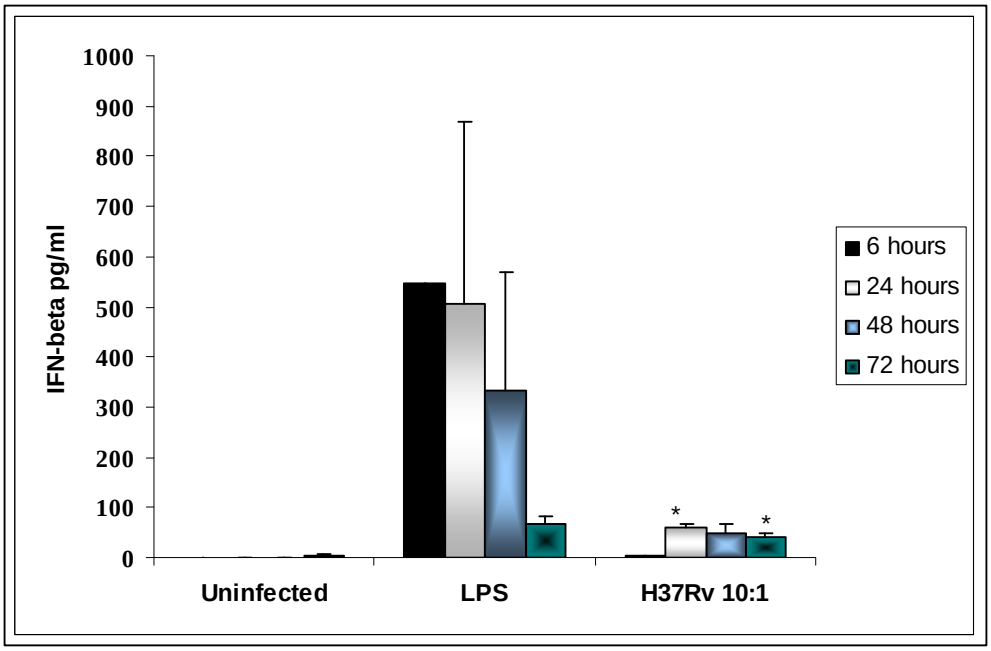


Figure 4.9 – Quantifying IFN beta secretion in the supernatants of *M. tuberculosis* infected BMDCs

BMDCs were cultured *in vitro* and day 6 unsorted cells were either left uninfected, stimulated with LPS or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at various time points post-infection for the quantification of IFN beta using ELISA. Results are the mean of ±SE of 3 independent experiments. Statistical analysis was preformed between *M. tuberculosis* infected group and uninfected group with the Student's t-test, *- p value <0.05.

4.9- RT profiler PCR expression profiles of type I IFN inducible genes in CD11c sorted BMDCs

To extend the analysis and obtain a more comprehensive list of type I IFN inducible genes, real time PCR was carried out as described in the materials and methods (section 2.5.2), using the RT profiler PCR array on a population of dendritic cells that was 95± 3% $CD11c^{+}$. This process also allowed for further validation of the type I IFN inducible genes in a sorted population of dendritic cells, thus excluding the possible influence of contaminating cell types.

4.9.1- Expression of type I IFN inducible genes in *M. tuberculosis* infected BMDCs (CD11c sorted)

BMDCs were sorted using CD11c magnetic beads and either left uninfected or H37Rv infected at a M.O.I of 10:1. RNA was isolated 24 hours post-infection and real time PCR was carried out the SYBR green method utilising the RT profiler PCR array system. All genes were normalised to the house keeping gene, GAPDH.

IFN inducible genes were divided into 4 gene clusters and the gene expression profiles are displayed in figures 4.10 and 4.11. Interferons and interferon related genes (Figure 4.10) were up regulated in response to *M. tuberculosis* infection. Exceptions to this statement include IL-4, IFN alpha 7 (Ifna7) and IFN alpha 11 (Ifna11), which were down regulated in response to infection. Multiple sub-types of IFN-alpha (ifna2, ifna4, ifna9) were up regulated in response to infection but this cytokine was not detected in the supernatants of infected BMDCs using ELISA. The role of all the different IFN alpha sub-types in dendritic cells is not yet fully understood. However, IFNa2 is known to induce CXCL9 and CXCL10 production from monocyte derived dendritic cells (Padovan, Spagnoli et al. 2002). Both of these chemokines were significantly up regulated in response to *M. tuberculosis* infection in BMDCs (Figure 4.4).

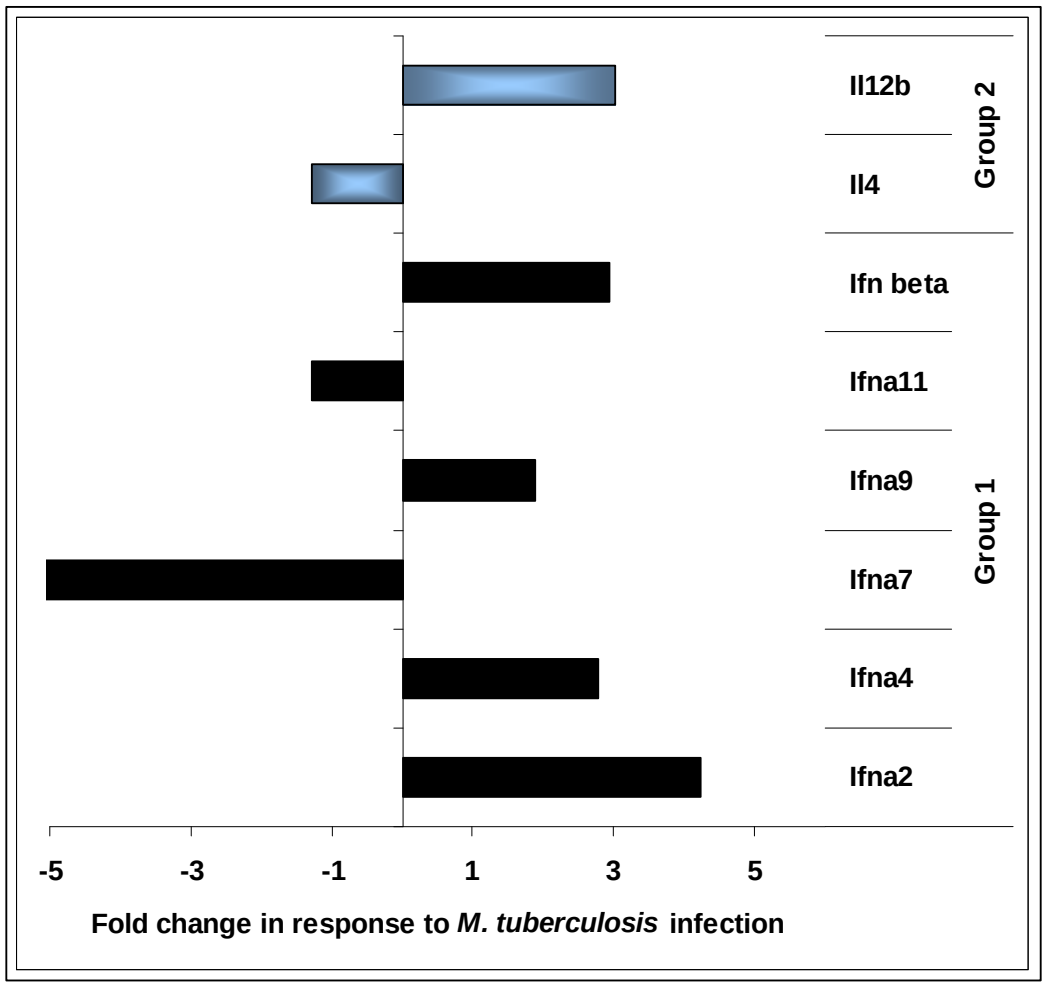


Figure 4.10- Real-time PCR expression profiles of IFN genes in *M. tuberculosis* infected BMDCs.

RNA was isolated from CD11c sorted uninfected BMDCS and *M. tuberculosis* infected BMDCs, converted to cDNA and real-time PCR was performed using RT profiler PCR array. Expression of individual interferon genes were normalised to the house keeping gene GAPDH. Fold change of a gene in response to *M. tuberculosis* infection relative to the uninfected sample is displayed. Genes were grouped into functional clusters: Group 1- type I Interferons, Group 2- Interferon related genes

The expression of interferon regulatory factor family (group 3) and IFN inducible genes (group 4) were also assayed and most of the genes from these clusters were up regulated in response to infection. Cellular immune response genes Ifit1, Ifit2 and G1p2 are significantly up regulated in response to infection. All the assayed IRFs were up regulated in response to infection with the exception of IRF-2 and IRF2bp2. IRF-2, described as a negative regulator of many type I IFN responsive genes is down regulated in response to infection. The co-repressors of IRF-2: Irf2bp1 and Irf2bp2 show contrasting levels of expression. It has been demonstrated that binding of Irf2bp1 (up regulated by 1.08 fold) is sufficient for minimal IRF-2 repression (Childs and Goodbourn 2003).

Quantification of type I IFN inducible genes using real-time PCR in CD11c sorted BMDCs, also made it possible to confirm the microarray analysis carried out using unsorted BMDCs (Figure 4.12). This provided an additional level of confirmation, of the IFN signature, that is observed in response to *M. tuberculosis* infection. Gene expression data obtained for CXCL10, Ifi44, Ifit1, Ifit2, IL12b (IL12p40) and IRF-7 was similar from both microarray analysis and real-time PCR (from CD11c sorted BMDCs). Although, the fold change values obtained using microarrays were much higher than that obtained using real time PCR (Figure 4.12).

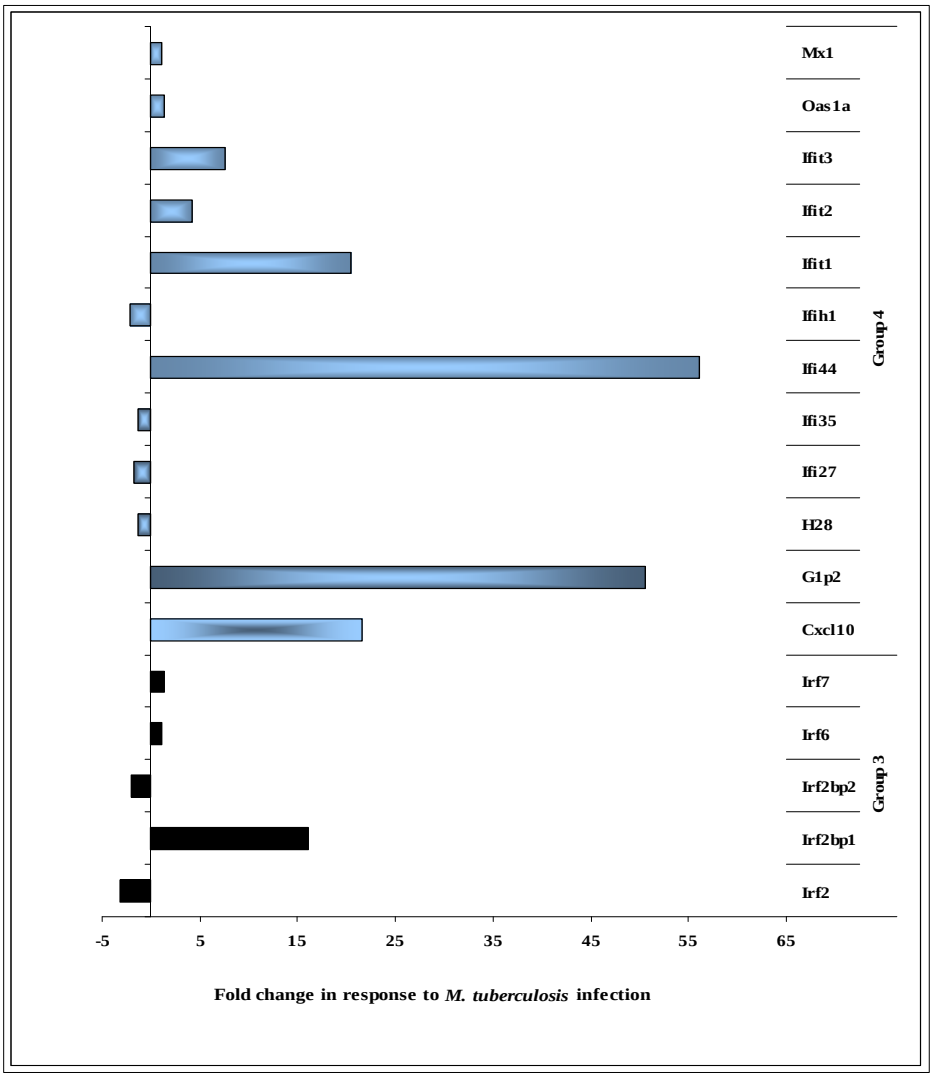


Figure 4.11- Real time PCR expression profiles of IFN induced genes in *M. tuberculosis* infected BMDCs.

RNA was isolated from CD11c sorted uninfected BMDCS and *M. tuberculosis* infected BMDCs, converted to cDNA and real-time PCR was performed using RT profiler PCR array. Expression levels of individual interferon genes were normalised to the house keeping gene GAPDH. Fold change of a gene in response to *M. tuberculosis* infection relative to the uninfected sample is displayed. Genes were grouped into functional clusters: Group 3- Interferon regulatory factors, Group 4- Interferon inducible genes.

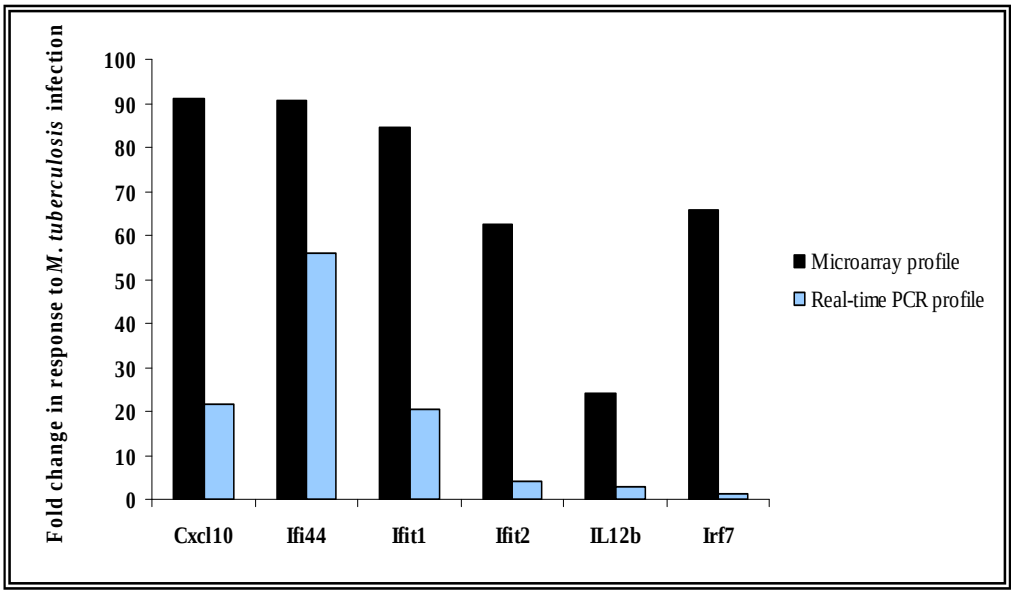


Figure 4.12 – Quantitative real-time PCR and microarray gene expression profiles of *M. tuberculosis* infected BMDCs.

RNA was extracted from CD11c sorted BMDCs 24 hour's post- infection, converted to cDNA and gene expression values as indicated were measured, by SYBR Green incorporation during PCR. For real time PCR, the expression of each gene was normalized to the internal control, GAPDH. Results obtained using real time PCR for each gene was compared with the fold induction values obtained using microarray analysis.

4.10- Discussion

Using high density oligonucleotide arrays, genes differentially expressed in response to *M. tuberculosis* infection of BMDCs, were identified. This global approach identified 522 genes that were up regulated and 163 genes which were down regulated in *M. tuberculosis* infected BMDCs. A complete list of the differentially regulated genes, in response to *M. tuberculosis* infection, is listed in Appendix- VII. These differentially regulated genes were, in large part, related to regulation of immune responses, signal transduction, cell adhesion and membrane transport. Specifically, the TLR signalling, JAK-STAT signalling and cytokine-cytokine receptor interaction pathways, were among the most significantly represented cellular response pathways in *M. tuberculosis* infected BMDCs.

Gene expression profiling revealed that BMDCs down regulated phagocytic receptors (mannose receptor and CD209a). CD209a is reported to be involved in the recognition of ManLAM from virulent mycobacteria leading to the increased production of IL-10. Thus, it is believed that engagement of this receptor could result in interfering with dendritic cell based host immune response thus enhancing the survival of the pathogen (van Kooyk and Geijtenbeek 2003). It seems contradictory that dendritic cells, which are efficient in taking up antigens, would down regulate phagocytic receptors and thus their phagocytic capacity in response to a pathogen like *M. tuberculosis*. This phenomenon is possibly explained by the fact, that when dendritic cells are activated by TLR ligands, there is a short burst in the uptake of antigens, which is followed by a long term down regulation of endocytic capacity, which coincides with maturation of dendritic cells (West, Wallin et al. 2004). Activated dendritic cells become efficient antigen presenting cells as opposed to effective phagocytic cells.

M. tuberculosis infection of BMDCs induced the expression of a large number of cytokine and chemokine genes. CXCL9 (up regulated 81.27 fold) and CXCL10 (up regulated 91.05 fold) are ligands for the receptor CXCR3 and are involved in preferentially attracting $CXCR3^+$ T cells to the site of infection (Lande, Giacomini et al. 2003). It has been previously shown that CXCL5, CXCL2 and CCL4 were more prominent in the lungs of mice challenged with *M. bovis* PPD beads. It is possible that secretion of chemokines CXCL2 (up regulated

by 5.4 fold) and CXCL5 (up regulated by 11.9 fold), which are known neutrophil chemoattractants, preferentially attract neutrophils to the site of infection (Chiu, Freeman et al. 2004).

Mature dendritic cells up regulate co-stimulatory molecules Tnfrsf5 (up regulated 12.5 fold) and CD86 (up regulated 3.5 fold), thus performing a critical role of providing co-stimulatory signals for the activation of T cells. Induction of inflammatory cytokines, like TNF-α (up regulated 6.4 fold) required for the integrity of the granuloma during mycobacterial infection and IL-12 (up regulated by a factor of 23.95 fold), which has been associated with the development of host Th-1 immunity and production of IFN-γ, represents a key part of the host immune response to *M. tuberculosis* infection. The up regulation of cytokines has also been substantiated at the protein level. In response to infection, there was increased production of both IL-12 and TNF-α at the protein level by BMDCs (Figures 3.4 and 3.6). The down regulation of phagocytic receptors and increased inflammatory cytokine production (both at the mRNA and protein levels), implies that BMDCs are activated in response to infection. This is accordance with previous reports that demonstrate activation of human dendritic cells in response to *M. tuberculosis* infection (Henderson, Watkins et al. 1997).

To extend the microarray analysis, a bioinformatics approach was used (TELiS), to identify the transcription factors regulating the changes in gene expression profiles. Binding motifs members of the NFκB family of transcription factors (NFκB p65, CREL), one of the most well documented transcription factors, which plays multiple roles in innate immune responses were significantly over represented in the promoters. TELiS also identified the transcription factor binding motif ISRE, which is a DNA binding motif for the IRF family of transcription factors. IRFs are known to have a principal role in the induction of type I IFN genes (Honda and Taniguchi 2006). Significant representation of NFκB and IRF binding motifs, suggests that these two transcription factors play a key role in regulating gene expression *in M. tuberculosis* infected BMDCs.

Since it was demonstrated that IRF binding sites were significantly represented in the promoters of differentially regulated genes, this was used as the

basis for the subsequent mining of type I IFN inducible genes from the microarray data. The function of type I IFNs has been well documented during viral infection but increasingly it has become clear that type I IFNs play a role in bacterial infections (Stetson and Medzhitov 2006). *M. tuberculosis* infection of human dendritic cells resulted in induction of early IFN beta production followed by a delayed IFN alpha production (Remoli, Giacomini et al. 2002). An array of type I IFN inducible genes and genes which are components of the type I IFN pathway including IRF-7, STAT-1, STAT-2 and Isgf3g, were significantly up regulated in response to *M. tuberculosis* infection of BMDCs. IRF-7, known as the "master regulator of the type I IFN response" (Honda, Yanai et al. 2005) and the components of the ISGF3 complex (STAT-1, STAT-2, Isgf3g) which is responsible for initiating the transcription of IFN inducible genes were all up regulated in *M. tuberculosis* infected BMDCs.

Although the expression of IFN alpha and IFN beta was confirmed using real time PCR (Figure 4.10) in *M. tuberculosis* infected BMDCs, only IFN beta was detected at the protein level with maximum production at 24 hours post infection (Figure 4.9). The differential expression of IFNAR possibly represents a mechanism by which dendritic cells modulate their responsiveness to different sub-type of type I IFNs (Severa, Remoli et al. 2006). The observed down regulation of these receptors at early time points is linked with their maturation status, as it has been previously that LPS matured human dendritic cells, down regulate IFNAR1 and IFNAR2 as early as 4 hours post-stimulation (Gauzzi, Canini et al. 2002).

Similarly, the effect of down regulation of IRF-3 at 4 hours post-infection and then the up regulation at 24 hours (Figure 4.8) on dendritic cell responses in unclear. IRF-3 is thought to be mainly responsible for the initial induction of the IFN beta gene, whereas IRF-7 is involved in the late phase of IFN induction. In this study, down regulation of IRF-3 at 4 hours post-infection has no effect on the induction of IFN beta, which is up regulated by 4 hours in *M. tuberculosis*, infected BMDCs (Figure 4.8).

The role of type I IFNs during mycobacterial infection remains unresolved. Previous studies have shown that hypervirulent, Beijing strains of mycobacteria, produce high levels of type I IFNs, which was associated with the failure to stimulate Th-1 type immunity (Manca, Tsenova et al. 2001). It has also been demonstrated that, IFN (alpha and beta) receptor knock-out mice have less bacterial loads in the spleens compared to wild-type mice and thus, type I IFNs was suggested to have a role in promoting mycobacterial growth (Stanley, Johndrow et al. 2007). In contrast, other studies have shown that type I IFNs may facilitate a protective inflammatory response as it is required for the expression of CXCL10, a known activator of NK and T cell recruitment (Lande, Giacomini et al. 2003). The up regulation of an array of type I IFN inducible genes in this study, strongly indicates the induction of the type I IFN pathway, as an integral part of the dendritic cell based response to *M. tuberculosis*.

Neutrophil specific genes including neutrophil elastase 2, myeloperoxidase, neutrophil specific defensin and neutrophil cytosolic factor 2 showed no significant change in expression in the microarray dataset in response to *M. tuberculosis* infection. In addition, key observations with the type I IFN family of genes were confirmed using $CD11c^{+}$ sorted cells and thus it can be concluded, that the observed gene expression changes are a consequence of dendritic cell based responses to *M. tuberculosis* infection.

In figure 4.13, a summary of the key responses of *M. tuberculosis* infected BMDCs is illustrated. As shown by microarray, real time PCR and bioinformatic analysis, the type I IFN pathway and IFN inducible genes were significantly induced by BMDCs in response to infection. Overall, the transcriptome of dendritic cells reveal a shift towards and inflammation and immune related functions in response to *M. tuberculosis* infection.

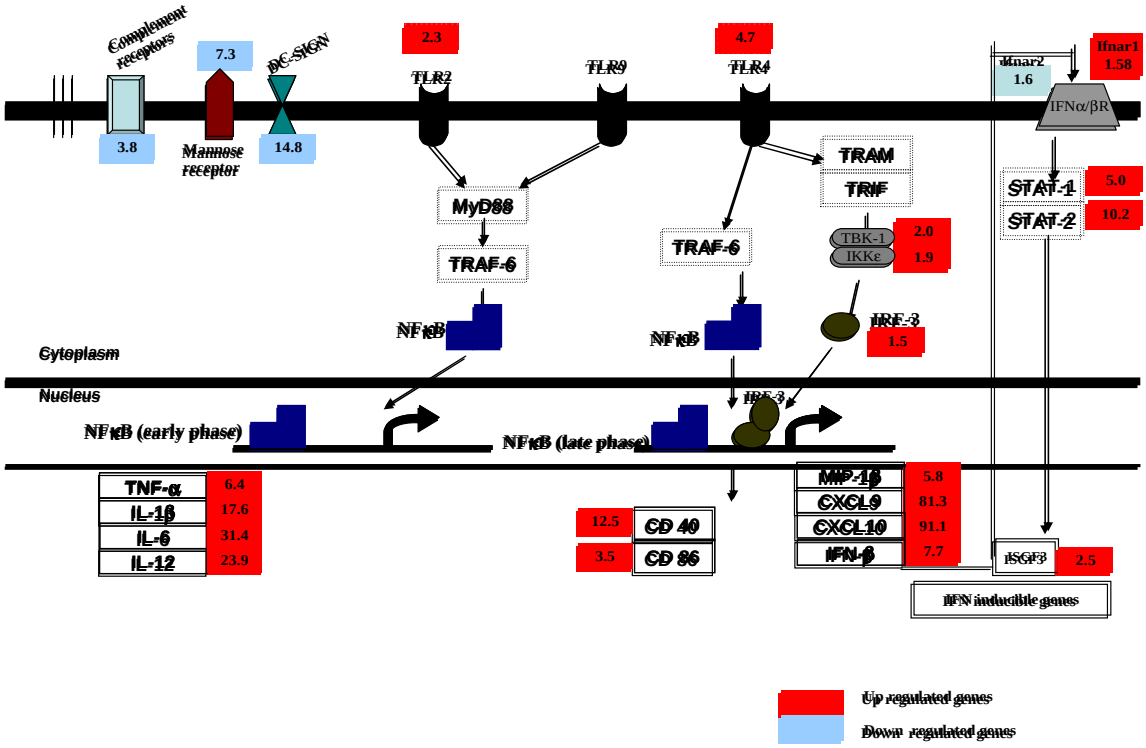


Figure 4.13- Overall schematics of differentially expressed immune response and signal transduction genes in response to *M. tuberculosis* infection (24 hours post-infection)

Data was mined from microarray and real-time PCR data.

Chapter 5

Profiling transcriptional responses of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

5.1- Introduction

In response to *M. tuberculosis* infection, an array of different signalling pathways which incorporated TLR signalling pathway and type I IFN pathway were differentially regulated (as described in chapter 4). Most TLRs signal through MyD88, a critical adaptor in the TLR signalling pathway but TLR4 is also able to signal via a MyD88 independent pathway utilising additional adaptors TRAM and TRIF. In addition, LPS stimulation of MyD88 deficient macrophages has identified a number of IFN inducible genes like IP-10 and GARG16 which are induced in a MyD88 independent manner (Kawai, Takeuchi et al. 2001). We hypothesised that *M. tuberculosis* infection could similarly induce the production of type I IFNs and IFN inducible genes independent of MyD88 in dendritic cells.

To test the hypothesis, microarrays were used to analyse the expression of genes that were differentially induced in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs. MyD88$^{-/-}$ mice possess a disrupted MyD88 gene (Adachi, Kawai et al. 1998). Bone marrow from these mice was used as the source of dendritic cells, to study the MyD88 independent induction of type I IFNs, in response to *M. tuberculosis* infection. Day 6 unsorted MyD88$^{-/-}$ BMDCs were infected with H37Rv (M.O.I 10:1) and 24 hours later, RNA was extracted as described in the material and methods (section 2.4.1). The uninfected BMDC group served as the control for the experiments. The analysis of the microarray data was carried out as previously described for the analysis of the wild-type BMDCs microarray data (chapter 4, section 4.1).

In this chapter, expression of co-stimulatory molecules and production of inflammatory cytokines by MyD88$^{-/-}$ BMDCs in response to infection are described first, followed by the gene expression profiles of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs, as determined by microarray analysis.

5.2- Expression of co-stimulatory molecules and MHC-II by MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection

BMDCs were generated in the presence of GM-CSF as described in the materials and methods (section 2.3.1). BMDCs were gated on FSC vs SSC and were 68 ±6% CD11c^{+}. As shown in figure 5.1, BMDCs generated from MyD88$^{-/-}$ mice expressed 74.42 ±4% CD80, 36.54 ±4% CD86 and 10.50 ±3% MHC-II in the absence of microbial stimulation. Two populations of BMDCs are observed at day 6, the first population which is CD80^{low} CD86^{low} and the second population which is CD80 $^{mid/high}$ CD86 $^{mid/high}$,as depicted in the histograms (Figures 5.1, A and B). The basal expression of MHC-II was lower in MyD88$^{-/-}$ BMDCs compared to wild-type mice (Figure 5.1 and Figure 3.1). Thus, BMDCs from MyD88$^{-/-}$ mice displayed an immature phenotype, with low expression levels of co-stimulatory and MHC-II molecules.

In order to determine whether these immature dendritic cells are capable of being activated in response to *M. tuberculosis* infection, day 6 BMDCs were either left uninfected (negative control), stimulated with LPS (positive control) or infected with H37Rv (M.O.I 10:1) and 24 hours later, the up regulation of activation markers was measured using flow cytometry. In response to infection BMDCs up regulated the expression of CD80 to 83.09 ±1.76%, CD86 to 81.12 ±1.71% and MHC-II to 74.15 ±11.4% and a similar degree of up regulation was observed in response to LPS stimulation (Figure 5.2, A, B and C). The expression of CD80, CD86 and MHC-II observed in MyD88$^{-/-}$ BMDCs was similar to wild-type BMDCs in response to *M. tuberculosis* infection (Figure 5.2, A, B, C and Figure 3.3, A, B, C).

Hence, MyD88$^{-/-}$ dendritic cells are activated in response to *M. tuberculosis* infection, with the degree of activation similar to that observed in wild-type BMDCs.

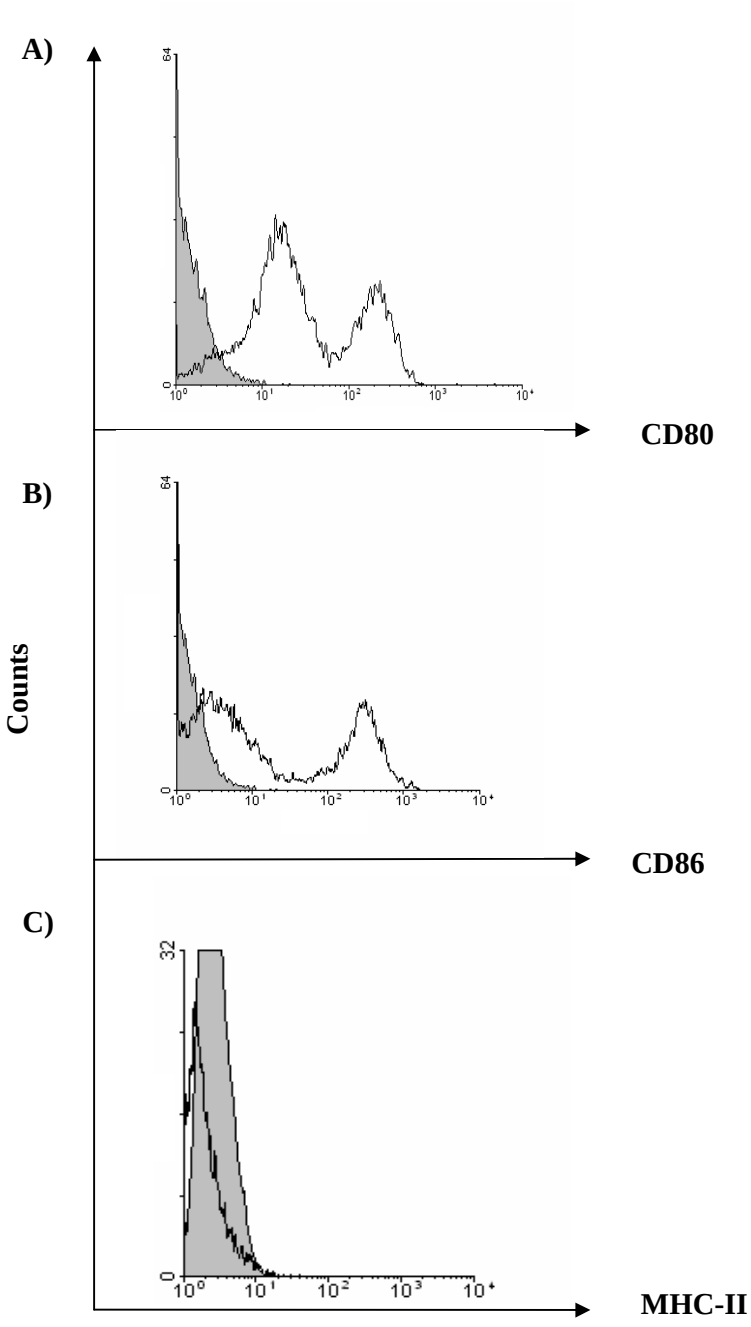


Figure 5.1- Expression of CD80, CD86 and MHC-II on MyD88$^{-/-}$ BMDCs

BMDCs were generated from MyD88$^{-/-}$ bone marrow cells by culturing in medium containing GM-CSF for 6 days. The expression of A) CD11c CD80 B) CD11c CD86 and C) CD11c MHC-II were analysed using flow cytometry. Data is representative of 3 independent experiments.

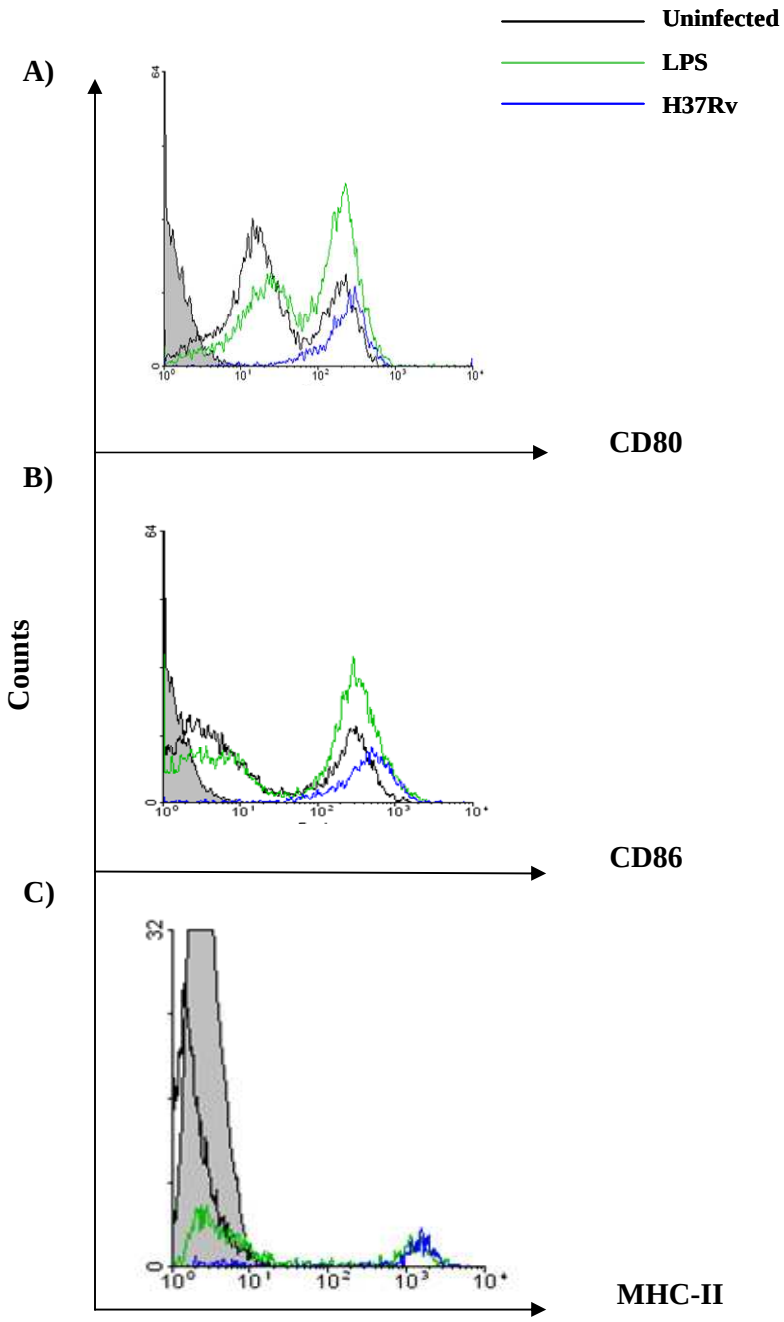


Figure 5.2- Up regulation of co-stimulatory and MHC-II by MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection.

Day 6 BMDCs were either LPS stimulated (1µg/ml), infected with H37Rv (M.O.I 10:1) or left uninfected and the expression of A) CD80 B) CD86 and C)MHC-II were assessed 24 hours later, using flow cytometry. The gray filled histograms represent isotype control staining. Data is representative of 3 independent experiments.

5.3- Analysis of inflammatory cytokine production by MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection

In order to evaluate the production of inflammatory cytokines by MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection, day 6 BMDCs were uninfected, stimulated with LPS (positive control) or infected with H37Rv 10:1 and supernatants were collected at various time points post-infection (6-72 hours).

5.3.1- Impaired production of TNF-α and IL-6 by MyD88$^{-/-}$ *M. tuberculosis* infected BMDCs

M. tuberculosis infection of MyD88$^{-/-}$ BMDCs induced production of cytokines (IL-6 and TNF-α) as shown in figure 5.3. However, cytokine production was severely impaired in response to infection of MyD88$^{-/-}$ BMDCs in comparison to wild-type BMDCs (Figure 3.4). TNF-α production by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs increased over time and subsequently decreased by 72 hours post infection (Figure 5.3, A). A similar trend was observed for IL-6 production, in response to infection and LPS stimulation (Figure 5.3, B). Uninfected MyD88$^{-/-}$ BMDCs produced negligible amounts of TNF-α and IL-6.

Interestingly, the production of TNF-α and IL-6 was delayed in MyD88$^{-/-}$ BMDCs with a maximum production at 48 hours post-infection (Figure 5.3); in comparison, production of these cytokines from wild-type BMDCs were detected by 6 hours, in response to *M. tuberculosis* infection (Figure 3.4). In addition, at all the time points examined there was considerably higher production of TNF-α and IL-6 by wild-type BMDCs infected with *M. tuberculosis* in comparison to MyD88$^{-/-}$ BMDCs (Figures 3.4 and 5.3).

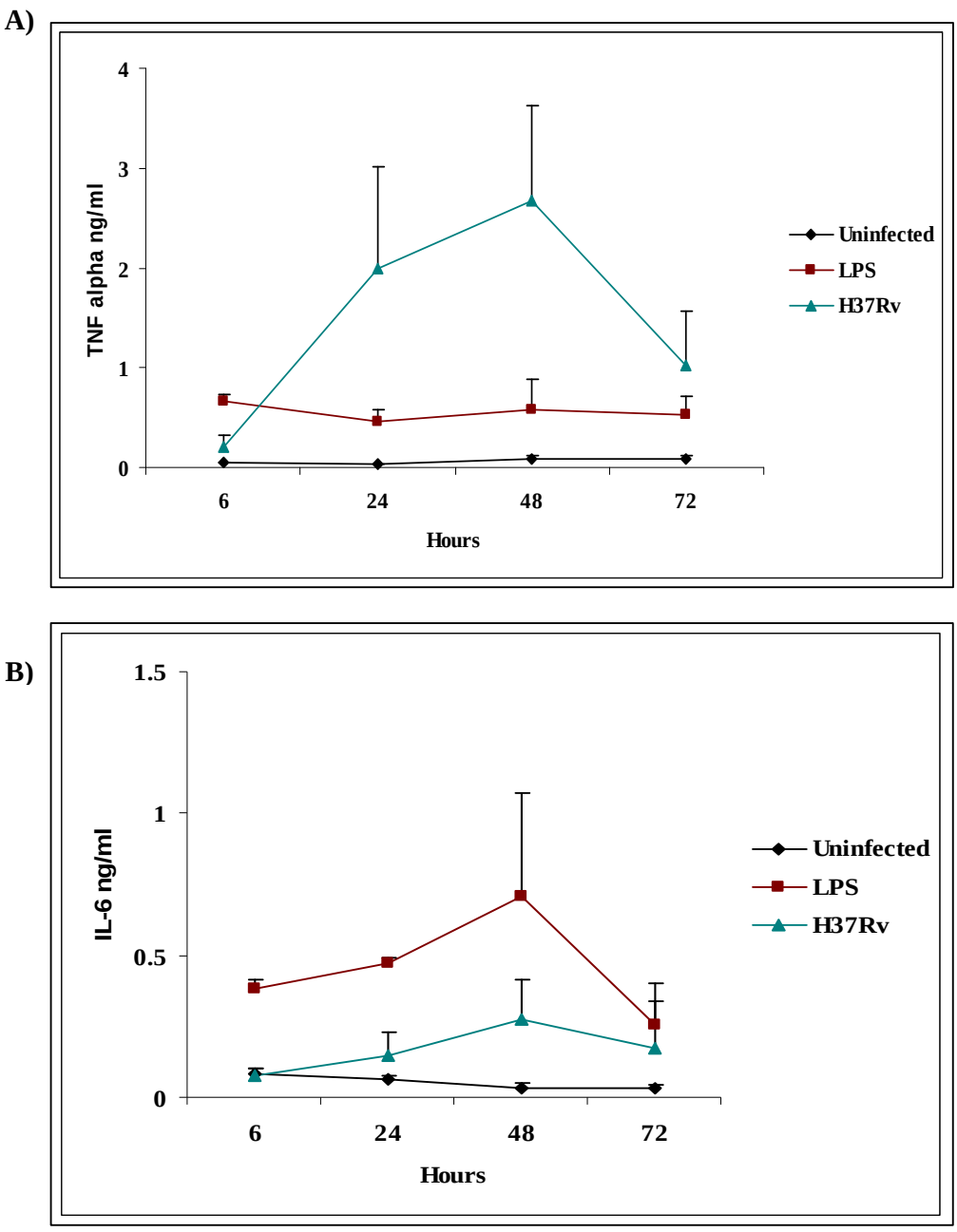


Figure 5.3- Production of TNF-α and IL-6 in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs.

Day 6 unsorted BMDCs were either left uninfected, stimulated with LPS (1μg/ml) or infected with H37Rv (M.O.I 10:1) and the production of **A)** TNF-α and **B)** IL-6 was measured using ELISA. Results are the mean of ±SE of 3 independent experiments.

5.3.2- Production of IL-12p40 by MyD88$^{-/-}$ *M. tuberculosis* infected BMDCs

IL-12p40 was produced by 24 hours in response to infection of MyD88$^{-/-}$ BMDCs and increased over the course of the experiment. But in the presence of LPS, IL-12p40 was produced steadily between 6-48 hours, with decreased production at later time points (72 hours) as shown in figure 5.4. In addition, the production of IL-12p40 by uninfected BMDCs also increased over the course of the experiment.

In contrast, wild-type BMDCs produced at least 3.2 fold higher IL-12p40 in comparison to MyD88$^{-/-}$ BMDCs at 24 hours post infection. In general, the production of IL-12p40 by MyD88$^{-/-}$ BMDCs followed a similar trend to that of the wild-type, although the level of IL-12p40 produced by wild-type BMDCs was higher than MyD88$^{-/-}$ BMDCs at all the time points examined, in response to infection (Figure 5.4 and Figure 3.6).

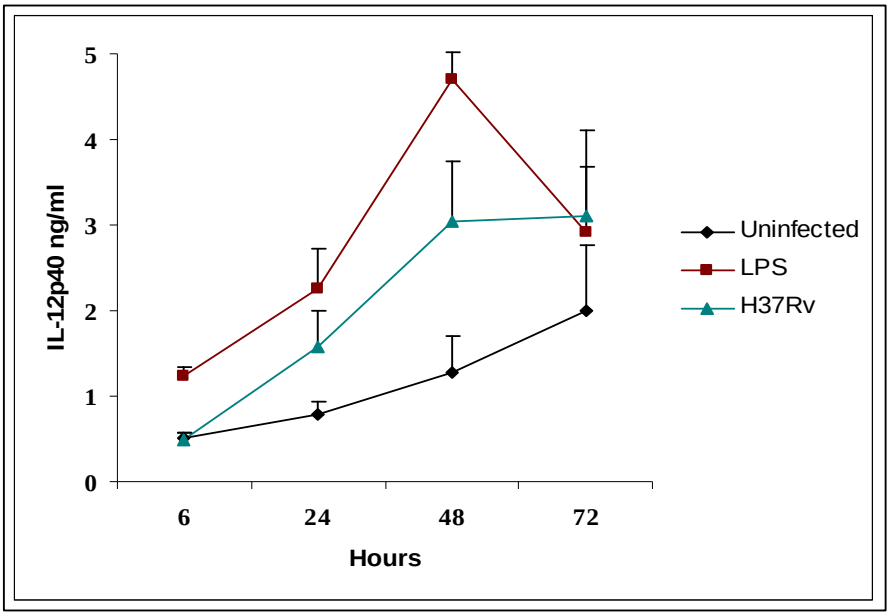


Figure 5.4- Production of IL-12p40 in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs.

Day 6 unsorted BMDCs were either left uninfected, stimulated with LPS (1µg/ml) or infected with H37Rv (M.O.I 10:1) and the production of IL-12p40 was measured using ELISA. Results are the mean of ±SE of 3 independent experiments.

5.3.3- Absence of IL-12p70 and IL-10 production by *M. tuberculosis* infected MyD88$^{-/-}$BMDCs

No measurable levels of IL-12p70 and IL-10 were detected in MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection or stimulation with LPS. This is in contrast to wild-type BMDCs which produced IL-12p70 and IL-10 in response to *M. tuberculosis* infection (Figures 3.5 and 3.6, A).

In conclusion, MyD88$^{-/-}$ BMDCs are impaired in their production of cytokines. Furthermore, the secretion of TNF-α and IL-6 was delayed, with a complete absence of IL-12p70 and IL-10 production by MyD88$^{-/-}$ BMDCs, in response to *M. tuberculosis* infection.

5.4- An overview of the KEGG biological pathways represented in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

In response to infection, 33 pathways were significantly represented in MyD88$^{-/-}$ BMDCS as identified from the microarray analysis. The top ten most significant pathways are listed in table 5.1. A more complete list of the highly represented KEGG pathways is listed in Appendix-V. Interestingly, the JAK-STAT signalling pathway, cytokine-cytokine receptor interaction and TLR signalling pathways were the most significantly represented in MyD88$^{-/-}$ BMDCs in response to infection (Table 5.1), which is similar to the KEGG pathway data obtained from wild-type BMDCs in response to infection (Table 4.1).

In *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs, biological pathways which are part of the immune response (B-cell receptor signalling pathway, T cell receptor signalling pathway, complement and coagulation cascades, leukocyte transendothelial migration), cell adhesion (focal adhesion) and other pathways (cell cycle, apoptosis) were highly represented (Table 5.1).

KEGG pathways	p-value
Jak-STAT signaling pathway	6.52E-11
Cytokine-cytokine receptor interaction	1.02E-09
Toll-like receptor signaling pathway	7.45E-09
B cell receptor signaling pathway	1.97E-06
T cell receptor signaling pathway	1.05E-05
Complement and coagulation cascades	4.31E-05
Focal adhesion	1.32E-04
Leukocyte transendothelial migration	2.94E-04
Cell cycle	3.27E-04
Apoptosis	4.93E-04

Table 5.1 – List of the top 10 significant biological pathways identified from microarray analysis of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs.

Genes which displayed differential regulation in response to *M. tuberculosis* infection were integrated within KEGG pathways. The p value indicates the statistical significance; the likelihood of the pathway being part of the cellular response generated by MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection.

5.5- Gene expression profiles of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

From the list of KEGG biological pathways (Table 5.1), the 3 most significantly represented pathways (JAK-STAT pathway, TLR signalling pathway and cytokine- cytokine receptor interaction pathway) and phagocytic and pattern recognition receptors are described in more detail in the following sections. In the following sections, fold change of genes identified as having a significant change in expression denotes that the fold change is statistically significant, with a p value of ≤0.05.

5.5.1- Differential expression of phagocytosis and pattern recognition receptors in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

In response to infection, components of the classical complement pathway (c1qa, c1qg, c1qb) and Fcγ receptors (Fcgr1, Fcgr2 and Fcgr3) were significantly down regulated in MyD88$^{-/-}$ BMDCs (Table 5.2, A). In contrast, C1qbp, which is involved in generating the first component of the serum complement system, is up regulated by a factor of 3.7 fold in response to infection of MyD88$^{-/-}$ BMDCs. The pattern recognition receptors (TLR1, TLR6 and TLR7) were also down regulated in MyD88$^{-/-}$ BMDCs in response to infection (Table 5.2, A). In comparison, these genes did not show a statistically significant change in expression, in wild-type BMDCs, in response to infection.

Nevertheless, phagocytic receptors (c1qr1 and mrc1) were down regulated in both wild-type and MyD88 deficient BMDCs by a factor of 3 fold or more, in response to infection (Table 5.2, B). Of interest, was the differential regulation of TLR2. TLR2 was up regulated by a factor of 2 fold in response to infection in wild-type BMDCs but down regulated by a factor of 5.6 fold in MyD88$^{-/-}$ BMDCs (Table 5.2, B).

The down regulation of TLR2 (Table 5.2, B) on MyD88$^{-/-}$ BMDCs was in response to *M. tuberculosis* infection and does not correlate with the difference in expression on uninfected BMDCs (Figure 5.5). Furthermore, the basal expression of TLR2 was marginally higher in MyD88$^{-/-}$ uninfected BMDCs (Figure 5.5).

In response to *M. tuberculosis* infection, MyD88 deficient BMDCs down regulate phagocytic receptors, which correlate with the down regulation of antigen uptake, as dendritic cells mature.

A)

Affymterix Id	Common name	Description	Fold change
1417381_at	C1qa	complement component 1, q subcomponent, alpha polypeptide	-180.83
1448274_at	C1qbp	complement component 1, q subcomponent binding protein	3.72
1417063_at	C1qb	complement component 1, q subcomponent, beta polypeptide	-751.88
1449401_at	C1qg	complement component 1, q subcomponent, gamma polypeptide	-353.36
1419482_at	C3ar1	complement component 3a receptor 1	-9.43
1436625_at	Fcgr1	Fc receptor, IgG, high affinity I	-7.75
1455332_x_at	Fcgr2b	Fc receptor, IgG, low affinity IIb	-4.17
1448620_at	Fcgr3	Fc receptor, IgG, low affinity III	-3.61
1449049_at	Tlr1	toll-like receptor 1	-5.52
1418162_at	Tlr4	toll-like receptor 4	-3.02
1421352_at	Tlr6	toll-like receptor 6	-7.57
1422010_at	Tlr7	toll-like receptor 7	-16.6

B)

Affymterix Id	Common name	Description	Fold change	
			Wild-type	MyD88-/-
1419132_at	Tlr2	toll-like receptor 2	2.33	-5.68
1419589_at	C1qr1	complement component 1, q subcomponent, receptor 1	-3.83	-12.13
1450430_at	Mrc1	mannose receptor, C type 1	-14.85	-86.95

Table 5.2- Gene expression profiles of pattern recognition and phagocytic receptors A) significantly, differentially expressed in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs B) significantly, differentially expressed in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection.

Differentially regulated phagocytic and pattern recognition receptor genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection, are displayed. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

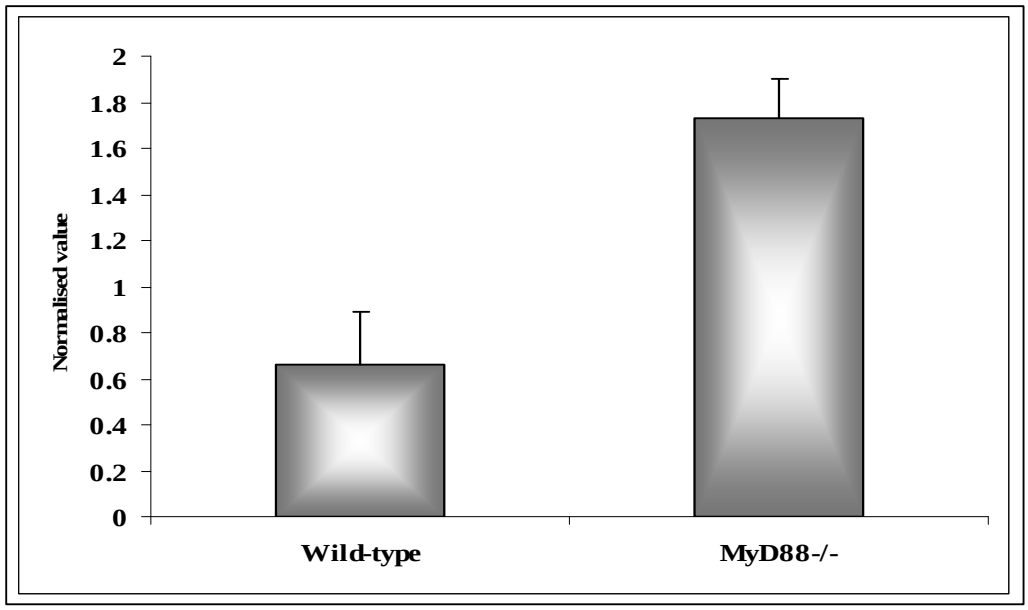


Figure 5.5- Basal expression of TLR 2 in wild-type and MyD88$^{-/-}$, uninfected BMDCs

Normalised TLR 2 values were obtained from microarray data. Results are an average of 3 independent experiments.

5.5.2- Differential expression of TLR signalling pathway genes *in M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

The expression of TLR signalling pathway genes, IRAK-4, LPS binding protein (LBP) were down regulated by a factor of >3 fold in MyD88$^{-/-}$ BMDCs in response to infection (Table 5.3, A) and moreover, these genes did not display a statistically significant change in expression in wild-type infected BMDCs. Expression of Nfkb1 (subunit p50 of NFκB) , TRAF-6 and Ikbkg was up regulated by a factor of 2.6 fold or more in MyD88$^{-/-}$ BMDCs, in response to infection. Ikbkg, also known as NEMO is the regulatory sub-unit of IκB kinase family and is involved in activating the NFκB complex (Akira and Takeda 2004).

Pik3r1 (p85 subunit) and CD86 (co-stimulatory molecule) were significantly up regulated by >2.5 fold in both wild-type and MyD88 deficient BMDCs in response to infection (Table 5.3, B). The up regulation of CD86 at the mRNA level correlates with increased expression of CD86 as determined by flow cytometry in MyD88$^{-/-}$ BMDCs infected with *M. tuberculosis* (Figure 5.2, B). The expression of Ikbke, an important component of the IFN mediated anti-viral responses is up regulated in wild-type BMDCs (1.95 fold) and down regulated (-3.98 fold) in MyD88 deficient BMDCs in response to infection (Table 5.3, B).

A)

Affymterix Id	Common name	Description	Fold change
1427691_a_at	Ifnar2	interferon (alpha and beta) receptor 2	-3.14
1435647_at	Ikbkg	inhibitor of kappaB kinase gamma	2.62
1451750_at	Irak4	interleukin-1 receptor-associated kinase 4	-5.98
1448550_at	Lbp	lipopolysaccharide binding protein	-7.57
1427705_at	Nfkb1	nuclear factor of kappa light chain gene	4.15
1435350_at	Traf6	Tnf receptor-associated factor 6	2.80

B)

Affymterix Id	Common name	Description	Fold change	
			Wild-type	MyD88-/-
1420404_at	Cd86	CD86 antigen	3.52	15.06
1417813_at	Ikbke	inhibitor of kappaB kinase epsilon	1.95	-3.98
1425515_at	Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.54	4.91

Table 5.3- Gene expression profiles of TLR pathway genes A) significantly, differentially expressed in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs B) significantly, differentially expressed in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection.

Differentially regulated TLR pathway genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection are displayed. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

5.5.3- Differential expression of JAK-STAT signalling pathway genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

A number of negative regulators (miz-1, pias1 and SOCS 6) of the JAK-STAT signalling pathways were down regulated in infected MyD88$^{-/-}$ BMDCs (Table 5.4, A), in contrast, other negative regulators of the JAK-STAT signalling pathway including cish and SOCS 3 were up regulated > than 4 fold in MyD88 deficient BMDCs, in response to *M. tuberculosis* infection. Additionally, a number of members of the JAKs and STATs family were also up regulated (Table 5.4, A) in infected MyD88 deficient BMDCs.

SOCS 2, STAT-1 and STAT-2 were significantly represented in both wild-type and MyD88 deficient BMDCs in response to infection (Table 5.4, B). SOCS 2 is the only negative regulator of the JAK-STAT pathway that was significantly represented in both wild-type and MyD88$^{-/-}$ BMDCs. SOCS 2, although a negative regulator of the JAK-STAT signalling pathway, is also known to be involved in enhancing STAT phosphorylation in response to cytokine treatment (Johnston 2004), thus it possible that SOCS 2 contributes to the delicate balance maintained in the induction of the JAK-STAT signalling pathway in response to infection.

A)

Affymterix Id	Common name	Description	Fold change
1448724_at	Cish	cytokine inducible SH2-containing protein	8.93
1421066_at	Jak2	Janus kinase 2	2.44
1428725_at	Miz1	protein inhibitor of activated STAT 2	-2.27
1455486_at	Pias1	protein inhibitor of activated STAT 1	-1.71
1456212_x_at	Socs3	suppressor of cytokine signaling 3	4.04
1452764_at	Socs6	suppressor of cytokine signaling 6	-4.78
1426587_a_at	Stat3	signal transducer and activator of transcription 3	2.38
1448713_at	Stat4	signal transducer and activator of transcription 4	5.38
1421469_a_at	Stat5a	signal transducer and activator of transcription 5A	4.9
1422103_a_at	Stat5b	signal transducer and activator of transcription 5B	2.1

B)

Affymterix Id	Common name	Description	Fold change	
			Wild-type	MyD88-/-
1441476_at	Socs2	suppressor of cytokine signaling 2	2.19	5.43
1420915_at	Stat1	signal transducer and activator of transcription 1	5.09	3.24
1421911_at	Stat2	signal transducer and activator of transcription 2	10.22	4.32

Table 5.4- Gene expression profiles of JAK-STAT signalling pathway genes A) significantly, differentially expressed in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs B) significantly, differentially expressed in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection.

Differentially regulated JAK-STAT signalling pathway genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection are displayed. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

5.5.4- Differential expression of cytokine and chemokine genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

M. tuberculosis infected MyD88$^{-/-}$ BMDCs significantly up regulated members of the CC family of chemokines (CCL17, CCL22) and CXC family (CXCL16) by a > 3 fold (Table 5.5, A). Down regulation of chemokine receptors CCR5 (-3.93 fold) and up regulation of CXCR4 (3.47 fold) signifies the transition of BMDCs from an immature to mature state (Allavena, Sica et al. 2000). Anti-inflammatory cytokines IL-4 and Tgfb2 were up regulated in MyD88 deficient BMDCs in response to infection. In contrast, the expression of IL-4 was down regulated in wild-type BMDCs, in response to *M. tuberculosis* infection, as shown by real-time PCR (Figure 4.10).

Other chemokines including, CXCL1, CXCL9 and CXCL10 and cytokines (IL12b, IL-6, Lif) were up regulated in both wild-type and MyD88 deficient BMDCs in response to infection (Table 5.5, B). Furthermore, the change in gene expression of CXCL9, CXCL10 and IL12b was considerably reduced in MyD88 deficient BMDCs (Table 5.5, B). But the reduced expression of CXCL10 at the mRNA levels is not reflected at the protein level, as in response to infection, MyD88 deficient BMDCs produce similar levels of CXCL10 to wild-type BMDCs (Figures 5.6 and 4.6). However, the reduced expression of IL12b (IL-12p40), as shown in table 5.5, B, correlates with the reduced IL-12p40 production from MyD88$^{-/-}$ BMDCs in response to infection (Figure 5.4).

Expression of genes (mRNA expression) does not always correlate with protein expression. This is highlighted by the expression of IL-6 (up regulated by 89 fold) in MyD88 deficient BMDCs in response to infection, as shown in table 5.5, B. This up regulation of IL-6 is not representative of the small amounts of IL-6, that are produced at 24 hours post-infection in infected MyD88$^{-/-}$ BMDCs (Figure 5.3, B).

A)

Affymterix Id	Common name	Description	Fold change
1419413_at	Ccl17	chemokine (C-C motif) ligand 17	21.53
1417925_at	Ccl22	chemokine (C-C motif) ligand 22	3.54
1419610_at	Ccr1	chemokine (C-C motif) receptor 1	-12.7
1421188_at	Ccr2	chemokine (C-C motif) receptor 2	-55.8
1422259_a_at	Ccr5	chemokine (C-C motif) receptor 5	-3.93
1449195_s_at	Cxcl16	chemokine (C-X-C motif) ligand 16	6.78
1448995_at	Cxcl4	chemokine (C-X-C motif) ligand 4	-3.11
1448710_at	Cxcr4	chemokine (C-X-C motif) receptor 4	3.47
1417932_at	Il18	interleukin 18	-4.54
1449864_at	Il4	interleukin 4	12.4
1450923_at	Tgfb2	transforming growth factor, beta 2	3.64

B)

Affymterix Id	Common name	Description	Fold change	
			Wild-type	MyD88-/-
1441855_x_at	Cxcl1	chemokine (C-X-C motif) ligand 1	2.46	2.65
1418930_at	Cxcl10	chemokine (C-X-C motif) ligand 10	91.05	7.22
1418652_at	Cxcl9	chemokine (C-X-C motif) ligand 9	81.27	12.14
1449497_at	Il12b	interleukin 12b	23.95	12.24
1448681_at	Il15ra	interleukin 15 receptor, alpha chain	5.21	5.46
1450297_at	Il-6	interleukin 6	31.49	89.89
1421207_at	Lif	leukemia inhibitory factor	2.94	10.46
1425444_a_at	Tgfbr2	transforming growth factor, beta receptor II	-1.99	-5.55

Table 5.5- Gene expression profiles of cytokine and chemokine genes A) significantly, differentially expressed in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs B) significantly, differentially expressed in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection.

Differentially regulated cytokine and chemokine genes were identified using the GCRMA algorithm and normalised fold change values in response to *M. tuberculosis* infection are displayed. Differentially regulated genes have a 1.4 fold change and a p value of ≤0.05.

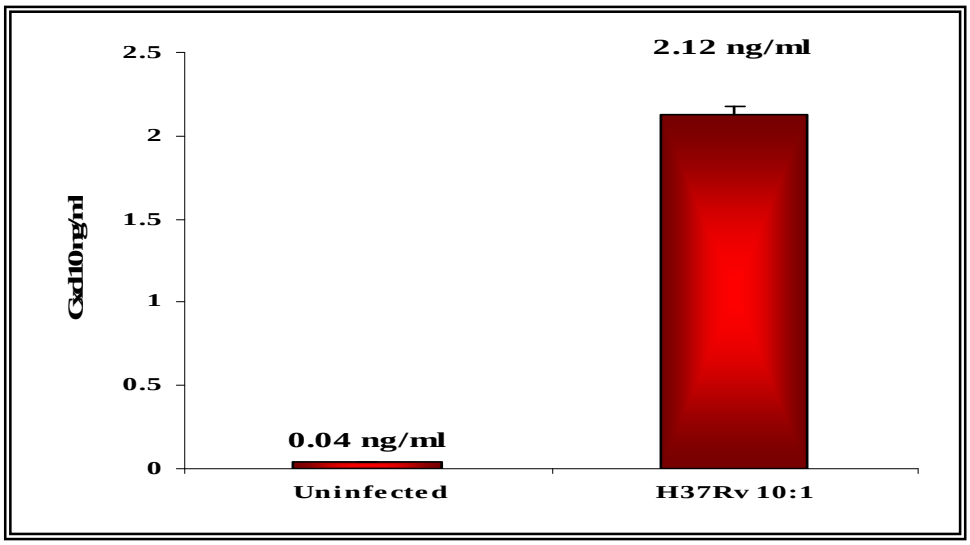


Figure 5.6- CXCL10 production by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs at 24 hours post-infection

Day 6 unsorted BMDCs were either left uninfected or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at 24 hours and CXCL10 were quantified by ELISA. Data shown are the mean ±SD of duplicate determinations in one experiment, representative of three independent experiments.

In conclusion, the up regulation of co-stimulatory molecules and chemokines in the absence of MyD88, in response to *M. tuberculosis* infection, suggest that BMDCs retain their ability to migrate in response to infection and function as efficient T cell stimulators. A complete list of the differentially regulated genes, in response to *M. tuberculosis* infection, in the absence of MyD88, is listed in Appendix- VIII.

5.6- Validation of microarray data from *M. tuberculosis* MyD88$^{-/-}$ BMDCs using real-time PCR

To confirm expression of a set of immunologically important genes, real time PCR was carried out as described previously (chapter 2, section 2.5.1). Briefly, RNA was extracted from day 6 unsorted MyD88$^{-/-}$ BMDCs, which were either uninfected or infected with *M. tuberculosis*. RNA was extracted at 24 hours post-infection to correlate with the time point of microarray analysis. Expression of individual genes was normalised to the internal control, 18S rRNA. The expression of cytokines (IL-10, IL-12p35 and TNF) was not detected by microarray analysis, however, these genes are important during mycobacterial infections (Cooper, Magram et al. 1997; Roach, Bean et al. 2002; Turner, Gonzalez-Juarrero et al. 2002) and thus the expression of these genes were quantified using real-time PCR. The expression of TLR 2 and TLR 4 were down regulated in MyD88$^{-/-}$ BMDCs, which is in contrast to the data obtained from wild-type BMDCs and thus to confirm this interesting observation, the expression of these genes were verified using real-time PCR.

Gene expression data obtained using real-time PCR and microarrays are displayed in figure 5.7. Cytokine and chemokine genes displayed a modest increase in expression levels in response to infection of MyD88$^{-/-}$ BMDCs as quantified using real time PCR (Figure 5.7, A). Although, the expression of IL-6 was increased by a factor of 89 fold, as determined from the microarray analysis, a much smaller increase in expression (3 fold) was detected using real-time PCR (Figure 5.6, A). IL-6 was induced 14 fold in wild-type BMDCs in response to infection (Figure 4.5), which is higher than the induction levels observed in MyD88$^{-/-}$ BMDCs. Nevertheless, the modest induction of IL-6 as quantified using real-time PCR, in MyD88$^{-/-}$ BMDCs correlates with the levels of IL-6 protein quantified using ELISA (Figure 5.3, B).

Transcripts for IL-10, IL-12p35 and TNF were not significantly different in infected vs uninfected samples in microarray anlaysis but an increase of 1.5 fold (IL-12p35) and 1.7 fold (TNF) was identified using real-time PCR (Figure 5.7, A). Since, real-time PCR is a more sensitive technique in comparison to

microarray analysis, it is possible that such small increases in expression is below the detection level of the microarrays.

The TLR 2 and STAT-1 gene expression profiles obtained using real time PCR were comparable to those obtained using microarray analysis, although the expression of TLR 4 was considerably underestimated by microarray analysis (decreased fold change -3 fold) in comparison to a down regulation of -142 fold quantified using real-time PCR (Figure 5.7, B).

In general, the fold change of gene expression detected by microarrays is higher than the gene expression values quantified using real-time PCR. Furthermore, real-time PCR is a useful technique for detecting expression of genes which are induced at very low levels (TNF and IL-12p35).

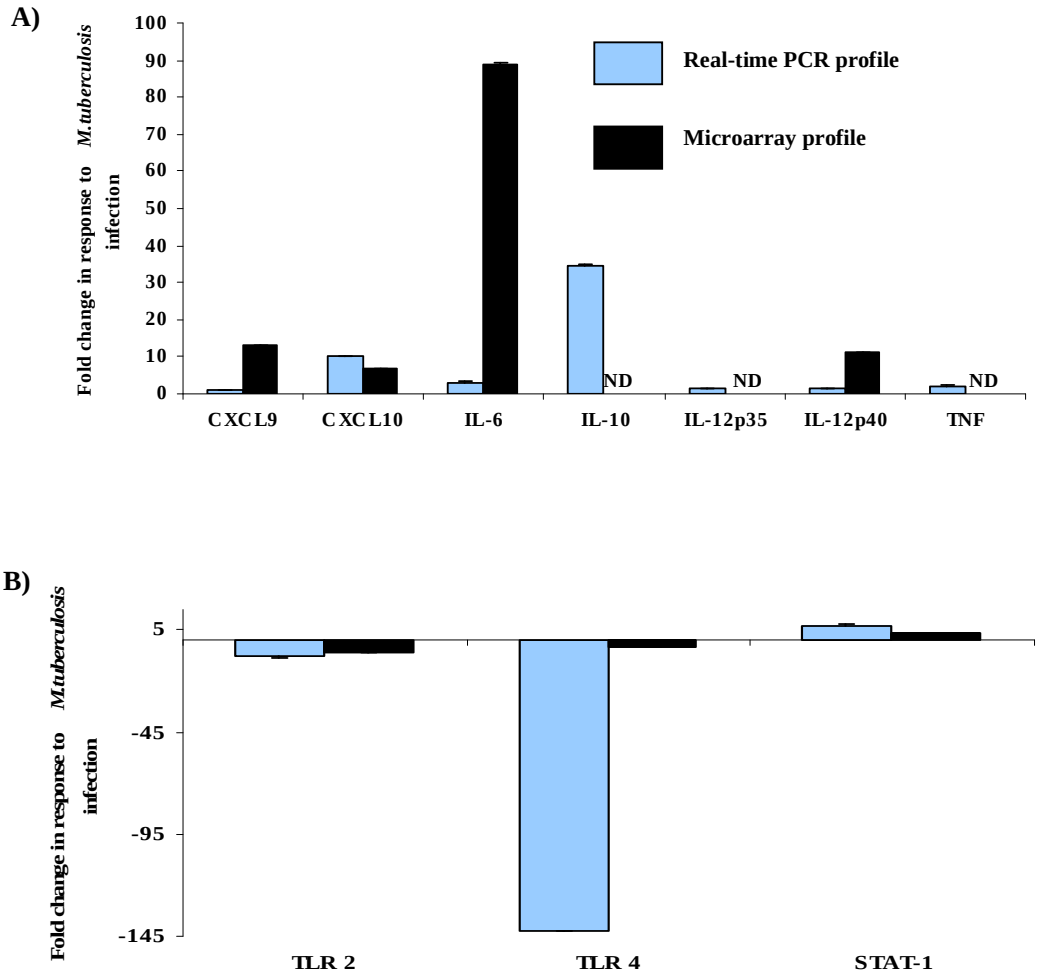


Figure 5.7- Quantitative real-time PCR and microarray profiles of A) cytokines and chemokines B) toll-like receptors and STAT-1 in *M.tuberculosis* infected MyD88$^{-/-}$ BMDCs.

Day 6 unsorted BMDCs were either left uninfected or infected with H37Rv at a M.O.I of 10: 1 and RNA was extracted at 24 hours post-infection. Gene expression data was determined using the SYBR green incorporation method. Expression of each gene was normalised to the endogenous control, 18S rRNA and subsequently fold change in response to infection was calculated relative to the uninfected sample. Results are representative of two independent experiments (mean ±SD of duplicate determinations in one experiment). Fold change values obtained for each gene using microarray analysis was compared to the real time PCR data. ND- no statistically significant change in gene expression was detected from microarray analysis.

5.7- *In silico* promoter analysis of differentially regulated genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

Genes differentially regulated in response to infection in MyD88$^{-/-}$ BMDCs were analysed using TELiS database, in order to identify the transcription factor binding motifs represented in the promoters of differentially regulated genes. Details of the database and the method are described in section 2.6. Listed in the table 5.6, are significant transcription factor binding motifs represented in the promoters of genes differentially regulated in response to infection, of MyD88$^{-/-}$ BMDCs.

Similar to the wild-type data (Table 4.2), ISRE and NFκB binding motifs were among the transcription factors that were significantly represented in the promoters of differentially regulated genes (Table 5.6).

Other transcription factor binding motifs that were significantly regulated belonged to the Ets family of transcription factors, Elk-1. Its activity is thought to be important for cell proliferation and blocking of apoptotic cell death. Myeloid zinc finger gene 1 (mzf1) has a role as a transcriptional regulator of hematopoiesis (Morris, Hromas et al. 1994).

From the TELiS data, it is evident that IRFs and NFκB are important regulating the transcriptional responses of MyD88 $^{-/-}$ BMDCs in response to *M. tuberculosis* infection.

Transcription factor binding motifs	P value	Z –test score
AP-2 (Activator protein 2)	$1.00x10^{-10}$	5.62
NFKB (Nuclear factor kappa B)	$1.10x10^{-5}$	4.40
CREL	$2.00x10^{-4}$	3.77
MZF1 (myeloid zinc finger 1)	$2.10x10^{-3}$	3.08
Elk1(member of ETS oncogene family)	$2.70x10^{-3}$	3.00
ISRE (Interferon stimulated response element)	$1.46x10^{-2}$	2.44
NFKAPPAB65 (Nuclear factor kappa B- p65)	$1.87x10^{-2}$	2.35

Table 5.6- Transcription factor binding motifs represented in *M. tuberculosis* infected MyD88 $^{-/-}$ BMDCs.

Significant transcription factor binding motifs, prevalent in the promoters of differentially regulated genes, in response to *M. tuberculosis* infection, was identified using the bioinformatic database TELiS. The positive values obtained with the Z-test indicate an increased prevalence of the individual transcription factor binding motifs and the p value represents the level of significance.

5.8- Profiling type I IFN inducible genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

Using microarray analysis it was possible to identify type I IFN inducible genes that were induced in the absence of MyD88. All the genes were mined from microarray data and were identified as type I IFN inducible genes using the same criteria, as previously described for wild-type type I IFN cluster of genes (Chapter 4, section 4.6).

5.8.1 -MyD88 independent induction of type I IFN genes in *M. tuberculosis* infected BMDCs: a comparative analysis between wild-type and MyD88$^{-/-}$ BMDCs

The type I IFN inducible genes were grouped together in 6 clusters (Table 5.7) and this set of genes were induced in both MyD88$^{-/-}$ and wild-type BMDCs in response to *M. tuberculosis* infection. All the names of the genes abbreviated in the text can be found in table 5.7. Normalised values of the genes, displayed in the table 5.7 are listed in Appendix- VI.

Genes involved in antiviral immunity (Gbp2, Ifit1, Isg20, Oasl1, Traf3), cytokines and chemokines (CXCL10, IL12b) and type I IFN pathway genes (IRF-7, STAT-1, STAT-2) were up regulated in both *M. tuberculosis* infected MyD88$^{-/-}$ and wild-type BMDCs. Traf-3 has a role in the regulation of IRF-3 activation via TLR pathways (Oganesyan, Saha et al. 2006) and Isg20, a 3'-5' exonuclease with specificity for ssRNA, was up regulated over 7 fold in MyD88$^{-/-}$ BMDCs (Table 5.7).

Other sub-groups consist of genes implicated in MHC-I processing and presentation (H2-Q10, Psme2, Tap1), and regulation of transcription (Adar, Ell2), had similar expression profiles in both wild-type and MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection.

The expression of CXCL10, IL12b and STAT-1 was confirmed using real time PCR, as shown previously in figure 5.7. From the microarray gene expression profiles it can be concluded that a large number of type I IFN inducible genes can be induced independent of MyD88 in response to *M. tuberculosis* infection.

Affymetrix ID	Fold change		Common name	Description	
	Wild-type	MyD88$^{-/-}$			
1423555_a_at	90.56	4.50	A430056A10Rik	interferon-induced protein 44	**Antiviral immunity**
1418240_at	3.97	2.90	Gbp2	guanylate nucleotide binding protein 2	
1450783_at	84.62	7.21	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	
1419569_a_at	32.59	10.24	Isg20	interferon-stimulated protein 20	
1424339_at	56.58	13.62	Oasl1	2'-5' oligoadenylate synthetase-like 1	
1418587_at	1.97	7.77	Traf3	Tnf receptor-associated factor 3	
1418930_at	91.05	7.22	Cxcl10	chemokine (C-X-C motif) ligand 10	**Cytokines and chemokines**
1449497_at	23.95	12.24	Il12b	interleukin 12b	
1417244_a_at	65.58	5.24	Irf7	interferon regulatory factor 7	**IRFs and Type I IFN pathway**
1420915_at	5.01	3.24	Stat1	signal transducer and activator of transcription 1	
1421911_at	10.22	4.32	Stat2	signal transducer and activator of transcription 2	
1426324_at	1.79	2.21	H2-Q10	histocompatibility 2, D region locus 1	**Antigen processing and presentation via MHC-I**
1417189_at	2.03	3.14	Psme2	proteasome (prosome, macropain) 28 subunit, beta	
1416016_at	2.9	3.34	Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	
1434268_at	2.58	2.11	Adar	adenosine deaminase, RNA-specific	**Regulation of transcription**
1450744_at	2.64	3.95	Ell2	elongation factor RNA polymerase II 2	
1435446_a_at	2.67	1.87	Chpt1	choline phosphotransferase 1	**Miscellenaeous**
1451426_at	11.86	4.05	D11Lgp2e	DNA segment, Chr 11, Lothar Hennighausen 2, expressed	
1422433_s_at	-1.78	-2.50	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	
1421207_at	2.94	10.46	Lif		
1452178_at	6.04	2.22	Plec1	plectin 1	
1434015_at	3.3	2.03	Slc2a6	solute carrier family 2 (facilitated glucose transporter), member 6	
1417961_a_at	8.49	2.55	Trim30	tripartite motif protein 30	
1418191_at	7.15	4.42	Usp18	ubiquitin specific peptidase 18	

Table 5.7- Expression of type I IFN inducible genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs- a comparative analysis to wild-type type I IFN gene profiles.

Expression profiles of type I IFN inducible genes were mined from the microarray data. Genes were grouped into functional clusters. Relative change in expression level in response to *M. tuberculosis* infection is displayed for each gene. Positive values for fold change indicate up regulation and negative value implies down regulation. All genes displayed have a p value of ≤ 0.05.

5.8.2- Profiling type I IFN inducible genes induced by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs but absent from infected wild-type BMDCs.

Type I IFN inducible genes induced in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs, with no statistically significant change in expression in wild-type BMDCs, are listed in table 5.8. All the gene names abbreviated in the text are in table 5.8.

The expression levels for the cytidine deaminase family member 1 (Apobec3), and mtmr9, which is involved in phospholipid de-phosphorylation was significantly up regulated over a factor of 3 fold but the type I IFN receptor, sububit 2 (IFNAR2) was down regulated in response to infection of MyD88$^{-/-}$ BMDCs (Table 5.8). Other genes which showed significant up regulation was, plek 2, involved in cell adhesion and spreading, psmb 7, a proteasome subunit which possess endopeptidase activity, tjp2, involved in the organisation of epithelial and endothelial intercellular junctions and stxbp6 which has roles in SNARE (soluble NSF attachment receptor) complex formation.

Affymetrix ID	Fold change	Common name	Description	Function
1417470_at	3.72	Apobec3	Apolipoprotein B editing complex 3	Miscellaneous
1427691_a_at	-3.14	Ifnar2	Interferon (alpha and beta) receptor 2	Type I IFN receptor
1433446_at	3.96	Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	Miscellaneous
1452254_at	2.71	Mtmr9	Myotubularin related protein 9	Miscellaneous
1449424_at	45.50	Plek2	Pleckstrin 2	Cell adhesion
1449424_at	2.23	Psmb7	Proteasome subunit, beta type 7	Peptidase activity
1425749_at	3.96	Stxbp6	Syntaxin binding protein 6 (amisyn)	SNARE complex formation
1434599_a_at	5.19	Tjp2	Tight junction protein 2	Organisation of intercellular junctions

Table 5.8 – Profiling type I IFN genes which showed significant expression in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs.

Expression of type I IFN inducible genes were mined from microarray data. Relative change in expression level in response to *M. tuberculosis* infection is displayed for each gene. All genes displayed has a fold change of >1.4 and p value of ≤0.05.

5.8.3- Profiling type I IFN inducible genes induced by *M. tuberculosis* infected wild-type BMDCs but absent from infected MyD88$^{-/-}$ BMDCs.

Type I IFN inducible genes, which showed a significant change in expression only in infected wild-type BMDCs were mined from the microarray data (Table 5.9).

Wild-type BMDCs in response to infection induced a cluster of genes which are classically known to be part of the innate immune response to viruses (Prkr, OAS1c, OAS2 and Mx2). Oas1c, the synthesis of which is induced by interferons, displayed a significant change in expression only in wild-type BMDCs, in response to infection. MHC-I genes (H2-Q8, H2-T10,H2-T24) induced by type I IFNs were also up regulated over 3 fold in wild-type BMDCs, in response to infection.

In conclusion, although a large proportion of type I IFN inducible genes was induced in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection, key anti-viral genes (Prkr, Mx2, OAS 1c and OAS2) displayed a significant change in expression only in wild-type BMDCs, thus possibly indicating an inefficiency by MyD88$^{-/-}$ mice to mount innate immune responses .

Affymetrix ID	Fold change	Common name	Description	
1422005_at	5.34	Prkr	eukaryotic translation initiation factor 2-alpha kinase 2	Antiviral immunity
1418686_at	5.48	Oas1c	2'-5' oligoadenylate synthetase 1C	
1426278_at	6.14	2310061N23Rik	interferon, alpha-inducible protein 27	
1425065_at	8.00	Oas2	2'-5' oligoadenylate synthetase 2	
1419676_at	11.78	Mx2	myxovirus (influenza virus) resistance 2	
1418293_at	62.53	Ifit2	interferon-induced protein with tetratricopeptide repeats 2	
1427736_a_at	5.84	Ccrl2	chemokine (C-C motif) receptor-like 2	Cytokine receptor
1421322_a_at	2.53	Isgf3g	interferon dependent positive acting transcription factor 3 gamma	Type I IFN pathway
1449875_s_at	3.05	H2-T10	histocompatibility 2, T region locus 10	Antigen processing and presentation via MHC-I
1430802_at	5.85	H2-Q8	histocompatibility 2, Q region locus 8	
1422160_at	6.47	H2-T24	histocompatibility 2, T region locus 24	
1451821_a_at	3.48	Sp100	nuclear antigen Sp100	Regulation of transcription
1425053_at	2.12	2610034N03Rik	isochorismatase domain containing 1	Miscellaneous
1421550_a_at	5.98	Trim34	tripartite motif protein 34	
1417314_at	50.13	H2-Bf	histocompatibility 2, complement component factor B	

Table 5.9 – Profiling type I IFN inducible genes which were significantly induced only in *M. tuberculosis* infected wild-type BMDCs

Expression of type I IFN inducible genes were mined from microarray data. Relative change in expression level in response to *M. tuberculosis* infection is displayed for each gene. All genes were grouped into function clusters and genes displayed have a fold change of >1.4 and p value ≤0.05.

5.9- Validation of type I IFN pathway genes expressed in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs using real time PCR

The expression of type I IFN receptor (IFNAR1 and IFNAR2), IFN beta, IRF-3 and IRF-7 was quantified using real-time PCR. IFNAR1, IRF-3, and IFN beta were not significantly represented in the microarray data and thus the expression of these genes was quantified using real-time PCR. As outlined in chapter 4, (section 4.7), the real-time PCR analysis was extended to incorporate expression of these genes at both 4 hours and 24 hours post-infection.

In brief, unsorted MyD88$^{-/-}$ BMDCs was infected with H37Rv at a M.O.I of 10:1 and RNA was extracted at 4 and 24 hours post-infection. Real-time PCR was carried out using the SYBR green incorporation method as described in materials and methods (section 2.5.1).

The expression of IFNAR1, IFNAR2, IFN beta and IRF-3 was down regulated at 4 hours post-infection in MyD88$^{-/-}$ BMDCs , the only exception being IRF-7, which was up regulated as early as 4 hours post-infection (Figure 5.8). Similarly, the expression of the type I IFN receptor subunits and IRF-3 was also down regulated in wild-type BMDCs but IFN beta and IRF-7 was up regulated at 4 hours post-infection (Figure 4.8). At 24 hours post-infection, expression of type I IFN receptors subunits was still down regulated but IFN beta , IRF-3 and IRF-7 were up regulated in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs (Figure 4.8). The down regulation of type I IFN receptors seem to be an inherent process that occurs during the maturation of dendritic cells and thus represents an intricate mechanism to regulate responsiveness to type I IFNs (Gauzzi, Canini et al. 2002).

The expression of IFN beta, IRF-3 and IRF-7 in MyD88$^{-/-}$ BMDCs was similar to wild-type BMDCs although, the up regulation of IFN beta was higher in wild-type BMDCs (Figures 4.8 and 5.8).

Although, the transcripts for IFN beta, IFNAR1, and IRF-3 were not significantly represented using microarrays, it was possible to quantify the expression of these genes using real-time PCR. Furthermore, the expression of IFNAR2 and IRF-7 from microarray data was confirmed using real-time PCR.

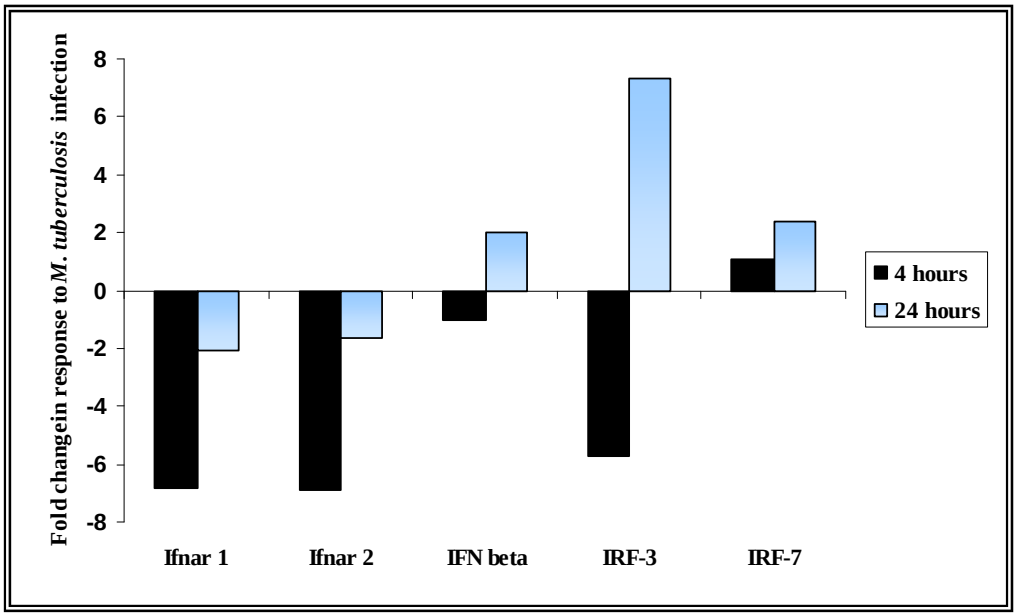


Figure 5.8 – Real-time PCR expression profiles of type I interferon genes in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs.

RNA was extracted from unsorted MyD88$^{-/-}$ BMDCs at 4 hours and 24 hours post- infection, converted to cDNA and real-time PCR was performed using the SYBR green detection method. All values were normalised to the house keeping gene- 18S rRNA. Fold induction of each gene in response to infection was calculated relative to its normalised value in the uninfected sample. Data is representative of 2 independent experiments.

5.10- Quantification of IFN beta production by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

IFN beta mRNA was induced by 24 hours in *M. tuberculosis* infected BMDCs (Figure 5.8). To analyse IFN beta production, unsorted MyD88$^{-/-}$ BMDCs were either left uninfected, stimulated with LPS (1μg/ml) or infected with H37Rv 10:1 and supernatants were collected between 6-72 hours post-infection and IFN beta production was measured using ELISA.

As shown in figure 5.9, IFN beta was detectable by 24 hours post-infection (115 ±3.86pg/ml), with decreased production at later time points. In contrast, IFN beta was detected by 6 hours in LPS stimulated MyD88$^{-/-}$ BMDCs (280 ±1 pg/ml) with a rapid reduction in IFN beta protein levels by 24 hours (Figure 5.9). Uninfected MyD88$^{-/-}$ BMDCs produced negligible amounts of IFN beta. In contrast, LPS stimulated wild-type BMDCs produced higher levels of IFN beta with a more gradual decrease in IFN beta production at later time points (Figure 4.9). In wild-type BMDCs, the induction of IFN beta in response to *M. tuberculosis* infection was not significantly different to MyD88$^{-/-}$ BMDCs at any of the time points examined (Figure 4.9 and Figure 5.9).

Thus, IFN beta was induced in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs at 24 hours, both at the mRNA (Figure 5.8) and protein level (Figure 5.9). Hence the induction of IFN beta is independent of MyD88 in *M. tuberculosis* infected BMDCs.

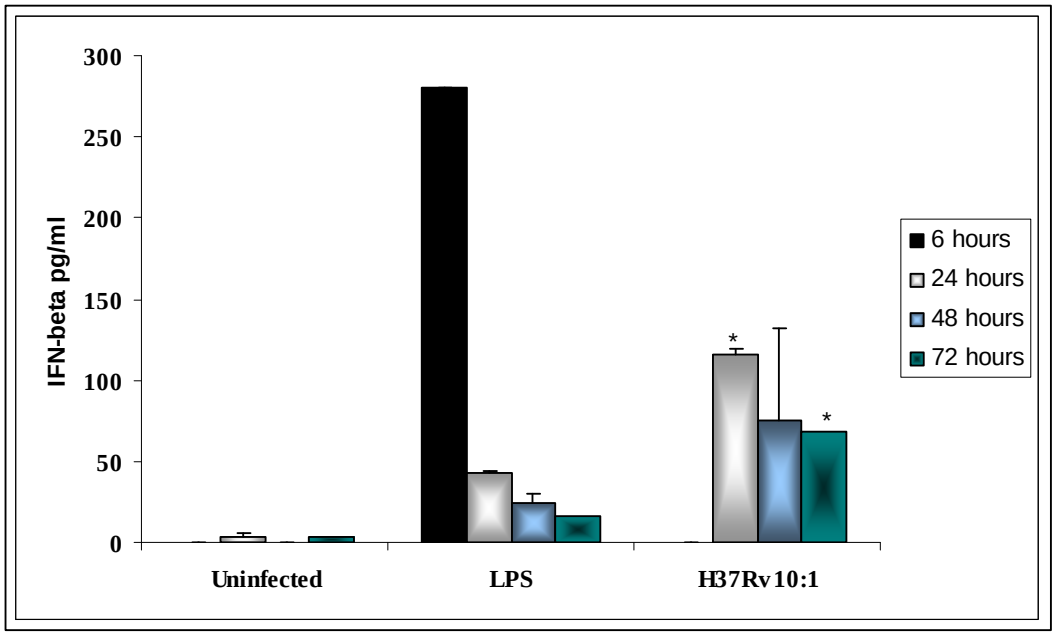


Figure 5.9- Induction of IFN beta in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs.

MyD88$^{-/-}$ unsorted BMDCs were cultured *in vitro* and day 6 cells were either left uninfected, stimulated with LPS (1µg/ml) or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at various time points post-infection for the quantification of IFN beta using ELISA. Results are the mean of ±SE of 3 independent experiments. Statistical analysis was preformed between *M. tuberculosis* infected group and uninfected group with the Student's t-test, *- p value <0.05.

5.11- Real-time PCR analysis of type I IFN inducible genes in CD11c sorted MyD88$^{-/-}$ BMDCs

In order to obtain a more comprehensive list and substantiate the expression of selected type I IFN inducible genes, obtained from microarray analysis using unsorted BMDCs (68 ±6% CD11c^{+}), real-time PCR was carried out using RNA extracted from a population of sorted dendritic cells (95 ±3% CD11c^{+}).

Day 6, BMDCs were sorted based on their CD11c expression and RNA was extracted from cells that were either left uninfected or infected with H37Rv (M.O.I 10:1) for 24 hours. Real-time PCR was carried out using the RT profiler PCR array system and all genes were normalised to the endogenous control, GAPDH.

Similar to the wild-type real-time PCR data (Figures 4.10 and 4.11), IFN inducible genes were sub-divided into 4 sub-groups and the gene expression profiles are displayed in figure 5.10 and figure 5.12. The type I IFNs , IFN alpha (IFNa2, a4, a9 and a11) and IFN beta were up regulated but IFN alpha sub-type IFNa7 was down regulated in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs (CD11c sorted). In contrast, the expression of IFNa7 and IFNa11 was down regulated in wild-type CD11c^{+} BMDCs (Figure 4.10) nonetheless, IFNa2, IFNa4 and IFNa9 were up regulated in both wild-type and MyD88$^{-/-}$ CD11c sorted BMDCs, in response to infection (Figures 4.10 and 5.10).

As shown in figure 5.11, low amounts of IFN alpha (14.31 ±1.59pg/ml) protein was detected at later time points post-infection (72 hours). This is in contrast to wild-type *M. tuberculosis* infected BMDCs where IFN alpha was not measurable at the protein level using ELISA.

Cytokines IL12b (IL-12p40) and IL-4 were both up regulated in response to infection of MyD88$^{-/-}$ CD11c sorted BMDCs (Figure 5.10), however, the expression of IL-4 was down regulated in wild-type *M. tuberculosis* infected CD11c sorted BMDCs (Figure 4.10). But similar to the wild-type, IL-4 protein

was not detected in the supernatants of *M. tuberculosis* infected MyD88$^{-/-}$ CD11c sorted BMDCs.

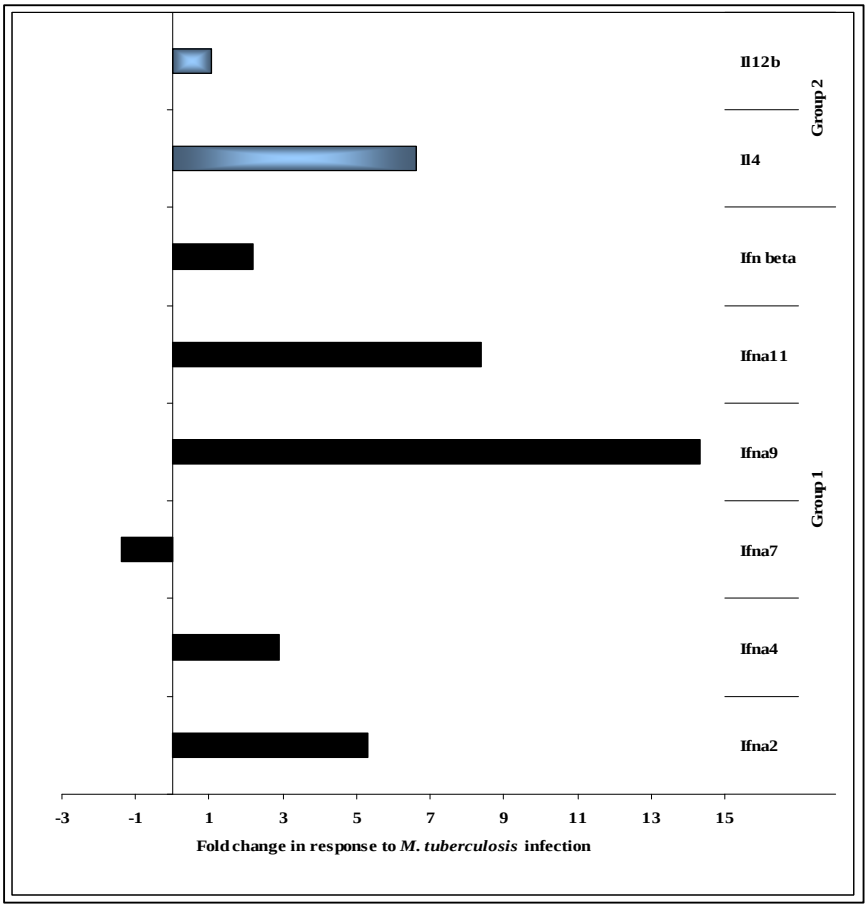


Figure 5.10- Real-time PCR expression profiles of type I IFNs and IFN inducible genes in CD11c sorted MyD88$^{-/-}$ BMDCs.

RNA was isolated from CD11c sorted uninfected BMDCs and *M. tuberculosis* infected BMDCs (24 hours post-infection), converted to cDNA and real-time PCR was performed using RT profiler PCR array. Expression of individual interferon genes were normalised to the house keeping gene GAPDH. Fold change of a gene in response to *M. tuberculosis* infection relative to the uninfected sample is displayed. Genes were grouped into functional clusters: Group 1- type I interferons, Group 2- Interferon related genes. Results are representative of two independent experiments.

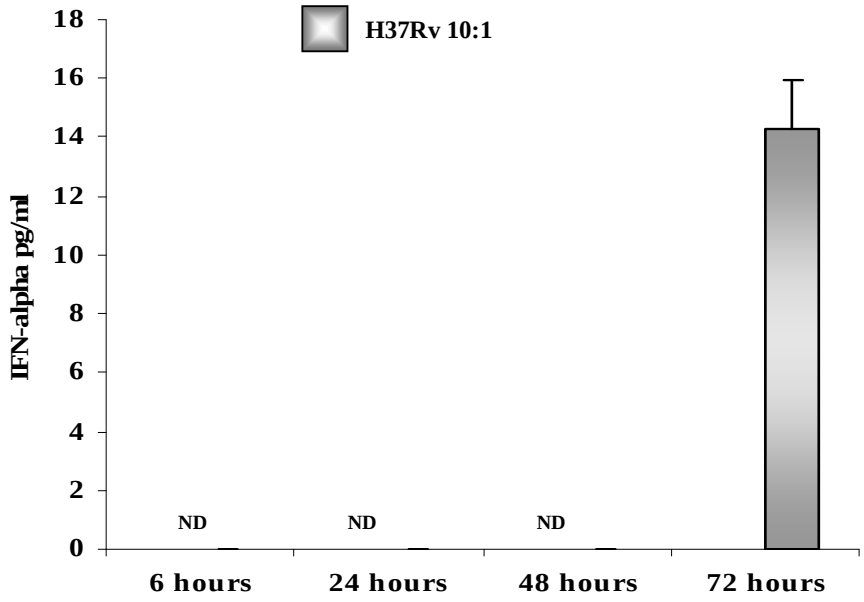


Figure 5.11- Induction of IFN alpha in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs

MyD88$^{-/-}$ BMDCs were cultured *in vitro* and day 6 cells were either left uninfected, or infected with H37Rv at a M.O.I of 10:1. Supernatants were collected at various time points post-infection for the quantification of IFN alpha using ELISA. Results are the mean of ±SE of 3 independent experiments.

Expression of interferon regulatory factors (group 3) was similar between MyD88$^{-/-}$ and wild-type CD11c sorted BMDCs with the exception of Irf2bp1, which was up regulated by a factor of 16 fold in wild-type BMDCs but was down regulated in MyD88$^{-/-}$ BMDCs in response to infection (Figures 4.11 and 5.12).

In group 4, key anti-viral genes, Mx1, OAS1a and cellular immune response genes, Ifit2, Ifit3, Ifi27 and H28 were down regulated in MyD88$^{-/-}$ BMDCs, in response to infection but were up regulated in wild-type BMDCs (Figure 4.11 and figure 5.12).

As outlined previously in chapter 4 (section 4.9.1), real-time PCR profiles of type I IFN inducible genes in CD11c sorted BMDCs also made it possible to validate the microarray analysis carried out using unsorted BMDCs. As shown in figure 5.13, gene expression data obtained for CXCL10, IL12b, Ifi44, Ifit1 and IRF-7 was similar in both microarray analysis and real-time PCR data (obtained from CD11c sorted BMDCs). Thus, gene expression profiles from real time PCR, carried out using CD11c sorted BMDCs and microarray analysis, with unsorted BMDCs, were comparable.

In general, the induction of type I IFNs (IFN alpha and IFN beta), IRF-7 and other type I IFN inducible genes substantiates the induction of the type I IFN pathway but the down regulation of key anti-viral genes and the general reduced fold change of type I IFN genes like CXCL10 suggests that there are crucial differences that exist between MyD88 deficient and wild-type BMDCs.

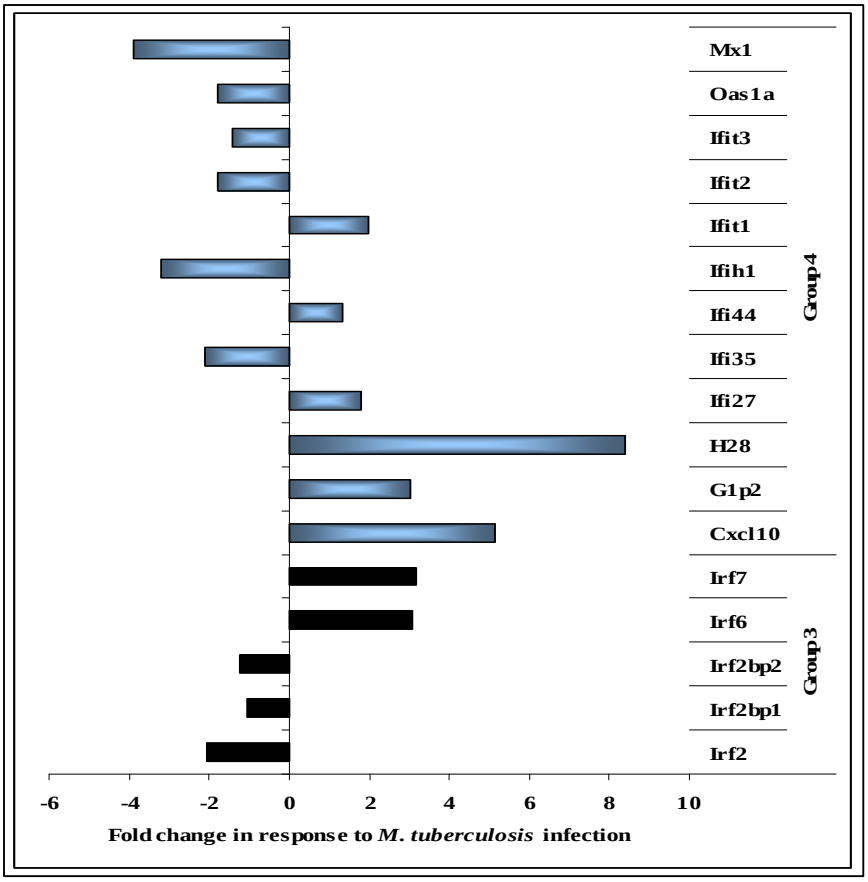


Figure 5.12 - Real-time PCR expression profiles of Interferon regulatory factors and type I IFN inducible genes in *M. tuberculosis* infected MyD88$^{-/-}$ CD11c sorted BMDCs

RNA was isolated from CD11c sorted uninfected MyD88$^{-/-}$ BMDCS and *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs, converted to cDNA and real-time PCR was performed using RT profiler PCR array. Expression of individual interferon genes were normalised to the house keeping gene GAPDH. Fold change of a gene in response to *M. tuberculosis* infection relative to the uninfected sample is displayed. Genes were grouped into functional clusters: Group 3-Interferon regulatory factors, Group 4- Interferon inducible genes. Results are representative of two independent experiments.

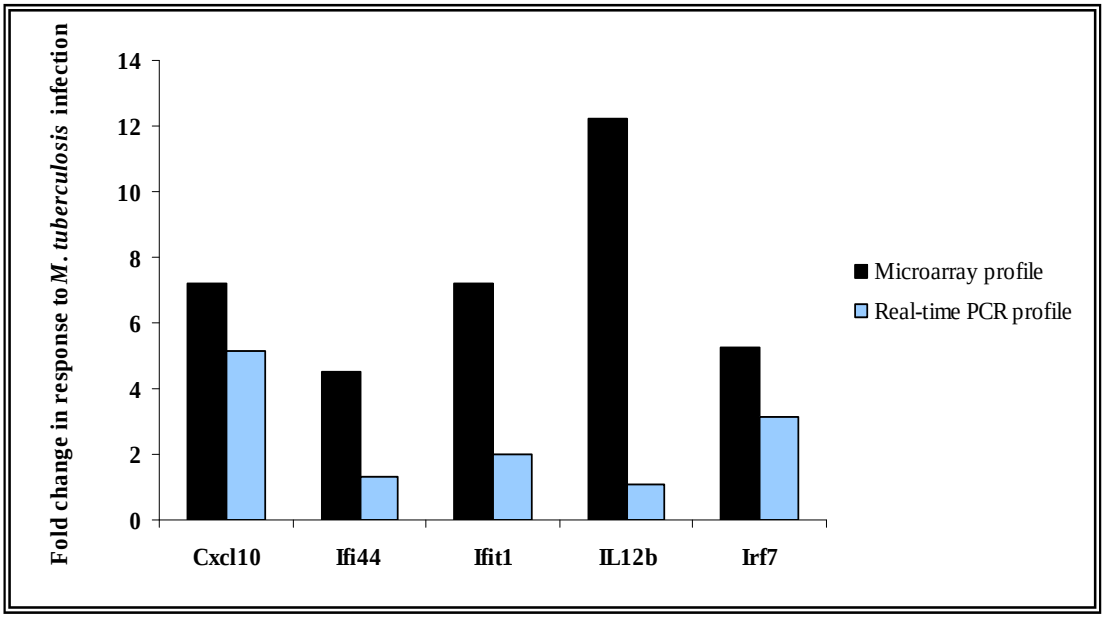


Figure 5.13 – Quantitative real-time PCR and microarray gene expression profiles of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs.

RNA was extracted from CD11c sorted BMDCs 24 hour's post- infection, converted to cDNA and gene expression values as indicated were measured, by SYBR Green incorporation during PCR. For real time PCR, the expression of each gene was normalized to the internal control, GAPDH. Results obtained using real-time PCR for each gene was compared with the fold induction values obtained using microarray anlaysis.

5.12- Discussion

In the absence of MyD88, dendritic cells in response to *M. tuberculosis* infection reveal no impairment in up regulating the co-stimulatory and MHC-II expression and this degree of up regulation is comparable to wild-type BMDCs. Hence, this feature of dendritic cell maturation is independent of MyD88. This correlates with previous studies which have shown that MyD88$^{-/-}$ dendritic cells, show similar up regulation of co-stimulatory and antigen presenting molecules in response to LPS and *M. tuberculosis* infection (Kaisho and Akira 2001; Fremond, Yeremeev et al. 2004).

However, MyD88 deficient BMDCs exhibit a profound impairment and delay in producing inflammatory cytokines. The reduced induction of cytokines in reflected at both the mRNA and protein level (Figures, 5.3, 5.4 and 5.7, A). IL-6 and TNF-α were highly induced in response to *M. tuberculosis* infection, of wild-type BMDCs by 6 hours post-infection, with the exception of IL-12p40, which peaked around 48 hours post-infection. In contrast, IL-6 and TNF-α peaked around 48 hours with a profound reduction in cytokine levels in MyD88$^{-/-}$ BMDCs. Additionally, the anti-inflammatory cytokine IL-10 and the bioactive IL-12p70 were not detected in response to infection of MyD88$^{-/-}$ BMDCs. Hence it appears that the induction of inflammatory cytokines is partially dependent on MyD88 and requires the MyD88 dependent pathway for optimal cytokine production, as seen in the wild-type BMDCs, in response to infection.

Gene expression analysis of wild-type BMDCs identified a large number of type I IFN inducible genes which were induced in response to *M. tuberculosis* infection. Previous reports have suggested that LPS stimulation and *M. tuberculosis* infection of macrophages can induce IFN inducible genes independent of MyD88 (Kawai, Takeuchi et al. 2001; Shi, Nathan et al. 2003). Since no similar studies have been performed with murine dendritic cells, the induction of type I IFN genes was determined in *M. tuberculosis* infected MyD88 deficient BMDCs using a combination of microarray, real-time PCR and bioinformatics approaches.

Following a similar pattern to wild-type BMDCs, the most significantly represented KEGG pathways were that of TLR signalling, JAK-STAT signalling and cytokine-cytokine receptor interaction pathway. Although a large proportion of genes within these pathways exhibited similarities between wild-type and MyD88 deficient BMDCs, some key differences did exist in the gene expression profiles.

Pattern recognition receptors TLR 2 and TLR 4 are important during mycobacterial infections. In response to infection, these receptors were down regulated in MyD88 deficient BMDCs and in contrast, these receptors were up regulated in wild-type BMDCs in response to *M. tuberculosis* infection. In MyD88 deficient BMDCs it is possible, that delayed kinetics in TLR gene expression is responsible for this observation. This is likely, as other aspects of dendritic cell responses such as cytokine production by MyD88 deficient BMDCs is induced with delayed kinetics, when compared to wild-type BMDCs, in response to infection.

In the absence of MyD88, a proportion of type I IFN inducible genes and type I IFNs were up regulated in response to infection. These genes were further divided into functional gene clusters and genes involved in anti-viral immunity, cytokines/chemokines and genes which are part of the type I IFN pathway were differentially expressed in response to infection. A large number of genes in these clusters were induced in the absence of MyD88.

But a detailed look, also highlighted certain differences in gene expression patterns, between wild-type and MyD88$^{-/-}$ BMDCs. Cellular immune response genes Ifit2, OAS1a and Mx1 were down regulated in response to infection, as shown by real-time PCR (Figure 5.12).

OAS encodes a family of 2'-5'oligoadenylate synthetase enzymes, which is induced in response to type I interferons and functions to activate RNase L that degrades viral RNA. The transcript for OAS1a was up regulated by 1.6 fold in wild-type mice but was down regulated by a factor of 1.8 fold in MyD88$^{-/-}$ BMDCs, OAS1c and OAS2 was up regulated by a more than a factor of 5 fold in

wild-type BMDCs and these genes did not display a significant change in expression, in MyD88$^{-/-}$ BMDCs. Mx proteins which are part of the family of GTpases, was up regulated 1.13 fold in wild-type BMDCs and down regulated by a factor of 3 fold in MyD88$^{-/-}$ BMDCs, as shown by real-time PCR. The expression of SOCS 3 (up regulated by 4.0 fold) is known to suppress IFN alpha induced antiviral proteins like 2'-5' oligoadenylate synthetase and Mx proteins. It is possible that up regulation of SOCS 3 contributes to the down regulation of these key anti-viral immune genes in MyD88$^{-/-}$ BMDCs.

Transcription of IFN beta appears to follow different kinetics of induction in MyD88$^{-/-}$ and wild-type BMDCs, with this gene being down regulated at 4 hours post-infection in MyD88$^{-/-}$ BMDCs but is up regulated at later time points. In contrast, there is an increased induction of IFN beta at 4 and 24 hours post-infection of wild-type BMDCs. At 24 hours, the induction of IFN beta and IFN alpha sub types in MyD88$^{-/-}$ mice, suggests MyD88 independence in inducing specific sub-sets of type I IFNs.

In line with the induction of type I IFN inducible genes, ISRE motifs were significantly present in promoters of differentially regulated genes in MyD88 deficient BMDCs, as shown by TELiS analysis. Similar to wild-type BMDCs, IRFs and NFκB transcription factors contribute to the regulation of cellular response in MyD88 deficient BMDCs.

An overview of the key immune responses of *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs is illustrated in figure 5.14. Previous studies have shown that *M. tuberculosis* mediates gene expression in macrophages largely independent of MyD88 (Shi, Evans et al. 2003). Similarly, *M. tuberculosis* infection of BMDCs also mediates the expression of a large proportion of genes independent of MyD88 but the overall optimum immune response, as highlighted by the defective cytokine production and down regulation of key antiviral genes, seem to be still dependent on MyD88.

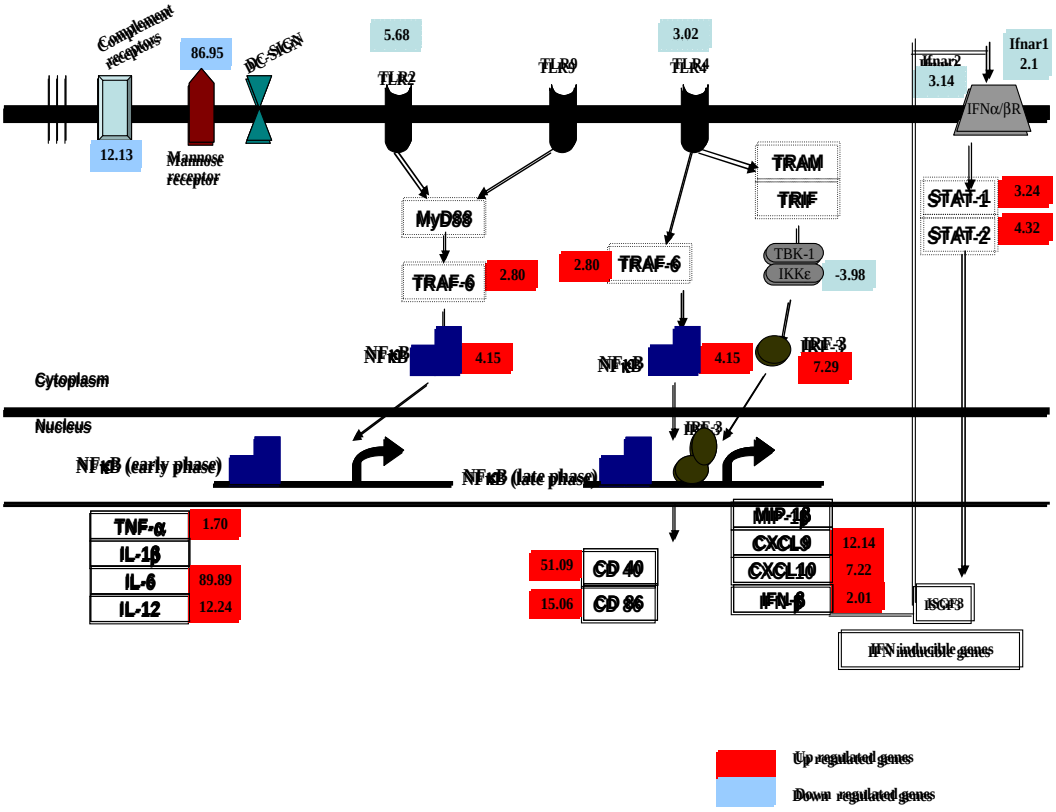


Figure 5.14- Overall schematics of differentially expressed immune response and signal transduction genes by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs (24 hours post-infection)

Gene expression values were obtained from microarray and real-time PCR data.

Chapter 6

Expression of IRF-3 and IRF-7 in *M. tuberculosis* infected BMDCs in the absence of MyD88

6.1- Introduction

The expression of a number of type I IFN inducible genes is dependent on a set of transcription factors. Transcriptional induction of type I IFN genes is mediated by two main transcription factors IRF-3 and IRF-7. These two transcription factors are structurally related and belong to the IRF family of transcription factors. IRF-3 is expressed constitutively, in its latent form, in the cytoplasm and undergoes nuclear translocation in response to viral stimulation. In contrast, IRF-7 is present in low levels in most cells and its expression is strongly induced in response to type I IFN signalling. Both these transcription factors undergo serine phosphorylation and dimerisation, before translocation to the nucleus. IRF 3 is classically thought to be important in the activation of IFN beta and IRF-7 is responsible for the activation of IFN alpha genes.

LPS, a TLR 4 ligand (Poltorak, He et al. 1998) and poly I:C, a TLR 3 ligand (Alexopoulou, Holt et al. 2001), are potent inducers of type I IFN responses. There is a lack of IFN beta mRNA induction in $IRF3^{-/-}$ dendritic cells in response to LPS stimulation. This led to the suggestion that IRF-3 is important in the LPS mediated IFN beta induction via TLR 4 (Sakaguchi, Negishi et al. 2003). Type I IFN induction is mediated by a positive feed back loop mechanism and recent studies have shown, that heterodimers of IRF-3/IRF-7 or the homodimer of IRF-7, are important in mediating the full induction of the type I IFN genes. $IRF\text{-}7^{-/-}$ mice were highly susceptible to viral infection more so than $MyD88^{-/-}$ mice, which coincide with decreased serum levels of IFNs. This paper also further demonstrated the dispensable role of IRF-3 during the induction of type I IFN genes in plasmacytoid dendritic cells via TLR9. Thus this key piece of work revealed the importance of IRF-7 in mediating innate anti-viral responses (Honda, Yanai et al. 2005).

In the previous chapters an account of the induction of the type I IFN pathway has been given, with particular reference to the induction of the type I IFN inducible genes. A proportion of these genes have been found to be

independent of the adaptor MyD88. Additionally, transcription factor binding motifs for IRFs, the ISRE sequence, was significantly present in the promoters of genes differentially regulated in response to *M. tuberculosis* infection, in both wild-type and MyD88$^{-/-}$ BMDCs. In light of this evidence and what is known from the literature, the expression of the two key transcription factors regulating the induction of type I IFN inducible genes was characterised at the protein level using western blotting.

6.2- Induction of IFN beta in response to stimulation by TLR3 and TLR4 ligands

In order to determine the ability of poly I:C (TLR 3 ligand) and LPS (TLR 4 ligand) to induce type I IFNs , day 6 unsorted BMDCs were stimulated with either LPS or poly I:C and the levels of IFN beta were assessed using ELISA at 24 hours post-stimulation.

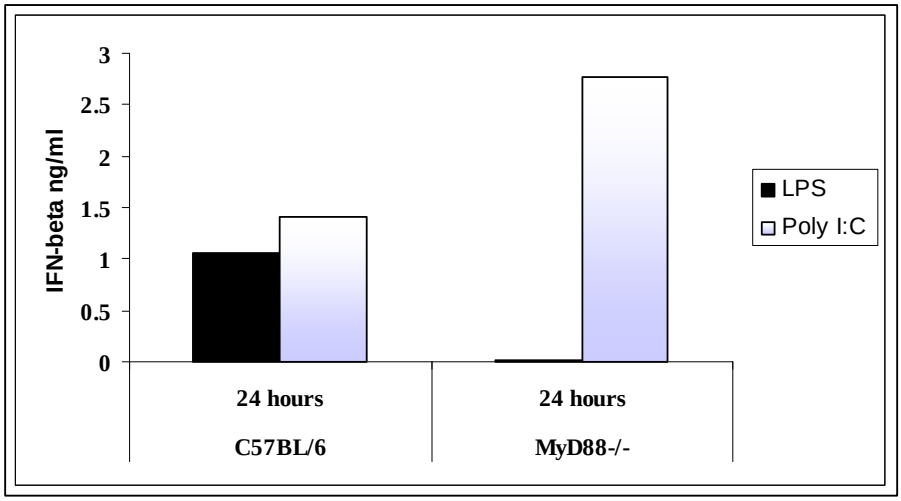


Figure 6.1- Induction of IFN beta in response to LPS and poly I:C stimulation of BMDCs.

Day 6 BMDCs were either stimulated with LPS or poly I:C or left un-stimulated and supernatants were collected at 24 hours. IFN beta levels were determined using ELISA. Data is representative of 2 independent experiments.

As shown in figure 6.1, LPS and poly I:C are capable of stimulating high levels of IFN beta, with poly I:C inducing higher levels of type I IFNs, especially in MyD88$^{-/-}$ BMDCs. LPS stimulated MyD88 deficient BMDCs produced much lower levels of IFN beta in comparison to poly I:C stimulation. Thus, both LPS and poly I:C were able to induce IFN beta production, with poly I:C being superior in inducing IFN beta production from BMDCs.

6.3 Activation of IRF-3 and IRF-7 in response to *M. tuberculosis* infection

Day 6 BMDCs from wild-type and MyD88$^{-/-}$ unsorted BMDCs were either left uninfected, stimulated with LPS, poly I:C or infected with H37Rv at a M.O.I of 10:1 and nuclear extracts were obtained 4 hours and 24 hours post-infection.

The expression of IRF-3 and IRF-7 was detected using western blots with antibodies against IRF-3 and IRF-7.

IRF-3 was expressed at low levels in its non phosphorylated form in wild-type uninfected cells and in contrast MyD88$^{-/-}$ uninfected cells showed a higher expression level of IRF-3 (Figure 6.2). Overall, IRF-3 was highly expressed in response to poly I:C stimulation with slightly weaker expression in response to LPS stimulation of wild-type BMDCs (Figure 6.2, A). At 4 hours, in response to *M. tuberculosis* infection, there was an increase in IRF-3 phosphorylation, as represented by the slower migrating band on the gel in wild-type BMDCs (Figure 6.2, A). In MyD88$^{-/-}$ BMDCs, the expression of IRF-3 was similar in response to LPS and poly I:C stimulation. IRF-3 expression was weaker in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs. At 24 hours post-infection, the expression of IRF-3 was decreased in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs (Figure 6.2, B). The phosphorylated form of IRF-3 was not detected in MyD88$^{-/-}$ BMDCs in response to infection.

IRF-7 was not expressed in uninfected wild-type cells and there was a faint expression of IRF-7, in MyD88$^{-/-}$ uninfected BMDCs. At 4 hours, IRF-7 was expressed at very low levels in response to LPS and poly I:C stimulation in wild-type and MyD88$^{-/-}$ BMDCs (Figure 6.3). There was no expression of IRF-7 in response to *M. tuberculosis* stimulation in both the wild-type and MyD88 knockout BMDCs at 4 hours. In contrast, there was an increase in expression of IRF-7 in response to *M. tuberculosis* infection at 24 hours as shown in figure 6.3, A and figure 6.3, B with an additional band appearing at 100kda.

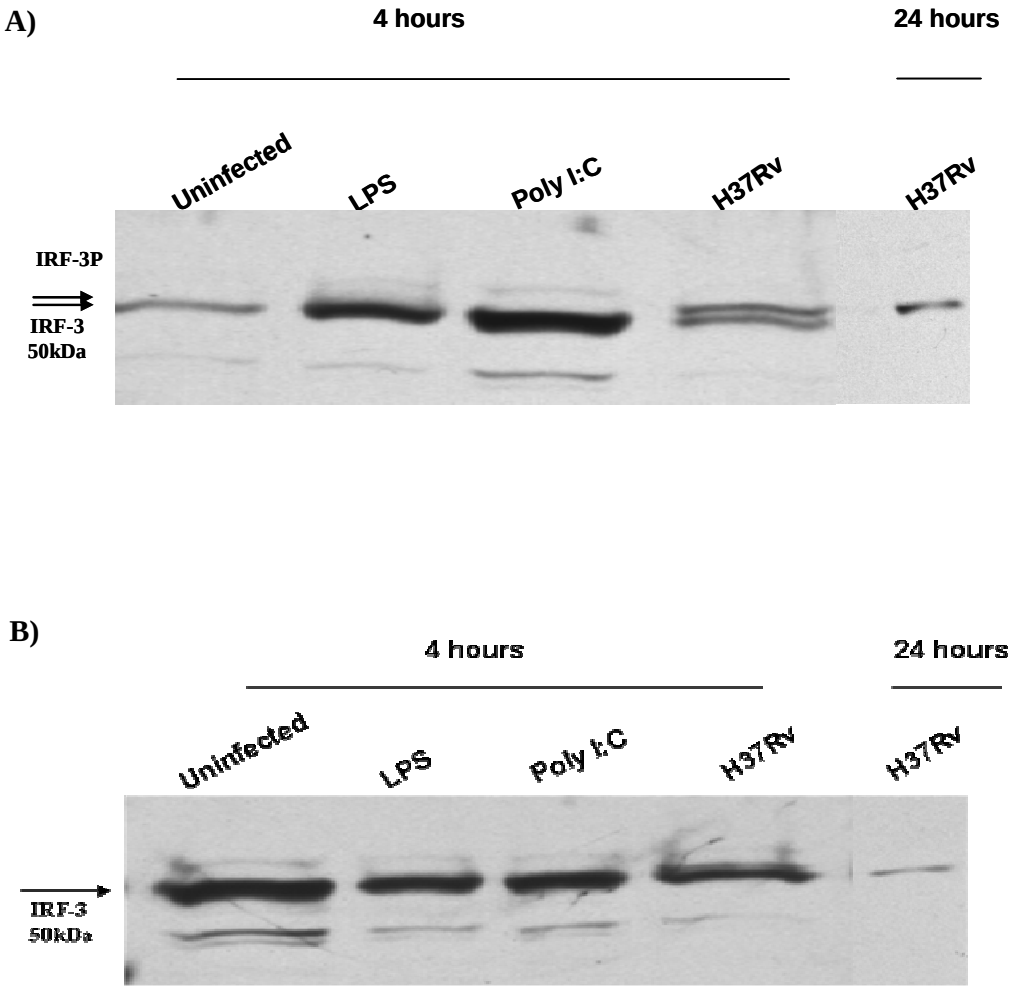


Figure 6.2- Expression of IRF-3 in response to *M. tuberculosis* infection of A) wild-type B) MyD88$^{-/-}$ BMDCs.

BMDCs were uninfected, stimulated with LPS, poly I:C or infected with H37Rv at a M.O.I of 10:1 and nuclear extracts were isolated 4 and 24 hours later. Nuclear extracts were analysed using gel electrophoresis and IRF-3 was detected using anti-IRF-3 antibody.

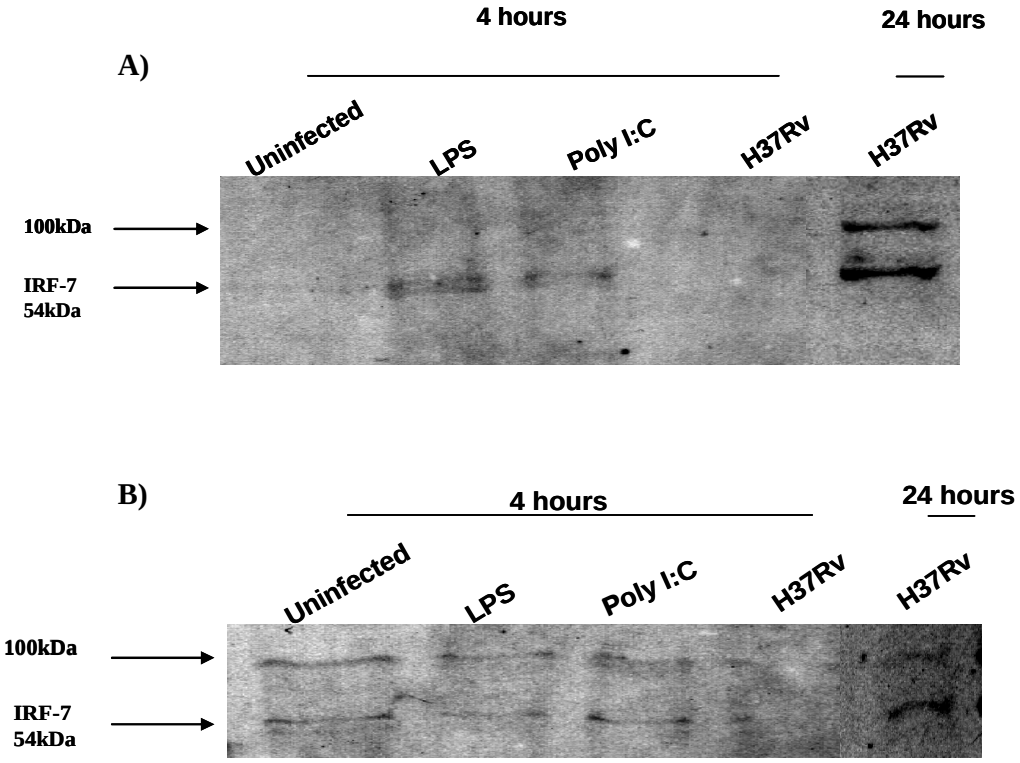


Figure 6.3- Expression of IRF-7 in response to *M. tuberculosis* infection of A) wild-type B) MyD88$^{-/-}$ BMDCs.

BMDCs were uninfected, stimulated with LPS, poly I:C or infected with H37Rv at a M.O.I of 10:1 and nuclear extracts were isolated at 4 and 24 hours later. Nuclear extracts were analysed using gel electrophoresis and IRF-7 was detected using anti-IRF-7 antibody.

6.4-Discussion

IRF-3 and IRF-7 are the two key transcription factors involved in the induction of type I IFN inducible genes. The presence of these two proteins was detected using western blots from nuclear lysates of BMDCs. Since LPS and poly I:C are capable of inducing IFN beta , they were selected as positive controls for the western blot experiments. There was expression of IRF-3 in both wild-type and MyD88$^{-/-}$ in response to LPS and poly I:C stimulation. IRF-3 was induced in the presence of *M. tuberculosis* infection with varying kinetics. There was a strong expression of IRF-3 in wild-type BMDCs with the appearance of the phosphorylated form of the protein at 4 hours post-infection, the overall expression of which decreased at 24 hours post-infection. The decrease in protein expression of IRF-3 is possibly due to the susceptibility of IRF-3 to proteasome mediated degradation. This is similar to the scenario during viral infections,where the phosphorylation of IRF-3 also signals initiation of protein degradation (Hiscott, Pitha et al. 1999). In MyD88$^{-/-}$ BMDCs there was a slight decrease in expression of IRF-3 in response to *M. tuberculosis* infection in comparison with uninfected BMDCs, which further decreased at later time points. It is possible that IRF-3 undergoes proteasome degradation even in MyD88$^{-/-}$ BMDCs at later time point's post-infection similar to the wild-type BMDCs.

IRF-7 is induced in *M. tuberculosis* infected cells in both wild-type and MyD88$^{-/-}$ BMDCs, albeit at lower levels in the absence of MyD88, at later time points post-infection (24 hours). IRF-7 is expressed as two forms; the faster migrating 54kDa sub-unit and the slower migrating 100kDa possibly representing the dimerised form of IRF-7. In wild-type cells the kinetics of expression of IRF-7 are quite clear, with no detectable expression at 4 hours and by 24 hours there is a clear induction with the appearance of the two forms of IRF-7. It is thought that the homodimer of IRF-7 with an approximate band size of 100kDa binds to specific sequences in the promoters of target genes and enhances their transcription (Marie, Smith et al. 2000). IRF-3 is induced at earlier time points post-infection with IRF-7 being expressed at later time points in response to *M. tuberculosis* infection, thus there is a delay in the expression of IRF-7 in response to *M. tuberculosis* infection.

Chapter 7

Priming of anti-mycobacterial immune responses in the absence of MyD88

7.1- Introduction

The adaptor MyD88 plays a crucial role in TLR signalling as characterised by the fact that a number of toll-like receptors like TLR 2, 7 and 9, solely depend on this adaptor for efficient signalling (O'Neill 2003). In addition, MyD88 deficient mice are known to have an impaired ability to activate antigen specific Th-1 immune responses and are susceptible to a number of pathogens including *Listeria monocytogenes* (Edelson and Unanue 2002) and *Toxoplasma gondii* (Scanga, Aliberti et al. 2002). As described previously in this study, microarray results showed that dendritic cell responses after *M. tuberculosis* infection were mediated through both MyD88 dependent and independent pathways; in particular type I IFN responses which were clearly induced in the absence of MyD88.

The experiments described in this chapter were carried out to investigate the priming of anti-mycobacterial immune responses in the absence of MyD88, in an intravenous model of *M. tuberculosis* infection.

7.2- *M. tuberculosis* infection and immune responses in the absence of MyD88

Wild-type (C57BL/6) and MyD88$^{-/-}$ mice were divided into two groups, one group received 10^6 colony forming units (CFU) of H37Rv as a single dose intravenously and the other group was left uninfected. The experimental procedure is described in materials and methods (section 2.8). Whole spleen cells were isolated at 14 days and 30 days post-infection and cultured *in vitro*, in the presence or absence of PPD or anti-CD3/CD28, which served as a positive control. Supernatants were collected at 24 and 72 hours post-stimulation, for quantification of IL-2 and IFN-γ respectively, by ELISA.

As shown in figure 7.1 and 7.2, spleen cells from wild-type and MyD88 $^{-/-}$ infected mice produced IL-2 and IFN-γ in response to PPD stimulation. Furthermore, the observed cytokine production in response to PPD stimulation was due to pre-exposure to mycobacterial antigens as spleen cells from naïve mice do not respond to PPD stimulation.

MyD88$^{-/-}$ T cells displayed a reduced capacity to proliferate in comparison, to wild-type T cells at both 14 and 30 days post-infection (Figure 7.1). In particular, differences were pronounced at 30 days post-infection at a higher PPD dose, in the absence of MyD88 (Figure 7.1, B). In response to a high dose of PPD stimulation, wild-type and MyD88$^{-/-}$ T cells produced IFN-γ at both 14 and 30 days post-infection. But in the absence of MyD88, T cells were defective in IFN-γ production in response to a low dose of antigen, at both 14 and 30 days post-infection, although the difference in IFN-γ production between wild-type and MyD88$^{-/-}$ T cells was less prominent at 30 days post-infection (Figure 7.2). Hence, the data indicates a MyD88 independent route of T cell priming with comparable IFN-γ responses when stimulated with a high antigen dose.

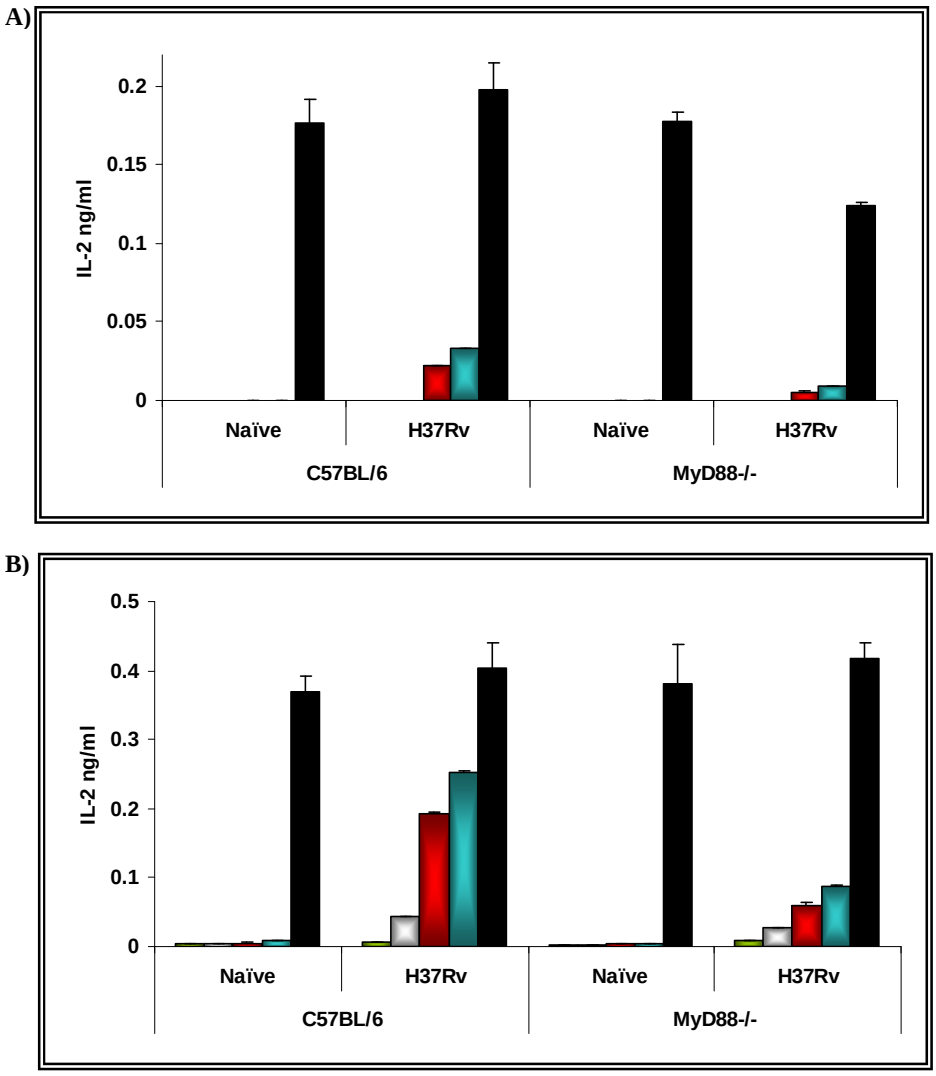


Figure 7.1- Production of IL-2 in response to PPD stimulation of spleen cells from *M. tuberculosis* infected mice.

Whole spleen cells were isolated at **A)** 14 days **B)** 30 days post-infection and stimulated *in vitro* with PPD. Levels of IL-2 were assayed using ELISA, 24 hours post-stimulation. Data are expressed as a mean ±SD of duplicate wells and are representative of two independent experiments.

Saline, PPD- 0.1µg/ml, PPD- 1.0 µg/ml, PPD-10µg/ml CD3/CD28

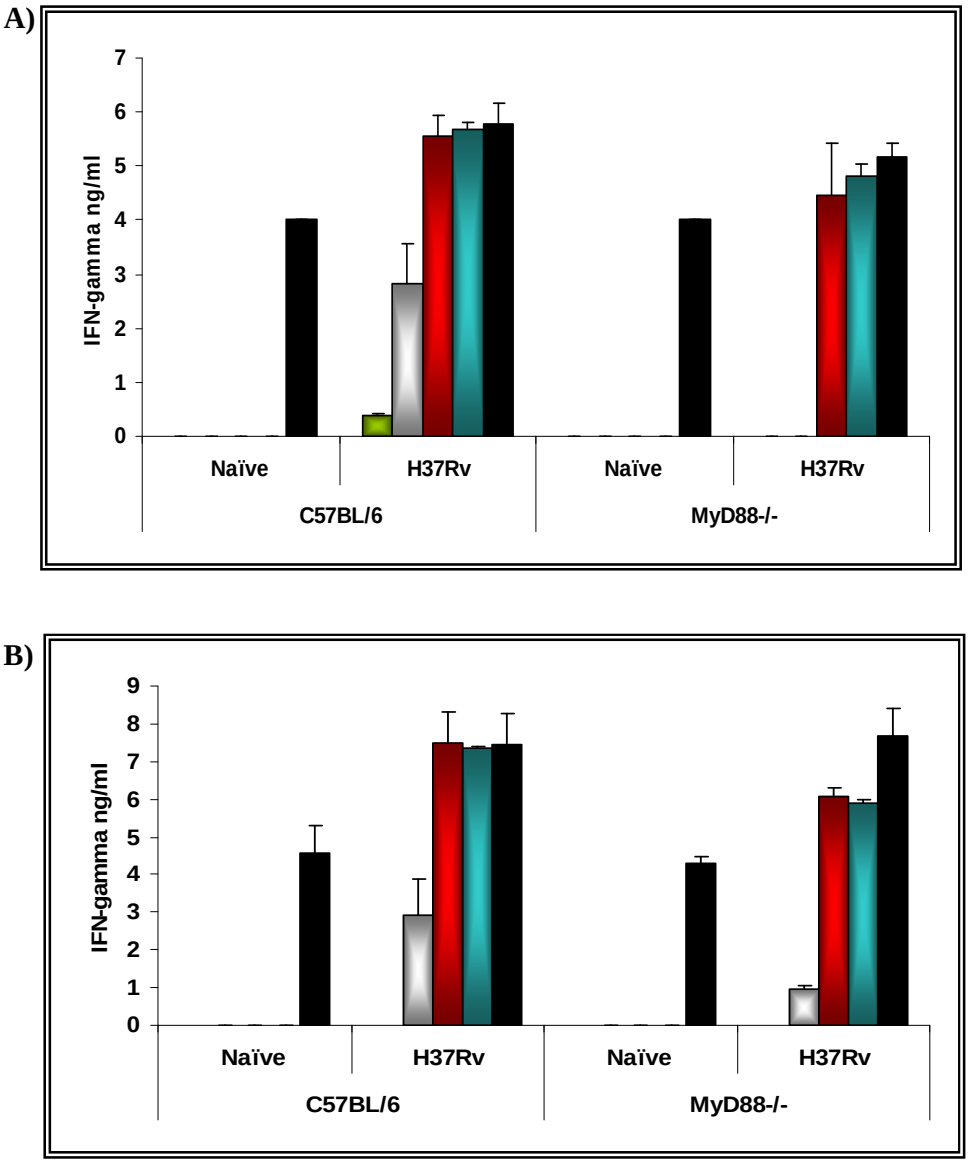


Figure 7.2 – Production of IFN-γ in response to PPD stimulation of spleen cells from *M. tuberculosis* infected mice.

Whole spleen cells were isolated ex-vivo at **A)** 14 days post-infection, **B)** 30 days post-infection and were stimulated using saline, PPD (at 0.1µg/ml, 1µg/ml and 10µg/ml) or anti-CD3/CD28 (1µg/ml). IFN-γ levels were measured in supernatants using ELISA, 72 hours post-stimulation. Results are representative of two independent experiments (mean ±SD of duplicate determinations in one experiment).

Saline, PPD- 0.1µg/ml, PPD- 1.0 µg/ml, PPD-10µg/ml
CD3/CD28

7.3- Determining *M. tuberculosis* bacterial loads in the spleens and lungs of wild-type and MyD88$^{-/-}$ mice

In the previous section, it was demonstrated that both MyD88 knockout mice and wild-type strains of mice were able to prime anti-mycobacterial specific T cells. To address the ability of MyD88$^{-/-}$ mice to control the growth of *M. tuberculosis*, wild-type and MyD88$^{-/-}$ mice were infected intravenously with 10^6 CFU of H37Rv and bacterial counts were determined at 14 and 30 days post-infection, in both lungs and spleens.

As shown in fig 7.3, there was significant increase in the bacterial load in the spleens of MyD88$^{-/-}$ mice at 14 days post-infection. At 30 days post-infection, at least 1 $\log_{10}$ higher bacterial counts were detected in the lungs of MyD88$^{-/-}$ mice. Additionally, at each time point examined there were considerably higher bacterial counts in MyD88$^{-/-}$ mice in comparison to wild-type mice.

Thus, MyD88$^{-/-}$ mice have increased bacterial burdens in both lungs and spleens in comparison to wild-type mice, even though they are able to mount specific T cell responses producing high levels of IFN-γ.

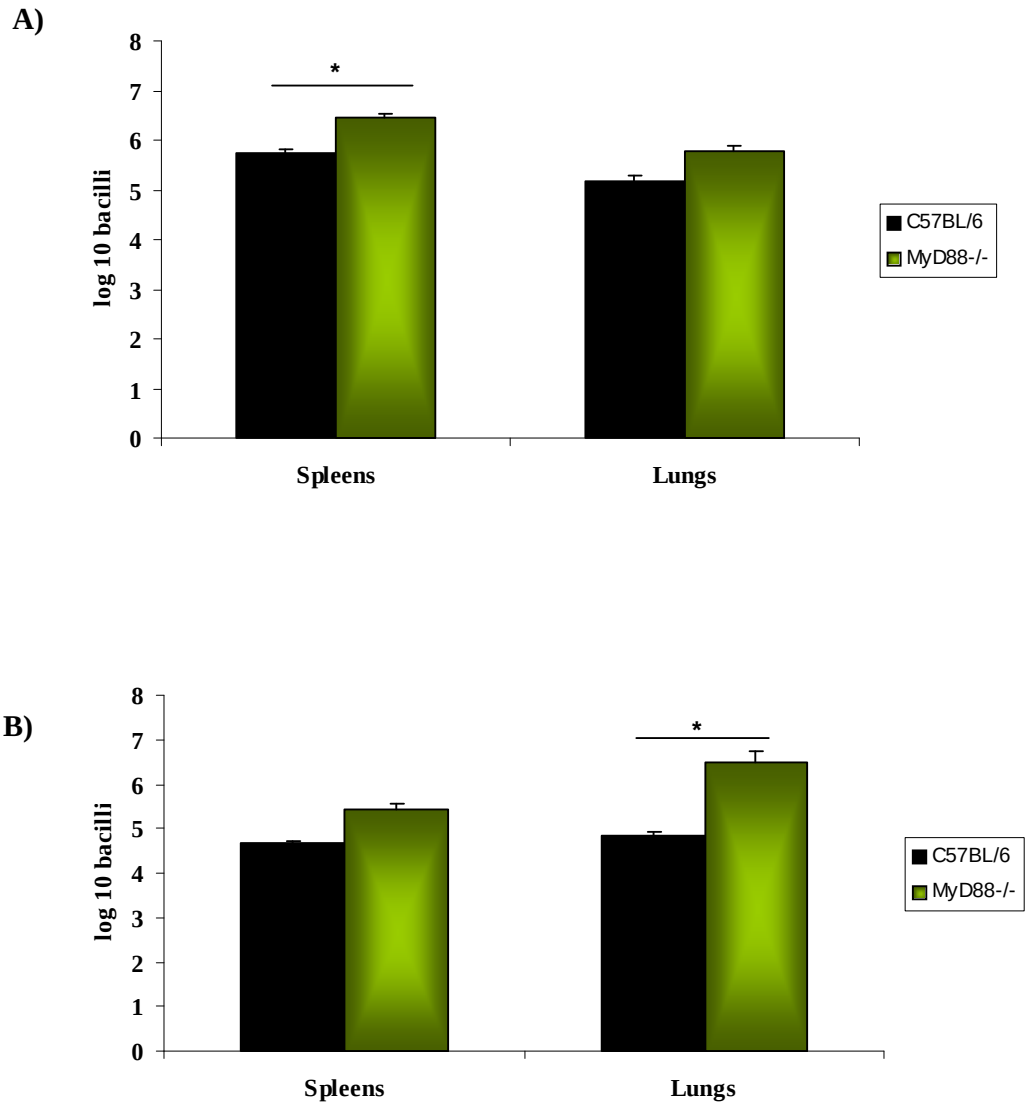


Figure 7.3 –Determining bacterial counts in wild-type and MyD88$^{-/-}$ mice. Mice were injected intravenously with 10^6 colony forming units of H37Rv. At **A)** 14 days and **B)** 30 days post-infection bacterial counts were determined. Results are expressed as the mean ±SEM of the bacterial load in each group expressed as $\log_{10}$ colony forming units (n=3 mice). *- p value < 0.05

7.4 Histological examination of lungs from MyD88 $^{-/-}$ *M. tuberculosis* infected mice

At 30 days post-infection, whole lungs from *M. tuberculosis* infected wild-type and MyD88$^{-/-}$ mice were fixed in 10% formalin and paraffin-embedded lung sections were stained using hematoxylin and eosin. Microscopic examination of the lungs of MyD88$^{-/-}$ mice 30 days post-infection revealed substantial interstitial inflammation and varying degrees of necrosis, nevertheless there was also evidence of few small granulomas (Figure 7.5 B). Lungs of wild-type mice showed no signs of necrosis and there was clear visible granuloma formation (Figure 7.4 B). Naïve wild-type and MyD88$^{-/-}$ mice, which were not infected with H37Rv showed no signs of granuloma formation or interstitial inflammation (Figures 7.4 A and 7.5 A).

Thus, in the absence of MyD88, there is uncontrolled growth of *M. tuberculosis* in the lungs, which is consistent with the increased bacterial counts in the lungs of these mice at 30 days post-infection (Figure 7.3, B).

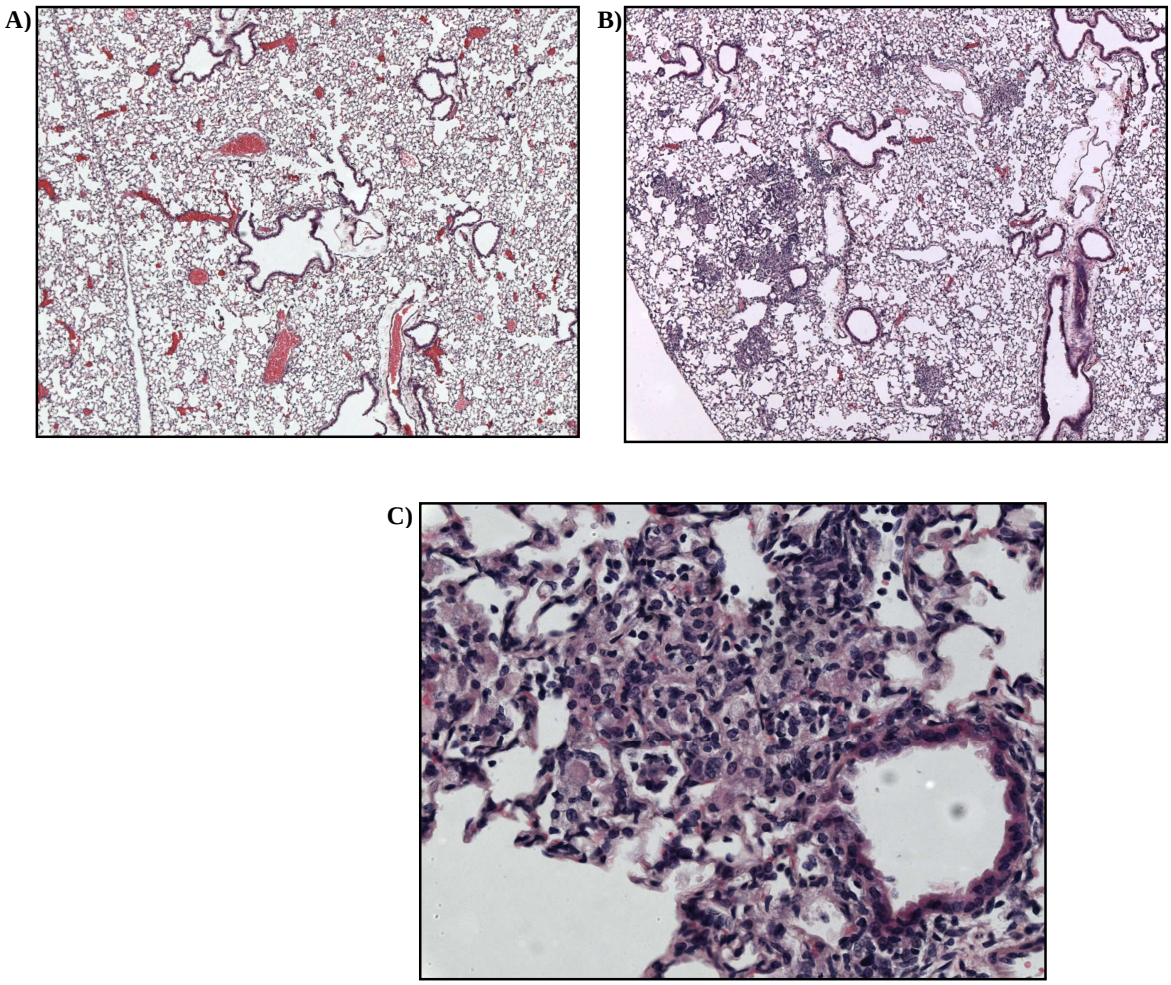


Figure 7.4 – Lung histology of wild-type *M. tuberculosis* infected mice

Lung tissue sections from **A)** naïve mice, **B)** H37Rv infected mice and **C)** *H37Rv* infected mice (higher magnification x 400), were analysed 30 days post-infection. Representative lung sections from n=3 mice are displayed.

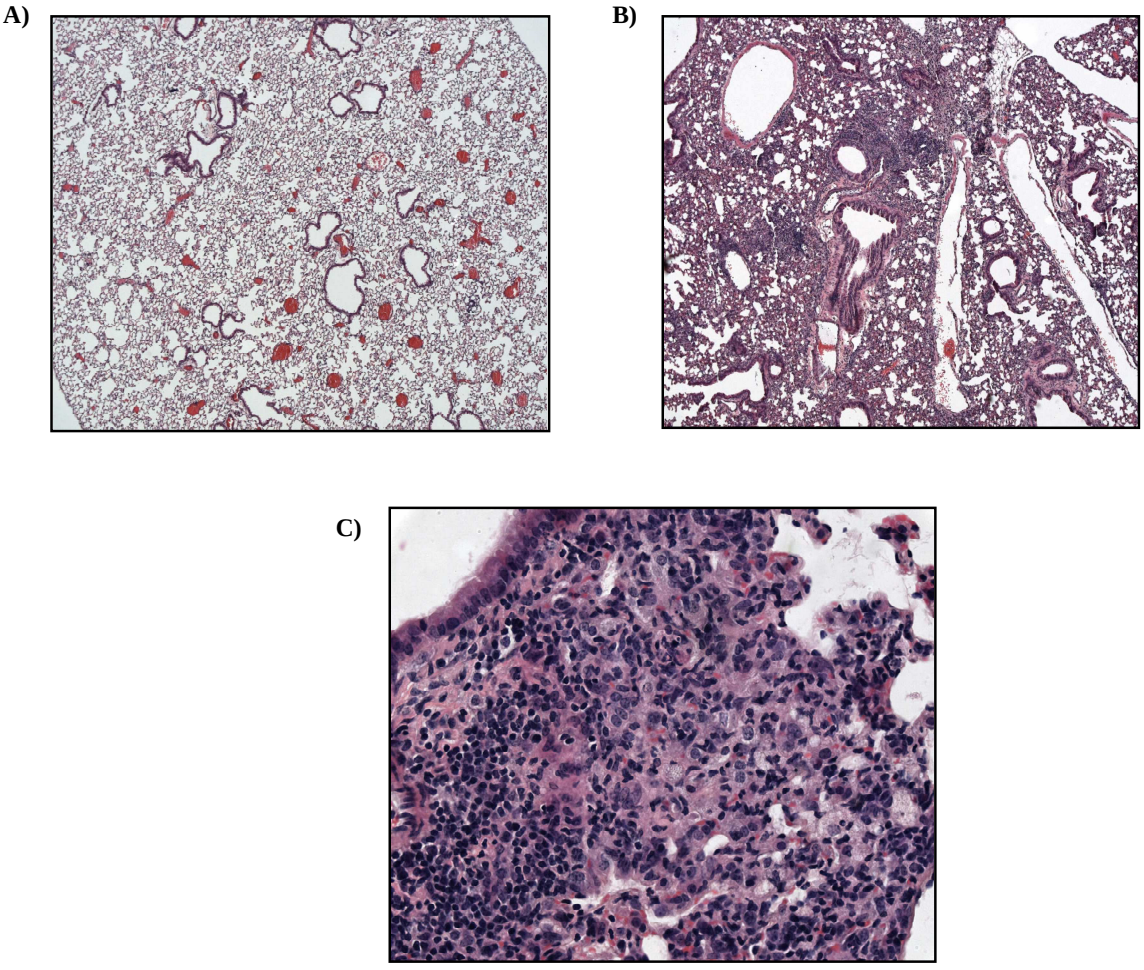


Figure 7.5 – Lung histology of MyD88$^{-/-}$ *M. tuberculosis* infected mice

Lung tissue sections from **A)** naïve mice, **B)** H37Rv infected mice and **C)** *H37Rv* infected mice (higher magnification x 400), were analysed 30 days post-infection. Representative lung sections from n=3 mice are displayed.

7.5- Discussion

The priming of a T cell immune response and the ability to control the growth of *M. tuberculosis* in the absence of MyD88 was addressed in this chapter. MyD88$^{-/-}$ mice were able to produce comparable IFN-γ responses to wild-type mice, although differences were observed when stimulating with a low antigen dose. However, T cells from MyD88$^{-/-}$ mice were impaired in their ability to proliferate at 30 days post infection although the difference was less obvious at 14 days post-infection. There is also a defect in controlling bacterial growth, as shown by the increased levels of bacterial counts in both lungs and spleens of MyD88$^{-/-}$ mice, at 14 and 30 days post-infection. This is reflected in the histological examination of MyD88$^{-/-}$ mice at 30 days post-infection, where there was increased inflammation and evidence of necrosis in the lungs which is suggestive of uncontrolled growth. Although, in the absence of MyD88 there is priming of T cells this is not protective, as demonstrated by the increased bacterial counts, thus the mice succumb to infection.

Chapter 8

Discussion

Dendritic cells are sentinels of the immune system and as antigen presenting cells; they play a key role in processing and presenting antigens to naïve T cells. Studies have reported that dendritic cells are infected with mycobacteria in the lungs and lymph nodes and furthermore, it has been proposed that dendritic cells contribute to the progression of disease by promoting dissemination of bacilli from the lungs to the draining lymph nodes (Humphreys, Stewart et al. 2006; Wolf, Linas et al. 2007). Previous work done in the lab has demonstrated that dendritic cells are capable of phagocytosing mycobacteria, thus leading to the priming of protective immune responses, which are comparable to or better than immune responses primed by BCG (Roy, De Silva et al. 2006) .

The priming of adaptive immune responses by dendritic cells not only requires the presentation of antigen on MHC-II but also the expression of co-stimulatory molecules on the surface and induction of inflammatory cytokines. These properties are regulated by signalling pathways triggered by the binding of microbial ligands to receptors on dendritic cells. However, there is very little information available regarding the signalling pathways in dendritic cells that play determinant roles in modulating the protective responses following *M. tuberculosis* infection. Thus, a detailed characterisation of the signalling pathways that are induced during the interaction of *M. tuberculosis* and dendritic cells will be crucial in understanding the pathogenesis of infection.

The aim of the work described in this thesis was to use a microarray based approach to map the pattern of transcriptional responses of *M. tuberculosis* infected BMDCs and particular emphasis was placed on identifying signalling pathways that are significantly represented during the interaction between the pathogen and dendritic cells. To assist in the dissection of this complex network, the responses in dendritic cells from wild-type mice were compared to responses from dendritic cells from MyD88$^{-/-}$ mice, a key adaptor of the TLR signalling pathway.

8.1- Transcriptional profiling of *M. tuberculosis* infected BMDCs

Transcriptional profiling of *M. tuberculosis* BMDCs was performed using microarrays. Using this approach it was possible to have a global overview of the changes in gene expression of BMDCs in response to *M. tuberculosis* infection, as 39,000 transcripts are represented on a single Affymetrix array.

For this present study, the experimental population of dendritic cells was isolated after 6 days of culture in GM-CSF. Using this method of culture, approximately 70% of the cells were dendritic cells as identified by their expression of CD11c. Using this protocol it was possible to generate a highly reproducible cell population which was suitable for microarray studies. To exclude any possible influence of contaminating cell types, key observations were confirmed using real-time PCR analysis on a population of dendritic cells that were further purified by positive selection using anti-CD11c magnetic beads.

All the microarray analysis was carried out at 24 hours post-infection, based on previous knowledge from the lab and time course analysis of cytokine induction. The signalling pathways under study are clearly dynamic processes and the absence of time course for the microarray analysis represents an important limitation. A multiplicity of infection of 10 bacteria per cell was selected for microarray studies, based on initial experiments measuring cytokine expression. This represents a relatively high dose of *M. tuberculosis*, but was found to generate clearly detectable and reproducible transcriptional changes.

8.2- Signalling pathways induced in *M. tuberculosis* infected BMDCs

An overview of the transcriptional pathways in both wild-type and MyD88$^{-/-}$ *M. tuberculosis* infected BMDCs, highlighted three major pathways: the TLR signalling pathway, the JAK-STAT signalling pathway and the cytokine-cytokine receptor interaction pathway. The role of TLR signalling in the immune response to mycobacteria has been extensively documented. Thus, significant

representation of the TLR signalling pathway is not surprising, as TLRs are important in recognising conserved microbial structures thus playing a critical role in the induction of innate immune responses in the host (Medzhitov 2001). Interestingly, the expression of TLR 2 and TLR 4 which are known to be important in recognising mycobacterial components were down regulated in the absence of MyD88 and up regulated in wild-type BMDCs in response to *M. tuberculosis* infection (Figures 4.5 and 5.7). The expression levels of TLRs have been previously demonstrated to vary between different populations of immune cells. Monocytes in response to LPS increase TLR 4 expression at 2-4 hour post-stimulation (Muzio, Bosisio et al. 2000). However, human dendritic cells were reported to down regulate TLRs during the course of maturation (Visintin, Mazzoni et al. 2001) and similarly, macrophages down regulated TLR4 expression in response to LPS at early time points, with expression levels almost equal to un-stimulated cells by 20 hours post-stimulation (Nomura, Akashi et al. 2000). So it is evident that the TLR expression varies quite considerably with different immune cells and, if down regulation of TLRs is a process associated with dendritic cell maturation, then the question remains as to why the wild-type BMDCs which clearly mature in response to *M. tuberculosis* infection, as indicated by the up-regulation of co-stimulatory molecules and production of inflammatory cytokines, up regulate TLR 2 and 4.

8.2.1- Activation of BMDCs in response to *M. tuberculosis* infection

TLR signalling results in the activation of NFκB driven expression of genes encoding a series of inflammatory cytokines and co-stimulatory molecules, which are involved in the interaction between mature dendritic cells and T cells. Using a bioinformatics approach it was possible to screen for known transcriptional response elements in the upstream region of induced genes (TELiS analysis). This analysis highlighted an important role for NFκB and IRFs. Consistent with the induction of NFκB signalling, genes encoding CD80, CD86 and MHC-II (Figure 3.3) were up regulated in response to *M. tuberculosis* infection. The induction of these activation markers was comparable between wild-type and MyD88$^{-/-}$ BMDCs (Figure 5.2) in response to infection. These

observations are in accordance with previous studies which demonstrated that up regulation of co-stimulatory molecules is independent of MyD88 (Kaisho and Akira 2001; Fremond, Yeremeev et al. 2004). In contrast to the observations in BMDCs, it has been previously reported that macrophages infected with *M. tuberculosis* or stimulated with the 19kDa lipoprotein are defective in their ability to up-regulate MHC-II and furthermore that these stimulants reduced IFN-γ mediated expression of CD86 expression on macrophages (Pai, Pennini et al. 2004).

Cytokines are important mediators of immune responses against various pathogens. In response to *M. tuberculosis* infection, significant levels of inflammatory cytokines were produced by wild-type BMDCs (Figures 3.4, 3.5 and 3.6). In contrast, there was impaired production of IL-12p40, IL-6 and TNF-α by *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs and IL-10 and IL-12p70 were undetectable in the absence of MyD88 (Figures 5.3 and 5.4). IL-10 expression at the mRNA and protein was similar in both wild-type and MyD88$^{-/-}$ *M. tuberculosis* infected macrophages (Shi, Blumenthal et al. 2005) but in response to *M. tuberculosis* infection, BMDCs up regulated IL-10 by a factor of 40.2 fold in wild-type BMDCs and 34.39 fold in MyD88$^{-/-}$ BMDCs, as shown by real-time PCR analysis, thus suggesting a MyD88 independent expression of IL-10. However, the production of IL-10 cytokine was dependent on the MyD88 signalling pathway as there was no measurable IL-10 in *M. tuberculosis* infected MyD88$^{-/-}$ BMDCs.

A recent report suggests that dendritic cells are able to induce high levels of IL-12 production by remodelling the IL-12p40 promoter in response to *M. tuberculosis* infection signalling via TLR 9 (Pompei, Jang et al. 2007). Induction of IL-6 and IL-10 production is predominantly mediated via TLR 2 in response to *M. tuberculosis* infection (Jang, Uematsu et al. 2004). MyD88 is an essential adaptor for signalling via a number of TLRs especially TLR 2 and TLR 9 and thus in the absence of MyD88 signalling via these TLRs is impaired. This could be a contributing factor for the reduction of IL-6 and the reason for the IL-10 being undetected in response to infection of MyD88$^{-/-}$ BMDCs.

High levels of IL-6 and TNF-α production following *M. tuberculosis* infection were detected as early as 6 hours post-infection in wild-type BMDCs but maximum cytokine production was not detected until 48 hours in MyD88$^{-/-}$ BMDCs (Figure 5.3), thus clearly indicating a severe delay in the production of these cytokines. Since NFκB has been strongly implicated in the transcriptional regulation of an array of cytokines including IL-6, the observed delay in cytokine production is possibly due to the delayed NFκB activation that is observed during induction of signalling via the MyD88 independent pathway (Covert, Leung et al. 2005). This delay in activation of NFκB has been attributed to the time required for IRF-3 mediated TNF-α expression, which subsequently binds to the TNF receptors and mediates NFκB activation (Covert, Leung et al. 2005).

The induction of TNF-α production in response to *M. tuberculosis* infection of BMDCs was considerably reduced in the absence of MyD88 in comparison to wild-type BMDCs, which correlates with the reduced induction of TNF-α mRNA in the absence of MyD88 (Figure 5.7, A). These results are suggestive of a MyD88 dependent pathway for the induction of TNF-α at the mRNA and protein level. The dependence on MyD88 for post-transcriptional control is in agreement with studies in *M. tuberculosis* infected macrophages but in contrast, the induction of TNF-α gene was found to be independent of MyD88 in infected macrophages (Shi, Blumenthal et al. 2005).

Studies have suggested that inhibition of phosphoinositide-3 kinase (PI3K) leads to the reduction in LPS induced secretion of TNF-α and similar results were also obtained for MyD88 independent induction of TNF-α in adenovirus infected BMDCs (Philpott, Nociari et al. 2004). PI3K(p85α regulatory subunit) was up regulated by a factor of 2.54 in wild-type BMDCs and 4.91 fold in MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection. Thus it is plausible that TNF-α induction in the absence of MyD88 occurs via a PI3K induction pathway during *M. tuberculosis* infection.

As illustrated in figure 8.1, it is reasonable to believe that in wild-type BMDCs, triggering the MyD88 dependent pathway of NFκB activation (Baltimore, 2005) accounts for the optimal cytokine production in response to infection. However, the delay in cytokine production observed in MyD88$^{-/-}$ BMDCs in response to infection correlates with delayed NFκB activation via the MyD88 independent pathway (Covert, Leung et al. 2005). With the increased expression of PI3K in MyD88$^{-/-}$ BMDCs in response to infection, it is plausible that signalling via PI3K contributes to the production of TNF-α in response to *M. tuberculosis* infection. But the receptor that is involved in the induction of PI3K signalling pathway, in the context of *M. tuberculosis* infection of BMDCs, has yet to be determined.

The production of pro-inflammatory cytokines such as IL-12 by dendritic cells clearly has a role in triggering an effective protective immune response to *M. tuberculosis* .This inflammatory response is balanced by anti-inflammatory cytokines such as IL-10, in order to limit the immunopathology during *M. tuberculosis* infection. Wild-type BMDCs are activated in response to infection, as shown by the production of inflammatory cytokines and up-regulation of CD80, CD86 and MHC-II. In the absence of MyD88, BMDCs up regulate co-stimulatory molecule expression and enhance their T cell stimulatory activity, but it is clear that optimal production of inflammatory cytokines is dependent on MyD88. Overall, these results confirm and extend previous reports of the role of TLR-signalling and NFκB mediated transcription of inflammatory cytokine response to *M. tuberculosis* infection.

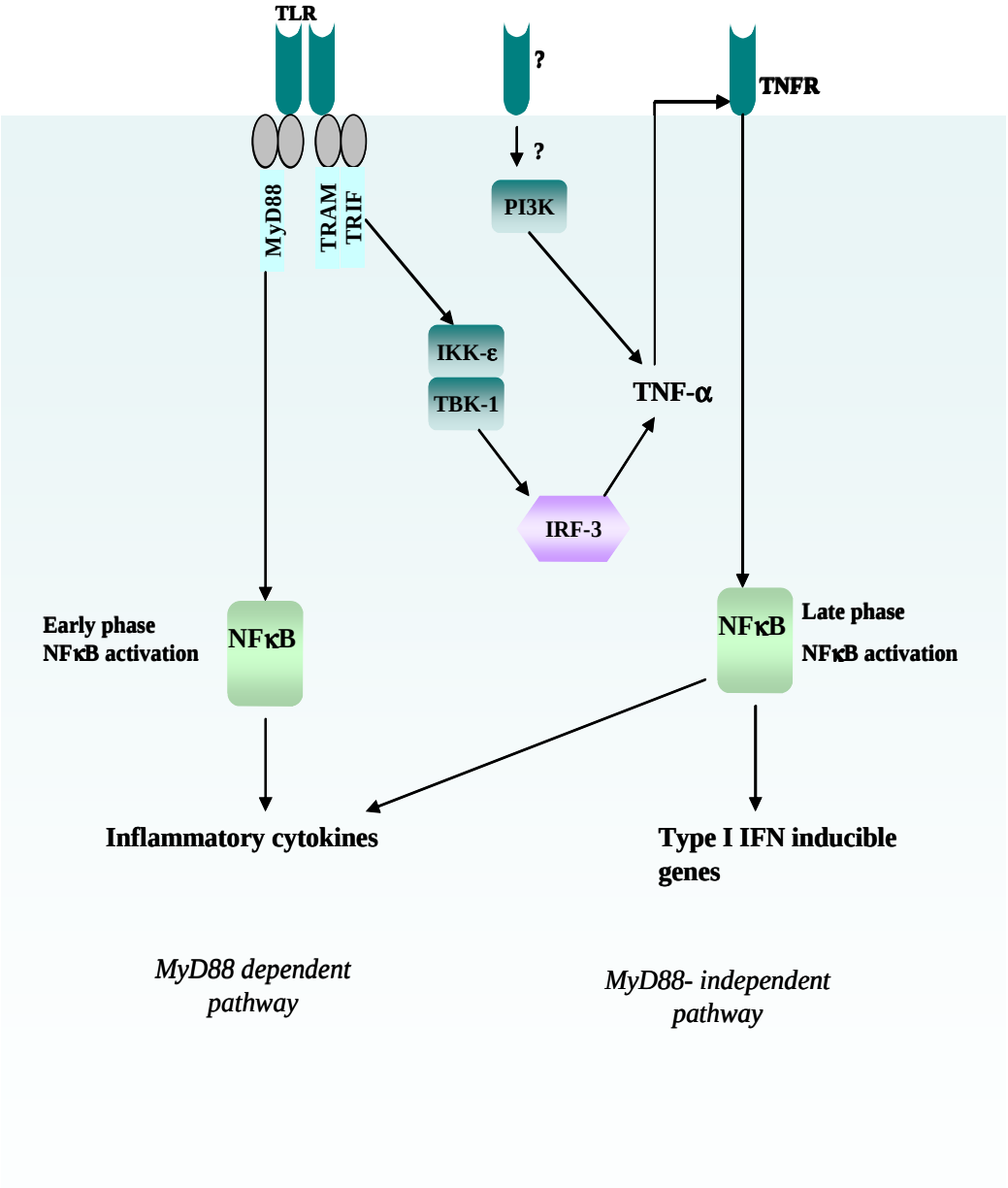


Figure 8.1- MyD88 dependent and independent pathway mediated activation of NFκB

Triggering the activation of NFκB can be mediated by the MyD88 dependent signalling pathway or independent of MyD88 via the induction of TNF-α.

8.3- Induction of IFN alpha and IFN beta in the absence of MyD88

While the role of NFκB mediated transcription in the response to mycobacterial infection is well known, the major contribution of IRFs to the transcription profile was unexpected. In this data, IRF mediated transcription of the type I IFN pathway was a significant feature of the microarray data obtained in response to *M. tuberculosis* infection. Type I IFNs are usually associated with responses to viruses with type II interferons being coupled to mycobacterial responses. Previous studies have demonstrated, that the Esx-1 secretion system is required for the induction of IFN beta in macrophages and a reduction in the ability to produce type I IFNs was associated with an increased capacity to limit the replication of *M. tuberculosis* (Stanley, Johndrow et al. 2007). Additionally it has been demonstrated that high levels of IFN alpha were induced by HN878 (Beijing family), a hypervirulent strain of *M. tuberculosis*. This increased induction of IFN alpha has been associated with the decreased induction of IL-12 (Manca, Tsenova et al. 2001) . The type I IFN response in murine dendritic cells in response to *M. tuberculosis* infection has not been clearly addressed.

Analysis of the microarray expression profile revealed a prominent IFN signature as part of the dendritic cell based immune responses, including up regulation of the major transcription factor IRF-7. Microarray results were corroborated with real-time PCR, which showed significant up regulation of IRF-7 and IRF-3 (the other key type I IFN pathway regulator) at 24 hours post-infection in both wild-type and MyD88$^{-/-}$ BMDCs. Analysis at different time points using real-time PCR demonstrated an initial transient down regulation of IRF-3, followed by the up regulation of IRF-3 at 24 hours post-infection (Figures 4.8 and 5.8). In contrast, the expression of IRF-3 was highly induced at the protein level at early time points, with decreased expression at 24 hours post-infection (Figure 6.2) in both wild-type and MyD88$^{-/-}$ BMDCs. This is associated with proteasomal degradation of IRF-3 (Hiscott, Pitha et al. 1999). IRF-7 was induced by 24 hours post-infection in both wild-type and MyD88$^{-/-}$ BMDCs, thus suggesting a delay in the induction of this transcription factor in comparison to IRF-3. This observation is consistent with the biphasic induction of type I IFNs,

where the level of IRF-7 is increased after the induction of the type I IFN mediated amplification loop (Marie, Durbin et al. 1998).

Type I interferons induce their synthesis by activating both STAT 1 and STAT 2, both of which are key players in the type I IFN pathway and are up regulated by a factor of 3 fold or more in both wild-type and MyD88$^{-/-}$ BMDCs (Table 5.7). Activation of the type I IFN signalling pathway led to the comparable production of IFN beta in both wild-type and MyD88$^{-/-}$ BMDCs (Tables 4.10 and 5.10). In contrast, IFN alpha production was detected at later time points post-infection only in MyD88$^{-/-}$ BMDCs (Figure 5.11), although IFN alpha was induced at the mRNA level in both wild-type and MyD88$^{-/-}$ dendritic cells (Figure 5.11). This induction of type I IFNs in BMDCs is similar to a study, which demonstrated that human dendritic cells can produce type I IFNs in response to *M. tuberculosis* infection (Remoli, Giacomini et al. 2002).

IRF-7 is critical in inducing IFN alpha gene expression (Honda, Yanai et al. 2005) and is necessary for the induction of late response IFN alpha genes (non-IFN alpha 4 genes) which requires ongoing protein synthesis. IFN alpha 4 (early response gene) expression and IFN alpha 2, 7 and 9 (late response gene) expression was comparable in both MyD88$^{-/-}$ and wild-type BMDCs in response to infection (Figures 4.10 and 5.10) Thus, it is plausible that the expression of IRF-7 even in the absence of MyD88 is responsible for the similar expression of IFN alpha genes in both wild-type and MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection.

The results demonstrate the overall dominance of the IRF and NFκB mediated transcription, which is retained even in the absence of MyD88, in response to *M. tuberculosis* infection. The induction of IFN alpha and beta in dendritic cells is independent of MyD88 in response to *M. tuberculosis* infection.

8.4- Expression of type I IFN inducible genes as an integral part of the dendritic cell based immune response

Detailed analysis of the microarray data revealed a subset of type I IFN inducible genes which were differentially regulated in wild-type and MyD88$^{-/-}$ BMDCs in response to *M. tuberculosis* infection. Key genes involved in anti-viral immunity were up regulated in wild-type BMDCs in response to infection which included the OAS family of genes which are important in the activation of RNase L leading to the degradation of viral RNA , Pkr (RNA dependent protein kinase) and Mx2 (family of GTPases), both which are activated by type I IFNs. In contrast, these genes did not exhibit a significant change in expression in infected MyD88$^{-/-}$ BMDCs (Table 5.9).

In the absence of MyD88, a subset of type I IFN inducible genes are still induced in response to *M. tuberculosis* some of which share similar expression patterns to wild-type mice. Type I IFN induced genes commonly induced in wild-type and MyD88$^{-/-}$ BMDCs include genes involved in signalling (STAT-1, STAT-2, IRF-7, Traf3, Ifih1), antiviral activities (G1p2, Isg20), cytokines/chemokines (CXCL10, Ebi3, IL12b, IL6, Ifnb1, Ifna2, Ifna4, Ifna7, Ifna9), MHC antigen processing and presentation (H2-q10, Tap1, Psme2) and immune response genes (Ifit1, Ifi35).

Although a number of IFN beta and IFN alpha genes and key signalling molecules (IRF-7) were induced in the absence of MyD88$^{-/-}$ there still exists an clear defect in the induction of OAS, Pkr, Mx proteins, which are well characterised and considered to be key anti-viral immune response genes. Previous studies have demonstrated that TLR3 and TLR 4 can induce expression of a set of IFN inducible genes in macrophages, which were classified as ‘primary response genes’- IFN beta, CXCL10, G1p2 and Ifit1 (up regulated by 2 hours in response to LPS stimulation in the presence of cycloheximide) or ‘secondary

response genes'-Mx1, Ifi1 and IRF-7 (induced after 2 hours, sensitive to cycloheximide treatment) (Doyle, Vaidya et al. 2002).

As illustrated in figure 8.2, type I IFN primary response genes are induced in both wild-type and MyD88$^{-/-}$ BMDCs in response to infection albeit at lower levels in the absence of MyD88. It is reasonable to believe that a delay or suppression in the amplification loop mechanism may lead to the down regulation or lack of expression of the secondary response genes in MyD88$^{-/-}$ BMDCs (Figure 8.2). This observation is further highlighted by the unchanged expression of the transcript for IRF-9 in MyD88$^{-/-}$ BMDCs. IRF-9 is part of the transcriptional complex in the amplification loop mediating the transcription of the secondary response genes. However, the expression of IRF-7 is comparable between wild-type and MyD88$^{-/-}$ BMDCs in response to infection.

In general, although minor differences do exist in the induction of type I IFN inducible genes, it can be concluded that the type I IFN response to *M. tuberculosis* infection is predominantly MyD88 independent.

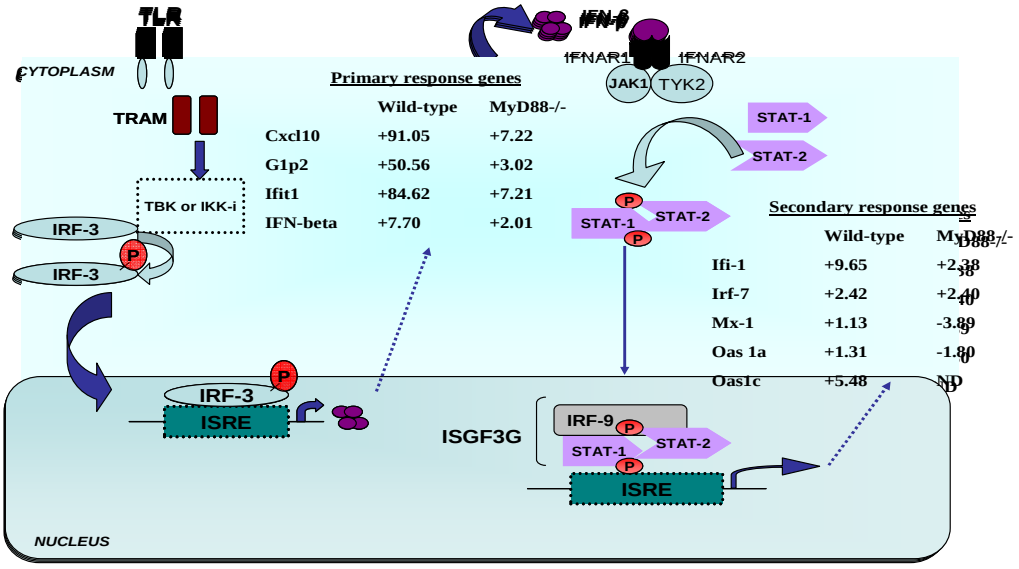


Figure 8.2- Induction of type I IFN primary and secondary response genes in *M. tuberculosis* infected BMDCs

IRF-3 induces the expression of a set of primary response genes including IFN beta which binds to the IFN (alpha and beta receptor) and induces the expression of secondary response genes. All the fold change values for the genes were obtained from microarray and real-time PCR data. ND- change in expression of the gene is not statistically significant.

8.5-Priming of anti-mycobacterial T-cells in the absence of MyD88

The induction of type I IFNs in the absence of MyD88 prompted the study of the role the MyD88 in priming anti-mycobacterial T cell responses. Innate immune responses were impaired in MyD88$^{-/-}$ mice infected with *M. avium* and during H37Rv infection even though there was priming of acquired immune responses (Fremond, Yeremeev et al. 2004; Scanga, Bafica et al. 2004). In contrast, (Sugawara, Yamada et al. 2003) found that MyD88$^{-/-}$ mice were not highly susceptible to *M. tuberculosis* infection. The difference between the report by Sugawara et al and others was attributed to the different *M. tuberculosis* strains that were used for infection and the background strain of the knockout mice.

In this study, in response to antigen stimulation, there was reduced proliferation of T cells from MyD88$^{-/-}$ mice when compared to T cells from wild-type mice, although T cells from MyD88$^{-/-}$ mice produced almost equivalent levels of IFN-γ to wild-type mice when stimulated with a high antigen dose (Figures 7.1 and 7.2).. However, there were significantly increased bacterial counts in the spleens at 14 days and lungs at 30 days post-infection which is also reflected in the histology. Increased necrotic lesions at 30 days in the lungs of MyD88$^{-/-}$ mice signified the uncontrolled growth of the bacteria in these mice. One of the reasons associated with the inability of controlling bacterial loads could be the defective cytokine production from key antigen presenting cells such as dendritic cells. The impaired cytokine production could also contribute to the inefficient T cell proliferation observed in MyD88$^{-/-}$ mice (Figure 7.1). Furthermore, the production of IL-6 has been suggested to be important in rendering pathogen primed T cells refractory to T cell mediated suppression (Pasare and Medzhitov 2003); the induction of this cytokine is severely impaired from MyD88$^{-/-}$ dendritic cells in response to infection (Figure 5.3, B). Recently, it has also been demonstrated that impaired signalling through the IL-1 receptor also contributes to the defective control of *M. tuberculosis* infection which is similar to that seen in MyD88$^{-/-}$ mice (Fremond, Togbe et al. 2007).

Thus, in the presence of a strong T cell response (IFN-γ production), MyD88$^{-/-}$ mice still had elevated levels of bacterial counts which were more prominent at 30 days post infection. This phenomenon could be attributed to the defective cytokine production from key antigen presenting cells like dendritic cells which are required for the long term control of the infection and also to the IL-1 receptor signalling pathway which is also impaired in the absence of MyD88.

8.6- Conclusions

Global gene expression analysis has enabled the identification of signalling pathways known to be important in the dendritic cell based immune responses including the TLR signalling pathway and type I IFN pathway, with IRFs and NFκB being the primary transcription factors responsible for the modulation of the transcriptional changes in response to infection. This is clearly a dynamic process and the focus of the present analysis on a single time point at 24 hours after infection probably underestimates the overall complexity. Restriction of analysis to the transcriptional responses also fails to capture information about changes in protein phosphorylation and subcellular location.

Analysis of the process of *M. tuberculosis* infection of dendritic cells by expression profiling identifies two major transcriptional themes: a prominent pro-inflammatory cytokine response leading from the activation of the NFκB which contributes to the induction of a Th-1 response associated with resistance to mycobacterial infection, and the induction of the type I IFN response which is via IRF-mediated pathways. Interestingly, the induction of type I IFNs is predominantly independent of MyD88 in *M. tuberculosis* infected BMDCs although the expression of important anti-viral genes is impaired in the absence of MyD88.

In the absence of MyD88, T cell stimulatory (up regulation of CD80, CD86 and MHC-II) and migration capability of mature dendritic cells is intact,

however, another aspect of dendritic cell maturation response including optimum inflammatory cytokine production from BMDCs is dependent on MyD88. In accord with previous macrophage studies, MyD88$^{-/-}$ BMDCs retain in large part their ability to recognise and respond to mycobacterial infections. It is reasonable to speculate that as yet unidentified receptors may contribute to the responses observed in a MyD88 independent manner, alongside signalling via the TRAM/TRIF pathway as described for TLR 4.

Thus, the type I IFN response appears to be an integral part of the dendritic cell based immune response and hence understanding the role of these cytokines during mycobacterial infections will be crucial.

In general, deciphering the mechanisms of interaction between mycobacteria and dendritic cells, with particular reference to the signalling pathways that are evoked, will aid in understanding the modulation of host mediated immune responses to this highly adapted intracellular pathogen. An understanding of these mechanisms of immune actions can be an important step forward in designing new therapeutic approaches as well as improving vaccine design.

8.7- Future perspectives

The work presented in this thesis provides an opportunity to characterise the type I IFN mediated response which has been demonstrated to be induced in the absence of MyD88 in *M. tuberculosis* infected BMDCs. The role of type I IFNs during mycobacterial infection is not well understood. It has been previously reported that the HN878, Beijing family of *M. tuberculosis* induced high levels of type I IFNs but in contrast, a recent report suggests that type I IFN production is not unique to the Beijing family but is also induced by other strains of *M. tuberculosis* (Manca, Tsenova et al. 2001; Ordway, Henao-Tamayo et al. 2007). It will be interesting to explore the nature of events responsible for the activation of the type I IFN pathway, as it has been reported that the induction of type I IFNs involves the esx-1 secretion system which is a major determinant of virulence in *M. tuberculosis* (Stanley, Johndrow et al. 2007).

If the type I IFN response is indeed deleterious to protective immunity, understanding the underlying recognition pathway for the induction of type I IFNs would be crucial. Interpreting the cross-talk between MyD88 independent and type I IFN pathways will be important in understanding the events that lead to the development of cell mediated immunity.

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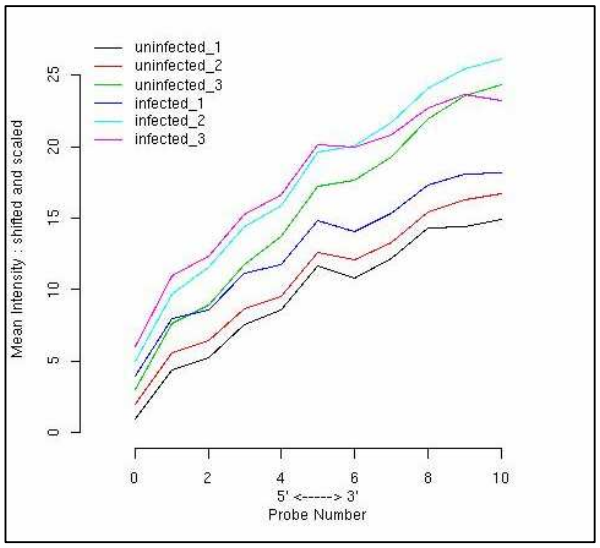
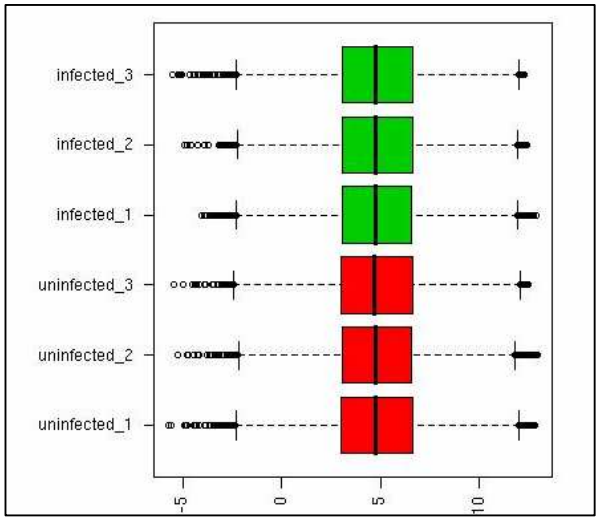
Appendix-I – Affymterix quality control plots- Wild-type BMDCs

RNA digestion plot of wild-type BMDCs



The RNA plots are typical of one cycle amplification and there are no apparent signs of RNA degradation thus indicating a good quality of RNA.

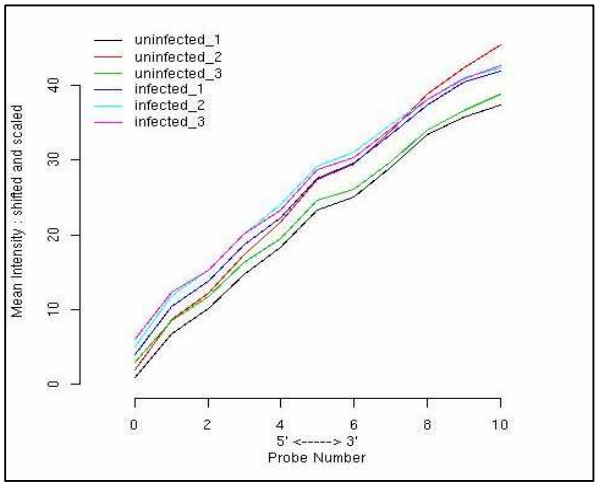
Box plot of wild-type BMDCs



The box plot indicates some variation in the mean intensity value but this was reduced after the normalisation. Generally, the replicates are in good agreement with each other.

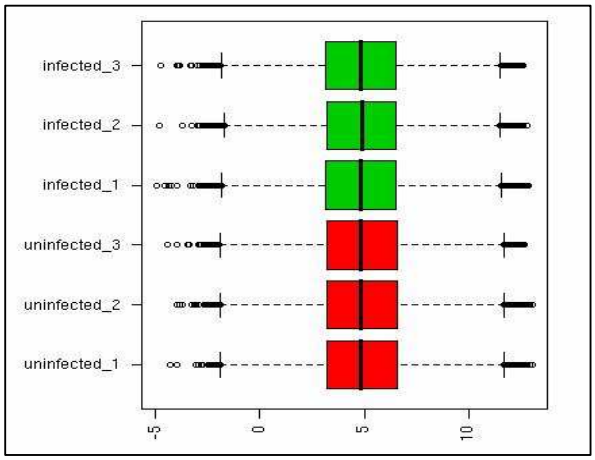
Appendix-II -Affymterix quality control plots- MyD88$^{-/-}$ BMDCs

RNA digestion plot of MyD88$^{-/-}$ BMDCs



The RNA plots are typical of two cycle amplification and the digestion plot indicates a good quality of RNA.

Box plot of MyD88$^{-/-}$ BMDCs



The box plots indicate that the replicates are in good agreement with each other.

Appendix- III- Western Blot reagents

NuPage LDS (Lithium dodecyl sulphate) Sample buffer

Glycerol-10%,
Tris Base-141 mM
Tris HCl-106 mM
LDS-2%
EDTA-0.51 mM
SERVA® Blue G250-0.22 mM
Phenol Red-0.175 mM
pH 8.5

Tris-Glycine Buffer SDS running buffer

25 mM Tris (pH 8.3)
192 mM glycine
0.1% SDS

10% Tris-HCL gels

10% resolving gel
4% stacking gel

Transfer buffer

25mM Tris (3.03g)
192mM glycine (14.4g)
20% v/v methanol (200ml)
Add distilled water to 1 litre (pH 8.3)

Appendix-IV- KEGG pathways- Wild-type BMDCs

KEGG pathway list identified from microarray analysis of *M. tuberculosis* infected BMDCs (wild-type)

Genes overlapping with Pathways	p value
Toll-like receptor signaling pathway	8.68E-12
Jak-STAT signaling pathway	1.17E-10
Cytokine-cytokine receptor interaction	2.06E-09
Type I diabetes mellitus	3.44E-06
Hematopoietic cell lineage	1.14E-04
Antigen processing and presentation	4.89E-04
Cell adhesion molecules (CAMs)	7.68E-04
Adipocytokine signaling pathway	1.01E-03
Type II diabetes mellitus	9.52E-03
ABC transporters	1.17E-02
Apoptosis	2.05E-02
Phosphatidylinositol signaling system	2.25E-02
B cell receptor signaling pathway	6.45E-02
mTOR signaling pathway	6.49E-02
T cell receptor signaling pathway	9.62E-02
Focal adhesion	1.10E-01
Natural killer cell mediated cytotoxicity	1.17E-01
MAPK signaling pathway	1.47E-01
Notch signaling pathway	1.76E-01
Complement and coagulation cascades	1.80E-01
Cell cycle	2.94E-01
ECM-receptor interaction	3.04E-01
Neuroactive ligand-receptor interaction	4.12E-01
Fc epsilon RI signaling pathway	5.36E-01
TGF-beta signaling pathway	5.50E-01
VEGF signaling pathway	5.71E-01
Leukocyte transendothelial migration	5.81E-01
Regulation of actin cytoskeleton	6.35E-01
Wnt signaling pathway	7.71E-01

Appendix-V- KEGG pathways- MyD88$^{-/-}$ BMDCs

KEGG pathway list identified from microarray analysis of *M. tuberculosis* infected BMDCs (MyD88$^{-/-}$)

KEGG pathways	p-value
Jak-STAT signaling pathway	6.52E-11
Cytokine-cytokine receptor interaction	1.02E-09
Toll-like receptor signaling pathway	7.45E-09
B cell receptor signaling pathway	1.97E-06
T cell receptor signaling pathway	1.05E-05
Complement and coagulation cascades	4.31E-05
Focal adhesion	1.32E-04
Leukocyte transendothelial migration	2.94E-04
Cell cycle	3.27E-04
Apoptosis	4.93E-04
Hematopoietic cell lineage	1.98E-03
Cell adhesion molecules (CAMs)	2.85E-03
ABC transporters	3.05E-03
Proteasome	6.12E-03
mTOR signaling pathway	1.54E-02
Natural killer cell mediated cytotoxicity	1.90E-02
ECM-receptor interaction	2.10E-02
SNARE interactions in vesicular transport	2.18E-02
Adipocytokine signaling pathway	2.22E-02
Regulation of actin cytoskeleton	2.56E-02
MAPK signaling pathway	3.91E-02
Fc epsilon RI signaling pathway	4.02E-02
Phosphatidylinositol signaling system	5.96E-02
Type II diabetes mellitus	7.29E-02
Type I diabetes mellitus	8.37E-02
Atrazine degradation	1.42E-01
Gap junction	1.51E-01
TGF-beta signaling pathway	1.76E-01
VEGF signaling pathway	2.14E-01
Tight junction	2.41E-01
Antigen processing and presentation	3.81E-01
Cell Communication	9.09E-01
Adherens junction	9.80E-01

Appendix- VI- Normalised gene values

Type I IFN inducible genes commonly represented in both C57BL/6 and MyD88$^{-/-}$ BMDCs

The normalised values for the type I IFN inducible genes (pooled from microarray and real-time PCR data) in both uninfected and H37Rv10:1 (infected) groups are displayed.

Affymetrix ID	Normalised gene values				Common name	Description	
	Wild-type		MyD88$^{-/-}$				
	Uninfected	Infected	Uninfected	Infected			
1423555_a_at	0.03	3.41	0.37	1.71	A430056A10Rik	interferon-induced protein 44	Antiviral immunity
1418240_at	0.5	1.98	0.48	1.41	Gbp2	guanylate nucleotide binding protein 2	
1450783_at	0.03	3.05	0.23	1.72	Ifit1	interferon-induced protein with tetratricopeptide repeats 1	
1419569_a_at	0.1	3.42	0.18	1.87	Isg20	interferon-stimulated protein 20	
1424339_at	0.05	2.84	0.13	1.87	Oasl1	2'-5' oligoadenylate synthetase-like 1	
1418587_at	0.7	1.4	0.21	1.67	Traf3	Tnf receptor-associated factor 3	
1418930_at	0.03	3.35	0.26	1.89	Cxcl10	chemokine (C-X-C motif) ligand 10	Cytokines and chemokines
1449497_at	0.16	1.91	0.20	2.47	Il12b	interleukin 12b	
1417244_a_at	0.03	2.25	0.32	1.71	Irf7	interferon regulatory factor 7	IRFs and Type I IFN pathway
1420915_at	0.33	1.79	0.53	1.35	Stat1	signal transducer and activator of transcription 1	
1421911_at	0.21	2.14	0.42	1.42	Stat2	signal transducer and activator of transcription 2	
1426324_at	0.78	1.4	0.67	1.49	H2-Q10	histocompatibility 2, D region locus 1	Antigen processing and presentation via MHC-I
1417189_at	0.73	1.47	0.45	1.43	Psme2	proteasome (prosome, macropain) 28 subunit, beta	
1416016_at	0.6	1.74	0.40	1.36	Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	
1434268_at	0.62	1.61	0.78	1.65	Adar	adenosine deaminase, RNA-specific	Regulation of transcription
1450744_at	0.59	1.56	0.42	1.69	Ell2	elongation factor RNA polymerase II 2	
1435446_a_at	0.58	1.56	0.73	1.36	Chpt1	choline phosphotransferase 1	Miscellenaeous
1451426_at	0.2	2.48	0.37	1.52	D11Lgp2e	DNA segment, Chr 11, Lothar Hennighausen 2, expressed	
1422433_s_at	1.32	0.74	1.70	0.68	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	
1421207_at	0.52	1.53	0.20	2.10	Lif		
1452178_at	0.37	2.25	0.56	1.26	Plec1	plectin 1	
1434015_at	0.48	1.6	0.61	1.24	Slc2a6	solute carrier family 2 (facilitated glucose transporter), member 6	
1417961_a_at	0.21	1.78	0.58	1.49	Trim30	tripartite motif protein 30	
1418191_at	0.31	2.22	0.34	1.53	Usp18	ubiquitin specific peptidase 18	

Appendix- VII- Complete gene list of differentially regulated genes in response to *M. tuberculosis* infection of wild-type BMDCs

All genes have a fold change of 1.4 and a p value ≤$_{0.05}$.

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1435529_at	136.3	2010002M12Rik	1440866_at	10.62		1426906_at	5.465	Ifi203
1417262_at	136	Ptgs2	1429947_a_at	10.56	Zbp1	1422293_a_at	5.354	4933402K10Rik
1418930_at	91.05	Cxcl10	1444350_at	10.53	9830137M10Rik	1422005_at	5.347	Prkr
1423555_a_at	90.56	A430056A10Rik	1421911_at	10.22	Stat2	1450033_a_at	5.346	Stat1
1450783_at	84.62	Ifit1	1422526_at	10.01	Acsl1	1421307_at	5.335	Car13
1418652_at	81.27	Cxcl9	1441887_x_at	9.822	Sh3bgrl	1425405_a_at	5.305	Adar
1450291_s_at	71.12	Ms4a4c	1449134_s_at	9.729	Spic	1439141_at	5.251	Gpr18
1420671_x_at	68.61	Ms4a4b	1424393_s_at	9.692	Adhfe1	1422668_at	5.228	Serpinb9b
1427381_at	65.92	Irg1	1418825_at	9.655	Ifi1	1434991_at	5.223	1110064L07Rik
1417244_a_at	65.58	Irf7	1451777_at	9.618	BC013672	1448681_at	5.215	Il15ra
1418293_at	62.53	Ifit2	1448918_at	9.557	Slco3a1	1447649_x_at	5.211	Dnajc1
1424339_at	56.58	Oasl1	1452879_at	9.505	Synpo2	1449317_at	5.206	Cflar
1431591_s_at	56.11	G1p2	1455656_at	9.39	Btla	1420915_at	5.099	Stat1
1417263_at	54.26	Ptgs2	1453757_at	9.388	2510038N07Rik	1445271_at	5.037	9230105E10Rik
1417314_at	50.13	H2-Bf	1420664_s_at	9.319	Procr	1419758_at	4.965	Abcb1a
1450322_s_at	48	Slfn4	1450643_s_at	9.295	Acsl1	1448881_at	4.955	Hp
1427102_at	44.39	Slfn4	1441206_at	9.191	Synpo2	1421998_at	4.951	Tor3a
1419043_a_at	43.63	AW111922	1415806_at	8.776	Plat	1421550_a_at	4.95	Trim34
1421009_at	42.1	2510004L01Rik	1436562_at	8.523	Ddx58	1441923_s_at	4.884	Edn3
1422095_a_at	40.94	Tyki	1417961_a_at	8.49	Trim30	1424857_a_at	4.884	Trim34
1419209_at	39.65	Cxcl1	1439620_at	8.429	Car13	1450214_at	4.878	Adora2b
1449009_at	38.22	Tgtp	1450454_at	8.005	Tor3a	1420466_at	4.79	Spt2
1421008_at	36.92	2510004L01Rik	1425065_at	8.005	Oas2	1425686_at	4.768	Cflar
1419042_at	36.25	AW111922	1419149_at	7.801	Serpine1	1460603_at	4.746	AA175286
1455451_at	33.16	AI449310	1451564_at	7.643	1600029O10Rik	1419534_at	4.739	Olr1
1419569_a_at	32.59	Isg20	1458458_at	7.575	Slfn5	1439825_at	4.703	BC023741
1450297_at	31.49	Il6	1456509_at	7.536	1110032F04Rik	1432548_at	4.7	1600029O10Rik
1435792_at	30.93	Csprs	1417141_at	7.456	Igtp	1435340_at	4.686	Jmjd2a
1419282_at	29.13	Ccl12	1434580_at	7.445	Enpp4	1452414_s_at	4.673	D19Ertd678e
1438868_at	28.77	Phf11	1450403_at	7.442	Stat2	1451798_at	4.638	Il1rn
1419604_at	27.05	Zbp1	1417420_at	7.37	Ccnd1	1460014_at	4.588	5031403H21Rik
1425890_at	25.9	Ly6i	1428660_s_at	7.214	Tor3a	1417193_at	4.577	Sod2
1436058_at	24.31	2510004L01Rik	1437176_at	7.181	AI451557	1448610_a_at	4.548	Sod2
1449497_at	23.95	Il12b	1431865_a_at	7.153	4933405K07Rik	1424448_at	4.547	Trim6
1427126_at	23.83	Hspa1b	1418191_at	7.15	Usp18	1448940_at	4.542	Trim21
1438676_at	23.76	AI595338	1421744_at	7.105	Tnfsf4	1434087_at	4.53	Mthfr
1427127_x_at	23.46	Hspa1b	1418612_at	7.073	Slfn1	1422953_at	4.528	Fpr-rs2
1447927_at	21.11	AI595338	1426276_at	7.067	9130009C22Rik	1453472_a_at	4.495	Slamf7
1420768_a_at	21.02	D11Lgp2e	1421308_at	7.043	Car13	1423566_a_at	4.494	Hsp105
1423467_at	20.41	Ms4a4b	1420591_at	6.996	Gpr84	1449168_a_at	4.465	Akap2
1455452_x_at	18.75	AI449310	1451780_at	6.966	Blnk	1448380_at	4.447	Lgals3bp
1452388_at	18.38	Hspa1a	1426774_at	6.944	Zc3hdc1	1421854_at	4.425	Fgl2
1450826_a_at	17.99	Saa3	1424923_at	6.705	Serpina3g	1419026_at	4.402	Daxx
1449399_a_at	17.6	Il1b	1451050_at	6.508	Nt5c3	1450295_s_at	4.354	D7Ertd458e
1451054_at	17.21	Orm1	1425719_a_at	6.491	Nmi	1417300_at	4.34	Smpdl3b
1436172_at	17.06		1422160_at	6.477	H2-T24	1454197_a_at	4.301	D19Ertd678e
1456494_a_at	16.95	LOC209387	1419607_at	6.424	Tnf	1430300_at	4.298	Sco1
1443698_at	16.92		1423017_a_at	6.351	Il1rn	1423905_at	4.284	D7Ertd458e
1425374_at	16.71	Oas3	1450672_a_at	6.335	Trex1	1456164_at	4.269	AW011738
1456288_at	15.62	Slfn5	1426278_at	6.146	2310061N23Rik	1423903_at	4.268	D7Ertd458e
1438716_at	15.32	LOC209387	1423883_at	6.116	Acsl1	1424289_at	4.236	BC010311
1426633_s_at	15.31	D7Ertd760e	1416515_at	6.063	Fscn1	1416572_at	4.21	Mmp14
1418666_at	14.48	Ptx3	1452178_at	6.043	Plec1	1439012_a_at	4.195	Dck
1435331_at	14.48	AI447904	1456890_at	6.026	Ddx58	1422397_a_at	4.171	Il15ra
1452318_a_at	13.98	Hspa1b	1432026_a_at	5.994	2510038N07Rik	1428838_a_at	4.137	Dck
1439831_at	13.66		1426092_a_at	5.987	Trim34	1429563_x_at	4.093	5830484A20Rik
1439114_at	13.51	9830118M07	1448229_s_at	5.876	Ccnd2	1447272_s_at	4.074	Atp10a
1441600_at	13.09		1430802_at	5.85	H2-Q8	1427348_at	4.032	BC036563
1460415_a_at	12.58	Tnfrsf5	1427736_a_at	5.842	Ccrl2	1428484_at	4.025	Osbpl3
1417172_at	12.41	Ube2l6	1421578_at	5.838	Ccl4	1418240_at	3.97	Gbp2
1459913_at	12.33	A330042I21Rik	1434070_at	5.759	Jag1	1434139_at	3.962	5330431N24Rik
1419728_at	11.98	Cxcl5	1417793_at	5.75	AI481100	1450034_at	3.934	Stat1
1451426_at	11.86	D11Lgp2e	1426670_at	5.745	Agrn	1435906_x_at	3.899	Gbp2
1419676_at	11.78	Mx2	1450808_at	5.67	Fpr1	1436899_at	3.856	2700019D07Rik
1420330_at	11.7	Clecsf9	1442130_at	5.636	Hsh2d	1439805_at	3.84	Nfat5
1449473_s_at	11.68	Tnfrsf5	1431095_a_at	5.601	2510038N07Rik	1422054_a_at	3.786	Skil
1439221_s_at	11.51	Tnfrsf5	1428735_at	5.588	Cd69	1449152_at	3.717	Cdkn2b
1422141_s_at	11.43	Csprs	1449591_at	5.587	Casp4	1453299_a_at	3.715	Pnp
1449926_at	11.42	Tnfsf7	1452231_x_at	5.584	Ifi203	1448728_a_at	3.692	AA408868
1419530_at	11.4	Il12b	1427091_at	5.53	AI481105	1448788_at	3.689	Mox2
1436555_at	11.35	AI158848	1449984_at	5.498	Cxcl2	1421571_a_at	3.687	Ly6c
			1418686_at	5.487	Oas1c	1426716_at	3.669	Tdrd7

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1426133_a_at	3.665	1500032H18Rik	1426415_a_at	2.809	Trim25	1434715_at	2.384	1600014C10Rik
1416897_at	3.644	BC003281	1424126_at	2.805	Alas1	1422140_at	2.383	Csprs
1418990_at	3.619	Ms4a4d	1417527_at	2.805	Ap3m2	1456251_x_at	2.378	Bzrp
1451330_a_at	3.601	Inpp5b	1426541_a_at	2.805	2310067E08Rik	1418115_s_at	2.374	Ifrg15
1449176_a_at	3.58	Dck	1435454_a_at	2.802	BC006779	1423160_at	2.374	Spred1
1420404_at	3.528	Cd86	1455320_at	2.796	AI480535	1458462_at	2.373	6030405P05Rik
1422006_at	3.51	Prkr	1449130_at	2.788	Cd1d1	1422664_at	2.371	Rab10
1424921_at	3.507	2310015I10Rik	1421855_at	2.77	Fgl2	1454889_x_at	2.367	C630016B22Rik
1431843_a_at	3.505	Nfkbie	1450696_at	2.766	Psmb9	1433617_s_at	2.363	B4galt5
1424290_at	3.5	BC010311	1445888_x_at	2.757	Adprtl3	1450155_at	2.362	Itga4
1451821_a_at	3.487	Sp100	1452834_at	2.752	2600010E01Rik	1419132_at	2.36	Tlr2
1448757_at	3.474	Pml	1426543_x_at	2.749	2310067E08Rik	1426084_a_at	2.36	MGC6357
1417333_at	3.468	Rasa4	1439226_at	2.745	5830472H07Rik	1448607_at	2.359	Pbef1
1445597_s_at	3.468	Hrasls3	1416724_x_at	2.744	Tcf4	1419069_at	2.358	Rabgef1
1425810_a_at	3.467	Csrp1	1445897_s_at	2.739	Ifi35	1450272_at	2.357	Tnfsf8
1451611_at	3.45	Hrasls3	1428324_at	2.732	Gpd2	1435415_x_at	2.356	Mlp
1437432_a_at	3.447	Trim12	1435526_at	2.72	A130072J07	1429193_at	2.353	4631416I11Rik
1460283_at	3.445	Mefv	1444777_at	2.717	Rai14	1449222_at	2.346	Ebi3
1458299_s_at	3.436	Nfkbie	1420816_at	2.708	Ywhag	1433515_s_at	2.345	Etnk1
1434431_x_at	3.418	Adora2b	1433699_at	2.706	Tnfaip3	1454731_at	2.337	Myo10
1446957_s_at	3.377	BC004022	1433944_at	2.702	A630025O09Rik	1428071_at	2.322	1110038D17Rik
1420500_at	3.342	Dnajc1	1455649_at	2.692	1700029M07Rik	1453228_at	2.32	Stx11
1424617_at	3.319	Ifi35	1422512_a_at	2.688	Ogfr	1418116_at	2.316	Ifrg15
1434015_at	3.305	Slc2a6	1421217_a_at	2.684	Lgals9	1415791_at	2.312	Rnf34
1424444_a_at	3.305	1600014C10Rik	1416123_at	2.682	Ccnd2	1433482_a_at	2.309	D3Ertd330e
1424609_a_at	3.276	Xdh	1427974_s_at	2.682	Cacna1d	1422511_a_at	2.304	Ogfr
1448698_at	3.269	Ccnd1	1435446_a_at	2.677	Chpt1	1416653_at	2.296	Stxbp3
1459151_x_at	3.262	Ifi35	1425811_a_at	2.669	Csrp1	1425050_at	2.296	2610034N03Rik
1425078_x_at	3.262	5830484A20Rik	1452225_at	2.662	2010106G01Rik	1435267_at	2.262	A430108E01Rik
1418077_at	3.244	Trim21	1418401_a_at	2.662	Dusp16	1424499_s_at	2.259	5730596K20Rik
1447930_at	3.233	Baz1a	1434148_at	2.66	Tcf4	1433592_at	2.255	Calm1
1417434_at	3.232	Gpd2	1456676_a_at	2.658	Pfkfb3	1440854_at	2.242	2810403A07Rik
1424607_a_at	3.214	Xdh	1459903_at	2.65	Sema7a	1447211_at	2.239	Nrip1
1430996_at	3.192	Etnk1	1450744_at	2.647	Ell2	1424826_s_at	2.233	Mtss1
1451544_at	3.176	Tapbpl	1426970_a_at	2.628	Ube1l	1423025_a_at	2.223	Schip1
1460453_at	3.151	2610315E15Rik	1437467_at	2.625	Alcam	1434149_at	2.218	Tcf4
1441089_at	3.135	Eif2c3	1429165_at	2.617	3110001I22Rik	1438948_x_at	2.215	Bzrp
1448958_at	3.124	Ifrg15	1438214_at	2.615	Trps1	1449996_a_at	2.201	Tpm3
1416432_at	3.116	Pfkfb3	1455070_at	2.614	AL118268	1441476_at	2.198	Socs2
1424041_s_at	3.112	C1s	1423401_at	2.611	Etv6	1436334_at	2.196	Synj1
1451160_s_at	3.103	D7Ertd458e	1455051_at	2.611	Rnf31	1442744_at	2.187	C79248
1426542_at	3.094	2310067E08Rik	1444029_at	2.61	5330431N24Rik	1428061_at	2.186	Hat1
1443536_at	3.086	9930009M05Rik	1456403_at	2.609	Pag	1460206_at	2.186	Grasp
1426587_a_at	3.079	Stat3	1437110_at	2.605	2810474O19Rik	1438682_at	2.184	Pik3r1
1426750_at	3.059	Flnb	1427247_at	2.585	D3Bwg0562e	1454809_at	2.179	9030406N13Rik
1445104_at	3.053	E230029C05Rik	1434268_at	2.584	Adar	1422286_a_at	2.175	Tgif
1449875_s_at	3.047	H2-T10	1450165_at	2.583	Slfn2	1433514_at	2.167	Etnk1
1426971_at	3.039	Ube1l	1450828_at	2.571	Synpo2	1420091_s_at	2.152	Zcwcc3
1448328_at	3.034	Sh3bp2	1428378_at	2.565	Zc3hav1	1435904_at	2.149	Eif2c3
1416530_a_at	3.031	Pnp	1416296_at	2.554	Il2rg	1442745_x_at	2.146	C79248
1418154_at	3.002	BC004022	1425515_at	2.545	Pik3r1	1426208_x_at	2.141	Plagl1
1421321_a_at	2.989	Net1	1452385_at	2.538	AA939927	1418929_at	2.136	Esrrbl1
1429809_at	2.966	8430438D04Rik	1421322_a_at	2.533	Isgf3g	1452013_at	2.135	Atp10a
1421207_at	2.944	Lif	1434706_at	2.533	5730538E15Rik	1423904_a_at	2.134	D7Ertd458e
1421098_at	2.928	AI586015	1436199_at	2.527	Trim14	1425053_at	2.124	2610034N03Rik
1456607_at	2.913	5730538E15Rik	1452833_at	2.505	Rapgef2	1437210_a_at	2.12	Brd2
1452925_a_at	2.908	1810015H18Rik	1437497_a_at	2.497	Hspca	1434290_at	2.119	E330008O22Rik
1416016_at	2.905	Tap1	1429488_at	2.486	Zdhhc21	1428843_at	2.115	1810015H18Rik
1433920_at	2.902	Sema4c	1429475_at	2.481	2810457I06Rik	1422962_a_at	2.105	Psmb8
1417483_at	2.894	AA408868	1450747_at	2.474	Keap1	1416695_at	2.105	Bzrp
1423870_at	2.891	2610208M17Rik	1441855_x_at	2.463	Cxcl1	1448377_at	2.095	Slpi
1450378_at	2.873	Tapbp	1431394_a_at	2.46	4921513O20Rik	1445037_at	2.091	6430510B20Rik
1427742_a_at	2.854	Copeb	1455282_x_at	2.455	Alas1	1431822_a_at	2.091	Azi2
1434253_s_at	2.835	C630016B22Rik	1426210_x_at	2.454	Adprtl3	1433590_at	2.089	Herc3
1449131_s_at	2.834	Cd1d1	1421236_at	2.45	Ripk2	1425329_a_at	2.086	Dia1
1438097_at	2.822	Rab20	1448751_at	2.441	Ap3m2	1435363_at	2.071	Plekhg1
1428346_at	2.821	Fln29	1433606_at	2.435	Mitc1	1433462_a_at	2.059	Pi4k2a
1433766_at	2.813	C330023M02Rik	1439755_at	2.424	Sipa1l1	1416929_at	2.057	Rbm12

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1416412_at	2.055	Nsmaf	1448530_at	0.523	Gmpr	1422438_at	0.316	Ephx1
1416755_at	2.055	Dnajb1	1416034_at	0.516	Cd24a	1436853_a_at	0.315	Snca
1452214_at	2.052	9130011J04Rik	1425718_a_at	0.516	Ivns1abp	1436448_a_at	0.311	Ptgs1
1418772_at	2.044	BC016423	1436026_at	0.515	1110032O19Rik	1437614_x_at	0.31	Zdhhc14
1435160_at	2.043	1110064P04Rik	1428500_at	0.513	2210419D22Rik	1420927_at	0.308	St6gal1
1416944_a_at	2.041	Tlk2	1423104_at	0.513	Irs1	1421562_at	0.306	Cd209c
1450456_at	2.038	Il21r	1415977_at	0.506	Isyna1	1438975_x_at	0.3	Zdhhc14
1434830_at	2.033	Mad	1426380_at	0.505	Eif4b	1438619_x_at	0.3	Zdhhc14
1423681_at	2.032	1300018I05Rik	1425444_a_at	0.503	Tgfbr2	1432198_at	0.296	6330414G02Rik
1434350_at	2.031	Axud1	1447707_s_at	0.501	Pde2a	1428574_a_at	0.292	Chn2
1423392_at	2.028	Clic4	1452277_at	0.5	6330406P08Rik	1430780_a_at	0.289	Pmm1
1433499_at	2.028	2010005J08Rik	1434328_at	0.498	Rpl15	1417481_at	0.283	Ramp1
1460315_s_at	2.02	Tbk1	1450084_s_at	0.496	Ivns1abp	1419589_at	0.281	C1qr1
1427310_at	2.016	Falz	1440195_at	0.493	9330147J08Rik	1431189_a_at	0.281	B430104H02Rik
1417189_at	2.012	Psme2	1455291_s_at	0.49	D6Ertd365e	1422837_at	0.28	Scel
1430353_at	2.009	4833409N03Rik	1436212_at	0.488	AI661017	1460672_at	0.268	2410002F23Rik
1428637_at	2.008	1810038L18Rik	1452933_at	0.486	C030004B10Rik	1456046_at	0.266	C1qr1
1434036_at	2.006	Mtss1	1421182_at	0.486	Clec2	1435203_at	0.265	1700052O22Rik
1435058_x_at	2.005	Stxbp3	1418123_at	0.485	Unc119	1418998_at	0.264	Kmo
1420617_at	2.001	Cpeb4	1432164_a_at	0.484	5730591C18Rik	1429324_at	0.262	1700012A16Rik
1421346_a_at	1.985	Slc6a6	1449183_at	0.483	Comt	1453552_at	0.262	2310014F07Rik
1421812_at	1.982	Tapbp	1427341_at	0.482	E130103I17Rik	1417872_at	0.26	Fhl1
1418587_at	1.977	Traf3	1452846_at	0.481	Ppfia4	1436323_at	0.252	2810001A02Rik
1454736_at	1.975	4921515A04Rik	1426516_a_at	0.48	Lpin1	1436322_a_at	0.252	2810001A02Rik
1420523_at	1.965	2610529H08Rik	1452770_at	0.474	Vkorc1	1426734_at	0.252	BC022623
1450642_at	1.961	3110001I20Rik	1415857_at	0.473	Emb	1449270_at	0.248	Plxdc2
1456114_at	1.96	Cds1	1430889_a_at	0.473	Tpmt	1419747_at	0.238	Asgr2
1431827_a_at	1.957	Tlk2	1425628_a_at	0.472	Gtf2i	1423231_at	0.236	Nrgn
1440984_at	1.952	5830435C13Rik	1416470_a_at	0.471	Rpn1	1425144_at	0.235	Klk21
1417813_at	1.95	Ikbke	1431190_x_at	0.471	B430104H02Rik	1418912_at	0.234	Plxdc2
1437926_at	1.94	E430012M05Rik	1416188_at	0.47	Gm2a	1418405_at	0.226	Hgfac
1452461_a_at	1.922	Mafb	1430656_a_at	0.469	2210409M21Rik	1437025_at	0.223	Cd28
1459896_at	1.915	D1Ertd251e	1438943_x_at	0.468	Rpn1	1420928_at	0.222	St6gal1
1424687_at	1.905	2700008B19Rik	1426378_at	0.468	Eif4b	1431110_at	0.201	5430431D22Rik
1451082_at	1.899	1300018I05Rik	1450048_a_at	0.468	Idh2	1421685_at	0.198	1810046I24Rik
1418911_s_at	1.895	Acsl4	1439257_x_at	0.465	Rpn1	1455885_at	0.188	6530401C20Rik
1435890_at	1.892	AW228836	1426379_at	0.46	Eif4b	1420715_a_at	0.174	Pparg
1430307_a_at	1.89	Mod1	1437046_x_at	0.456	4930504E06Rik	1425182_x_at	0.165	Klk9
1436333_a_at	1.886	Synj1	1420381_a_at	0.454	Rpl31	1454254_s_at	0.152	1600029D21Rik
1460376_a_at	1.881	Cox15	1417664_a_at	0.449	Ndrg3	1460003_at	0.138	AI956758
1425523_at	1.868	2610015J01Rik	1429856_at	0.447	2610042G18Rik	1450430_at	0.134	Mrc1
1438270_at	1.854	1700105P06Rik	1448241_at	0.445	Gm2a	1433719_at	0.0914	Slc9a9
1420901_a_at	1.823	Hk1	1425407_s_at	0.445	Clecsf6	1419605_at	0.071	Mgl1
1428227_at	1.819	2610008J04Rik	1456438_x_at	0.443	Rpn1	1426172_a_at	0.0673	Cd209a
1450973_s_at	1.81	Mapkbp1	1447521_x_at	0.441	D15Wsu169e	1438467_at	0.0593	Mgl2
1426324_at	1.788	H2-Q10	1422603_at	0.438	Rnase4	1425295_at	0.0566	Ear11
1442757_at	1.78	Chdc1	1438321_x_at	0.436	4930504E06Rik			
1428593_at	1.775	1700029F09Rik	1434874_x_at	0.433	Centb1			
1427040_at	1.763	Kdt1	1420770_at	0.427	Klk21			
1451805_at	1.751	Phip	1454646_at	0.425	E430026E19Rik			
1420618_at	1.748	Cpeb4	1450095_a_at	0.423	Acyp1			
1423393_at	1.74	Clic4	1437052_s_at	0.417	Slc2a3			
1434782_at	1.688	D5Ertd591e	1431133_at	0.417	Arhgap18			
1415740_at	1.682	Psmc5	1439703_at	0.416	Mox2r			
1429797_at	1.657	5730596K20Rik	1455898_x_at	0.415	Slc2a3			
1424813_at	1.64	LOC218805	1434470_at	0.41	Syt13			
1453582_at	1.637	Chka	1422477_at	0.408	Cables1			
1433668_at	1.635	Pnrc1	1416619_at	0.405	4632428N05Rik			
1454668_at	1.612	1810009A16Rik	1441907_s_at	0.404	C1qr1			
1426270_at	1.612	Smc5l1	1452841_at	0.399	Pgm2l1			
1425120_x_at	1.575	1810023F06Rik	1416389_a_at	0.397	Chc1l			
1428659_at	0.631	Phf7	1451867_x_at	0.397	Arhgap6			
1449878_a_at	0.623	Slc12a6	1422823_at	0.396	Eps8			
1455911_x_at	0.62	Np15	1422084_at	0.392	Bmx			
1456480_at	0.61	9330186A19Rik	1452956_a_at	0.386	D12Ertd647e			
1418560_at	0.607	Pdha1	1421026_at	0.385	Gna12			
1427139_at	0.598	Adamts10	1415856_at	0.38	Emb			
1428577_at	0.596	Ppfia4	1448471_a_at	0.378	Ctla2b			
1441937_s_at	0.591	Pink1	1417399_at	0.377	Gas6			
1450649_at	0.584	Gng10	1416319_at	0.376	Adk			
1435397_at	0.582	BC038156	1449366_at	0.369	Mmp8			
1427138_at	0.576	0610010D24Rik	1456333_a_at	0.366	Arhgap6			
1420638_at	0.566	Prps2	1434873_a_at	0.364	Centb1			
1422433_s_at	0.562	Idh1	1450097_s_at	0.363	Gna12			
1455972_x_at	0.561	Hadhsc	1423414_at	0.36	Ptgs1			
1423801_a_at	0.56	Aprt	1451486_at	0.359	1200006F02Rik			
1419206_at	0.558	Cd37	1452299_at	0.35	8030445B08Rik			
1452202_at	0.558	Pde2a	1426584_a_at	0.337	Sdh1			
1435006_s_at	0.557	Abcb7	1451715_at	0.337	Mafb			
1448182_a_at	0.555	Cd24a	1417704_a_at	0.335	Arhgap6			
1428181_at	0.542	Etfb	1438431_at	0.332	Abcd2			
1453109_at	0.533	2810429K17Rik	1427098_at	0.332	8030445B08Rik			
1428612_at	0.532	Apg7l	1438666_at	0.33	AI194318			
1460733_at	0.529	AA407659	1419748_at	0.326	Abcd2			
1458559_at	0.526	A730028G07Rik	1425733_a_at	0.323	Eps8			
1424771_at	0.524	E130307C13	1422824_s_at	0.32	Eps8			

Appendix- VIII- Complete gene list of differentially regulated genes in response to *M. tuberculosis* infection of MyD88$^{-/-}$ BMDCs

All genes have a fold change of 1.4 and a p value ≤$_{0.05}$.

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1436823_x_at	222.4	Hbb-y	1418936_at	18.36	Maff	1435645_at	10.79	Mmd
1436717_x_at	192.5	Hbb-y	1452196_a_at	18.31	Nckap1	1449152_at	10.78	Cdkn2b
1427624_s_at	108.3	Iltifb	1426657_s_at	17.18	Phgdh	1421317_x_at	10.68	Myb
1450297_at	89.89	Il6	1426724_at	17.13	Cnn3	1420572_at	10.62	Ms4a3
1449926_at	81.23	Tnfsf7	1416052_at	16.56	Prps1	1421207_at	10.46	Lif
1438658_a_at	70.25	Edg3	1421775_at	16.48	Fcer1a	1436188_a_at	10.41	Ndrg4
1436870_s_at	64.99	AU041783	1422053_at	15.73	Inhba	1460239_at	10.35	Tm4sf13
1440882_at	59.45	Lrp8	1449168_a_at	15.61	Akap2	1421308_at	10.28	Car13
1421262_at	56.56	Lipg	1427054_s_at	15.46	5033411B22Rik	1419569_a_at	10.24	Isg20
1439221_s_at	51.09	Tnfrsf5	1418003_at	15.45	1190002H23Rik	1451064_a_at	10.19	Psat1
1428947_at	50.5	2010001M09Rik	1455956_x_at	15.41	Ccnd2	1420505_a_at	10.17	Stxbp1
1448804_at	46.65	Cyp11a1	1437173_at	15.4	Edg3	1456509_at	10.14	1110032F04Rik
1449424_at	45.5	Plek2	1434403_at	15.07	Spred2	1432478_a_at	9.821	4930534K13Rik
1447831_s_at	44.6	Mtmr7	1449858_at	15.06	Cd86	1455810_a_at	9.804	E130112L23Rik
1427126_at	44.06	Hspa1b	1431422_a_at	15.02	Dusp14	1420691_at	9.785	Il2ra
1460415_a_at	41.85	Tnfrsf5	1421307_at	14.79	Car13	1423413_at	9.708	Ndrg1
1417184_s_at	41.72	Hbb-b1	1428535_at	14.76	1110056P05Rik	1420710_at	9.667	Rel
1422188_s_at	41.1	Tcrg-V4	1418547_at	14.65	Tfpi2	1452789_at	9.588	Snn
1450521_a_at	39.93	Tcrg-V4	1450194_a_at	14.63	Myb	1420150_at	9.584	4930422J18Rik
1422189_x_at	38.87	Tcrg-V4	1418937_at	14.35	Dio2	1421744_at	9.58	Tnfsf4
1417785_at	36.38	Pla1a	1417220_at	14.31	Fah	1429272_a_at	9.55	2210421G13Rik
1447669_s_at	35.69	Gng4	1447863_s_at	14.06	Nr4a2	1422533_at	9.542	Cyp51
1422706_at	35.24	Tmepai	1425569_a_at	13.96	Slamf1	1416008_at	9.485	Satb1
1436759_x_at	34.94	Cnn3	1432826_a_at	13.95	Cd80	1422734_a_at	9.445	Myb
1449473_s_at	34.12	Tnfrsf5	1417976_at	13.71	Ada	1460469_at	9.291	Tnfrsf9
1442347_at	34.05	AI848122	1427381_at	13.61	Irg1	1450290_at	9.175	Pdcd1lg2
1427127_x_at	33.29	Hspa1b	1425505_at	13.58	Mylk	1430156_at	9.11	4930520O04Rik
1420664_s_at	33.06	Procr	1429668_at	13.52	E130119J07Rik	1455812_x_at	9.089	Slitl2
1437308_s_at	32.98	F2r	1420404_at	13.46	Cd86	1452366_at	9.088	4732435N03Rik
1418449_at	32.11	Lad1	1416271_at	13.33	Perp	1448724_at	8.992	Cish
1454269_s_at	31.73	4930519L02Rik	1416007_at	13.31	Satb1	1436101_at	8.978	2810473M14Rik
1436836_x_at	31.73	Cnn3	1450259_a_at	13.29	Stat5a	1460466_at	8.818	1700047I17Rik
1422639_at	30.14	Calcb	1417928_at	13.28	Pdlim4	1449328_at	8.79	Ly75
1420994_at	29.33	B3gnt5	1449090_a_at	12.94	Yes	1416121_at	8.757	Lox
1428034_a_at	28.77	Tnfrsf9	1424289_at	12.93	BC010311	1448752_at	8.647	Car2
1455570_x_at	28.68	Cnn3	1453470_a_at	12.78	Gna13	1455143_at	8.52	Nlgn2
1428301_at	27.7	2610042L04Rik	1460227_at	12.77	Timp1	1424008_a_at	8.499	2400008B06Rik
1450188_s_at	27.69	Lipg	1440612_at	12.72	9930104E21Rik	1435226_at	8.498	4930534K13Rik
1423135_at	25.52	Thy1	1438354_x_at	12.66	Cnn3	1451804_a_at	8.42	Lrrc16
1456380_x_at	24.21	Cnn3	1424290_at	12.63	BC010311	1423091_a_at	8.369	Gpm6b
1424923_at	24.08	Serpina3g	1418175_at	12.46	Vdr	1449317_at	8.35	Cflar
1430127_a_at	23.88	Ccnd2	1449864_at	12.4	Il4	1434140_at	8.338	Mcf2l
1428735_at	23.04	Cd69	1449497_at	12.24	Il12b	1450922_a_at	8.318	Tgfb2
1452731_x_at	22.91	2610042L04Rik	1422924_at	12.17	Tnfsf9	1450869_at	8.27	Fgf1
1419413_at	21.53	Ccl17	1418652_at	12.14	Cxcl9	1421266_s_at	8.267	Nfkbib
1425570_at	21.36	Slamf1	1416811_s_at	12.07	Ctla2b	1439620_at	8.267	Car13
1434745_at	21.09	Ccnd2	1423289_a_at	11.96	1810029B16Rik	1422668_at	8.266	Serpinb9b
1448229_s_at	20.58	Ccnd2	1428396_at	11.72	4930431E10Rik	1416939_at	8.218	Pyp
1450753_at	20.54	Nkg7	1448788_at	11.51	Mox2	1449033_at	8.194	Tnfrsf11b
1416122_at	20.37	Ccnd2	1429566_a_at	11.28	Hipk2	1448730_at	8.189	Cpa3
1416123_at	20.31	Ccnd2	1420413_at	11.25	Slc7a11	1423418_at	8.183	Fdps
1427878_at	20.12	0610010O12Rik	1438325_at	11.15	Evi1	1416645_a_at	8.138	Afp
1417149_at	19.67	P4ha2	1450173_at	11	Ripk2	1460242_at	8.08	Daf1
1454714_x_at	19.25	Phgdh	1418176_at	10.94	Vdr	1449402_at	8.072	Chst7
1428834_at	18.89	Dusp4	1449824_at	10.92	Prg4	1419530_at	8.071	Il12b
1450852_s_at	18.49	F2r	1460307_at	10.85	Akt3	1418726_a_at	8.069	Tnnt2

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1438274_at	8.057	A630026H08Rik	1428669_at	6.139	Bmyc	1451109_a_at	4.938	Nedd4
1426411_a_at	7.958	Strbp	1455183_at	6.137	Stk38l	1434260_at	4.937	Fchsd2
1456471_x_at	7.956	Phgdh	1422473_at	6.134	Pde4b	1422567_at	4.929	Niban
1431008_at	7.947	0610037M15Rik	1433699_at	6.085	Tnfaip3	1456321_at	4.926	3830408G10Rik
1435792_at	7.932	Csprs	1427893_a_at	6.084	Pmvk	1423041_a_at	4.92	Bzw1
1422437_at	7.924	Col5a2	1451471_at	6.073	3230401I01Rik	1425514_at	4.917	Pik3r1
1421073_a_at	7.917	Ptger4	1421679_a_at	6.068	Cdkn1a	1436196_at	4.914	C030046G05
1455656_at	7.9	Btla	1430221_at	6.058	9130008F23Rik	1423466_at	4.909	Ccr7
1451596_a_at	7.89	Sphk1	1433454_at	6.05	AW539457	1417441_at	4.906	Dnajc12
1418736_at	7.884	B3galt3	1437635_at	6.045	Esdn	1449773_s_at	4.901	Gadd45b
1452757_s_at	7.868	Hba-a1	1423049_a_at	6.023	Tpm1	1428902_at	4.89	1110020P09Rik
1449037_at	7.823	Crem	1419470_at	6.008	Gnb4	1437486_at	4.878	Rai3
1449149_at	7.773	Traf3	1442130_at	5.974	Hsh2d	1417444_at	4.871	E2f5
1460378_a_at	7.683	Tes	1441706_at	5.974	Dscaml1	1423903_at	4.869	D7Ertd458e
1424086_at	7.677	D130038B21Rik	1418643_at	5.968	Tm4sf13	1418402_at	4.833	Adam19
1419202_at	7.603	Cst7	1424246_a_at	5.939	Tes	1448130_at	4.833	Fdft1
1420401_a_at	7.461	Ramp3	1418219_at	5.916	Il15	1435455_at	4.824	C79267
1418926_at	7.456	Zfhx1a	1422054_a_at	5.915	Skil	1451335_at	4.823	Plac8
1419469_at	7.416	Gnb4	1423122_at	5.864	Avpi1	1422474_at	4.814	Pde4b
1444585_at	7.406	4933429I20Rik	1421457_a_at	5.857	Samsn1	1421256_at	4.793	Gzmc
1430352_at	7.405	8430417A20Rik	1444606_at	5.855	Efna2	1420013_s_at	4.791	Lss
1431777_a_at	7.402	Hmgn3	1448713_at	5.854	Stat4	1457342_at	4.779	A630026H08Rik
1458299_s_at	7.387	Nfkbie	1423158_at	5.852	Gnpnat1	1423162_s_at	4.777	Spred1
1421936_at	7.312	Dapp1	1416236_a_at	5.848	Eva	1455048_at	4.776	Igsf2
1450350_a_at	7.312	Jundm2	1429347_at	5.843	Bcl2l14	1437419_at	4.776	Bmp2k
1445597_s_at	7.306	Hrasls3	1443536_at	5.84	9930009M05Rik	1423904_a_at	4.755	D7Ertd458e
1421260_a_at	7.291	Srm	1454809_at	5.832	9030406N13Rik	1429786_a_at	4.754	D10Ertd749e
1452614_at	7.271	LOC229672	1452061_s_at	5.824	Strbp	1425902_a_at	4.747	Nfkb2
1416658_at	7.269	Frzb	1420692_at	5.805	Il2ra	1434267_at	4.745	Nek1
1418718_at	7.261	Cxcl16	1448265_x_at	5.801	Eva	1421828_at	4.74	Kpna3
1431843_a_at	7.253	Nfkbie	1452914_at	5.777	2410024N18Rik	1428323_at	4.708	Gpd2
1418930_at	7.228	Cxcl10	1417398_at	5.77	Rras2	1423392_at	4.707	Clic4
1438511_a_at	7.209	1190002H23Rik	1426615_s_at	5.753	Ndrg4	1448162_at	4.706	Vcam1
1447585_s_at	7.194	Mrps6	1438037_at	5.743	2510038N07Rik	1437111_at	4.696	C230027N18Rik
1441092_at	7.186	9330159M07Rik	1432026_a_at	5.714	2510038N07Rik	1456584_x_at	4.69	Phgdh
1424991_s_at	7.166	Tyms	1427038_at	5.706	Penk1	1450971_at	4.69	Gadd45b
1426750_at	7.152	Flnb	1429667_at	5.702	E130119J07Rik	1418868_at	4.68	En2
1448192_s_at	7.094	Prps1	1418843_at	5.684	Slc30a4	1424414_at	4.673	Ogfrl1
1417558_at	7.081	Fyn	1448873_at	5.66	Ocln	1415922_s_at	4.669	Mlp
1450646_at	7.07	Cyp51	1441536_at	5.654	Hmgcs1	1460458_at	4.665	1810049K24Rik
1459903_at	7.053	Sema7a	1424761_at	5.65	BC011487	1453472_a_at	4.656	Slamf7
1460206_at	7.048	Grasp	1417289_at	5.643	Plekha2	1430640_a_at	4.654	Prkar2b
1419534_at	7.041	Olr1	1448918_at	5.628	Slco3a1	1424824_at	4.654	9630044O09Rik
1457248_x_at	7.031	Hsd17b7	1449109_at	5.616	Socs2	1421065_at	4.647	Jak2
1454607_s_at	7.016	Psat1	1448844_at	5.615	1810044O22Rik	1455181_at	4.645	Rasa2
1419759_at	6.984	Abcb1a	1427025_at	5.607	Mtmr7	1417273_at	4.64	Pdk4
1453590_at	6.959	Arl8	1434794_at	5.604	Rhof	1429549_at	4.634	Col27a1
1429809_at	6.951	8430438D04Rik	1448325_at	5.587	Myd116	1451985_at	4.629	Lrrk1
1416515_at	6.938	Fscn1	1418346_at	5.568	Insl6	1438306_at	4.624	3110001E11Rik
1418990_at	6.93	Ms4a4d	1429140_at	5.564	9830002I17Rik	1420888_at	4.612	Bcl2l1
1449231_at	6.912	Zfp296	1452925_a_at	5.486	1810015H18Rik	1455833_at	4.605	AU041783
1453365_at	6.903	8430421H08Rik	1445452_at	5.469	Traf1	1448885_at	4.581	Rap2b
1422601_at	6.901	Serpinb9	1429564_at	5.463	5830443C21Rik	1451037_at	4.577	Ptpn9
1448865_at	6.841	Hsd17b7	1448681_at	5.462	Il15ra	1449368_at	4.574	Dcn
1423602_at	6.841	Traf1	1418687_at	5.462	Arc	1450976_at	4.54	Ndrg1
1449195_s_at	6.784	Cxcl16	1418371_at	5.447	6720463E02Rik	1453127_at	4.527	Ppp2cz
1425506_at	6.779	9530072E15Rik	1423543_at	5.435	Swap70	1455649_at	4.513	1700029M07Rik
1450337_a_at	6.774	Nek8	1436895_at	5.431	Centd1	1453228_at	4.505	Stx11
1448765_at	6.741	Fyn	1423161_s_at	5.411	Spred1	1416038_at	4.479	AL033314
1433939_at	6.72	A730046J16	1423488_at	5.398	Mmd	1419060_at	4.47	Gzmb
1448656_at	6.711	Cacnb3	1429197_s_at	5.392	5830411O09Rik	1450846_at	4.464	Bzw1
1443923_at	6.695	Akap13	1435966_x_at	5.392	Mrpl13	1448050_s_at	4.463	Map4k4
1440955_at	6.661	LOC333088	1448451_at	5.371	Ak2	1437992_x_at	4.455	Gja1
1444402_at	6.654	C230027N18Rik	1415846_a_at	5.363	Ldh3	1434593_at	4.447	Eif5a2
1428512_at	6.642	2700087I09Rik	1434671_at	5.35	B230337E12Rik	1430259_at	4.445	Tnfrsf11a
1422804_at	6.631	Serpinb6b	1429110_a_at	5.338	2810405F18Rik	1448605_at	4.441	Rhoc
1418734_at	6.599	H2-Q1	1435927_at	5.325	E030003N15Rik	1451160_s_at	4.432	D7Ertd458e
1415810_at	6.558	Uhrf1	1460565_at	5.264	Slc41a1	1422785_at	4.431	Rasa2
1444233_at	6.527	Gpr132	1429196_at	5.258	5830411O09Rik	1448777_at	4.423	Mcm2
1452291_at	6.525	Centd1	1449591_at	5.254	Casp4	1438018_at	4.422	Hook1
1437056_x_at	6.481	1810049K24Rik	1442427_at	5.228	9630026M06Rik	1416730_at	4.419	Rcl1
1436598_at	6.469	Icos	1438097_at	5.223	Rab20	1425806_a_at	4.415	Surb7
1455647_at	6.433	AW320017	1420843_at	5.197	Ptprf	1423040_at	4.41	Bzw1
1436768_x_at	6.431	E130112L23Rik	1434599_a_at	5.195	Tjp2	1423285_at	4.406	Coch
1448452_at	6.423	Icsbp1	1419550_a_at	5.184	Stk39	1418322_at	4.4	Crem
1418507_s_at	6.404	D130043N08Rik	1419031_at	5.179	Fads2	1420760_s_at	4.387	Ndrg1
1431591_s_at	6.4	G1p2	1420499_at	5.176	Gch	1429239_a_at	4.386	Stard4
1448126_at	6.389	Tera	1452815_at	5.171	P2ry10	1422664_at	4.385	Rab10
1434758_at	6.388	1810049K24Rik	1421469_a_at	5.127	Stat5a	1435432_at	4.382	Centg2
1454992_at	6.362	Slc7a1	1456635_at	5.113	5830484A20Rik	1416067_at	4.381	Ifrd1
1422303_a_at	6.341	Tnfrsf18	1417703_at	5.093	Pvrl2	1416036_at	4.356	Fkbp1a
1444089_at	6.335	Spnb2	1423948_at	5.085	Bag2	1451376_at	4.349	5730596K20Rik
1426223_at	6.332	2810439F02Rik	1455899_x_at	5.069	Socs3	1424499_s_at	4.348	5730596K20Rik
1434875_a_at	6.326	Hmgn3	1428869_at	5.053	Nolc1	1418076_at	4.348	St14
1424204_at	6.31	Mrpl13	1438400_at	5.045	4632411B12Rik	1425145_at	4.346	Il1rl1
1433453_a_at	6.309	AW539457	1450850_at	5.02	Vil2	1436305_at	4.346	Al853829
1427053_at	6.296	5033411B22Rik	1417856_at	4.994	Relb	1448301_s_at	4.345	Serpinb1a
1416229_at	6.284	Rfk	1428543_at	4.987	Ppat	1424498_at	4.344	5730596K20Rik
1449799_s_at	6.264	1200008D14Rik	1418030_at	4.984	Slco3a1	1451122_at	4.329	Idi1
1428720_s_at	6.249	2010309G21Rik	1460354_a_at	4.973	Mrpl13	1452741_s_at	4.328	Gpd2
1439766_x_at	6.243	Vegfc	1455434_a_at	4.972	Ktn1	1434381_at	4.326	BC060631
1438322_x_at	6.209	Fdft1	1422177_at	4.972	Il13ra2	1426597_s_at	4.324	C79267
1421653_a_at	6.196	Igh-VJ558	1421236_at	4.969	Ripk2	1423156_at	4.324	Gnpnat1
1418835_at	6.172	Phlda1	1418635_at	4.955	Etv3	1455450_at	4.322	Ptpn3
1451330_a_at	6.16	Inpp5b	1451828_a_at	4.952	Acsl4	1428073_a_at	4.306	Nup88
1435245_at	6.151	Gls2	1424440_at	4.946	Mrps6	1435878_at	4.28	Stk38l

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1415993_at	4.277	Sqle	1452214_at	3.803	9130011J04Rik	1423402_at	3.433	Creb1
1416318_at	4.275	Serpinb1a	1453782_at	3.797	3021401C12Rik	1418128_at	3.432	Adcy6
1424067_at	4.269	Icam1	1456358_at	3.79	Etv3	1430185_at	3.432	5830460E08Rik
1460541_at	4.265	AI643885	1428861_at	3.784	4631422O05Rik	1454849_x_at	3.431	Clu
1416714_at	4.253	Icsbp1	1455250_at	3.781	Sh3bp4	1430634_a_at	3.43	Pfkp
1422705_at	4.25	Tmepai	1425515_at	3.781	Pik3r1	1444619_x_at	3.404	Psmb8
1448140_at	4.246	2810413N20Rik	1448571_a_at	3.778	Gmfb	1436181_at	3.403	LOC211914
1428395_at	4.239	4930431E10Rik	1438016_at	3.776	BC068171	1421103_at	3.4	Bmp2k
1425811_a_at	4.231	Csrp1	1437304_at	3.772	Cblb	1457656_s_at	3.398	C230085N15Rik
1417511_at	4.226	Lyar	1430295_at	3.77	Gna13	1450908_at	3.392	Eif4e
1423137_at	4.225	Rala	1423299_at	3.768	Txnl1	1420522_at	3.38	2610529H08Rik
1448604_at	4.225	AA407809	1449044_at	3.766	Eef1e1	1453240_a_at	3.379	2900054P12Rik
1416796_at	4.222	Nck2	1426607_at	3.765	3110070M22Rik	1437174_at	3.378	A330080J22Rik
1417434_at	4.221	Gpd2	1434388_at	3.765	A630029F06	1426482_at	3.372	Prkrir
1452364_at	4.217	D11Ertd530e	1425927_a_at	3.759	Atf5	1448271_a_at	3.372	Ddx21
1458634_at	4.207	Cd47	1419270_a_at	3.756	Dutp	1416268_at	3.371	Ets2
1460249_at	4.205	Lnx2	1416782_s_at	3.756	DXImx39e	1449300_at	3.371	BC003236
1441947_x_at	4.188	BC033915	1448793_a_at	3.746	Sdc4	1428593_at	3.37	1700029F09Rik
1425773_s_at	4.187	Nmnat	1430445_at	3.729	1700026G02Rik	1447926_at	3.368	2810410P22Rik
1424303_at	4.184	AV216087	1417470_at	3.727	Apobec3	1448968_at	3.366	D7Wsu128e
1426221_at	4.181	5830475I06Rik	1418587_at	3.722	Traf3	1430752_at	3.362	C330006D17Rik
1454931_at	4.179	Cri2	1435693_at	3.713	BC012256	1423269_a_at	3.362	Nedd4l
1428141_at	4.178	Gga2	1460441_at	3.709	Zxda	1438852_x_at	3.358	Mcm6
1422861_s_at	4.167	1110001A05Rik	1451050_at	3.708	Nt5c3	1456429_at	3.356	Malt1
1434339_at	4.164	2610318I01Rik	1448274_at	3.706	C1qbp	1421198_at	3.356	Itgav
1441075_at	4.16	LOC329416	1424412_at	3.704	Ogfrl1	1449433_at	3.352	P2rx5
1427705_a_at	4.152	Nfkb1	1460719_a_at	3.703	P2rx1	1422018_at	3.344	Hivep2
1423143_at	4.146	Gtpbp4	1450333_a_at	3.702	Gata2	1452666_a_at	3.341	1110063G11Rik
1425197_at	4.14	Ptpn2	1427324_at	3.7	LOC330189	1452661_at	3.34	Tfrc
1448175_at	4.127	Ehd1	1423724_at	3.696	D10Ertd749e	1416069_at	3.338	Pfkp
1419029_at	4.127	Ero1l	1430287_s_at	3.692	2310008M14Rik	1415925_a_at	3.336	Nup62
1418626_a_at	4.124	Clu	1434350_at	3.691	Axud1	1448226_at	3.335	Rrm2
1431072_a_at	4.118	2610529H08Rik	1456251_x_at	3.688	Bzrp	1421107_at	3.334	Stk4
1424671_at	4.116	Plekhf1	1451950_a_at	3.683	Cd80	1423681_at	3.334	1300018I05Rik
1433526_at	4.115	Klhl8	1433675_at	3.682	Rnu22	1423828_at	3.33	Fasn
1435766_at	4.114	1600022A19Rik	1419254_at	3.682	Mthfd2	1429682_at	3.323	4930431B09Rik
1450845_a_at	4.108	Bzw1	1457658_x_at	3.679	Anxa4	1455940_x_at	3.322	Wdr6
1417171_at	4.098	Itk	1426299_at	3.675	9130017C17Rik	1423720_a_at	3.316	Sara1
1434600_at	4.095	Tjp2	1423194_at	3.674	Arhgap5	1449287_at	3.316	Srms
1436871_at	4.094	Sfrs7	1416305_at	3.664	Sh3bp4	1416011_x_at	3.316	Ehd1
1460439_at	4.091	BC033915	1450970_at	3.66	Got1	1422675_at	3.309	Smarce1
1456174_x_at	4.079	Ndrg1	1417523_at	3.659	Plek	1416695_at	3.309	Bzrp
1455618_x_at	4.079	1300010A20Rik	1451069_at	3.659	Pim3	1427689_a_at	3.308	Tnip1
1450957_a_at	4.078	Sqstm1	1453181_x_at	3.649	Plscr1	1456739_x_at	3.305	Armcx2
1449108_at	4.075	Fdx1	1454962_at	3.648	SPIRE1	1423078_a_at	3.302	Sc4mol
1452316_at	4.065	1110020M19Rik	1438945_x_at	3.645	Gja1	1425350_a_at	3.301	Myef2
1434364_at	4.064	Map3k14	1450923_at	3.645	Tgfb2	1427062_at	3.298	9930104E21Rik
1424638_at	4.062	Cdkn1a	1451012_a_at	3.64	Csda	1419472_s_at	3.296	Nudc
1423316_at	4.059	2610033C09Rik	1418666_at	3.637	Ptx3	1423054_at	3.29	Wdr1
1423060_at	4.053	Pa2g4	1426609_at	3.635	2810028N01Rik	1420500_at	3.29	Dnajc1
1427922_at	4.052	1110046L09Rik	1453482_at	3.626	A430105C05Rik	1420548_a_at	3.289	2310008H09Rik
1455771_at	4.052	Bzrap1	1448297_a_at	3.621	Tnk2	1441380_at	3.284	2810439F02Rik
1419402_at	4.042	Mns1	1418401_a_at	3.62	Dusp16	1436746_at	3.279	Prkwnk1
1456212_x_at	4.037	Socs3	1448508_at	3.612	AI429613	1418744_s_at	3.278	Tesc
1437325_x_at	4.033	Pycs	1433523_at	3.603	D930005D10Rik	1418831_at	3.276	Pkp3
1434442_at	4.024	D5Ertd593e	1418932_at	3.6	Nfil3	1424839_a_at	3.274	2810405F18Rik
1448133_at	4.013	C87860	1453076_at	3.597	9130211I03Rik	1423769_at	3.274	Ptcd2
1436506_a_at	4.009	1110008H02Rik	1449232_at	3.596	Gata1	1429189_at	3.272	1110007C02Rik
1418093_a_at	4.006	Egf	1422615_at	3.591	Map4k4	1454873_at	3.271	C130032F08Rik
1438664_at	4.004	Prkar2b	1455854_a_at	3.588	AW551225	1417640_at	3.27	Cd79b
1428164_at	3.999	Nudt9	1423036_at	3.584	Txnl5	1438650_x_at	3.27	Gja1
1460207_s_at	3.983	E2f5	1421082_s_at	3.583	Banf1	1448154_at	3.27	Ndrg2
1424724_a_at	3.978	D16Ertd472e	1437296_at	3.576	Pkn2	1437100_x_at	3.25	Pim3
1440739_at	3.974	Vegfc	1423581_at	3.567	Nmt2	1456748_a_at	3.247	Nipsnap1
1433974_at	3.968	Sephs1	1438761_a_at	3.56	Odc	1421623_at	3.24	Il12rb2
1425749_at	3.964	Stxbp6	1435338_at	3.554	AI504062	1423745_at	3.239	1110031B06Rik
1420948_s_at	3.959	4833408C14Rik	1417925_at	3.547	Ccl22	1434580_at	3.232	Enpp4
1450744_at	3.957	Ell2	1426612_at	3.546	Tipin	1416852_a_at	3.229	Ncdn
1435679_at	3.946	Optn	1417166_at	3.543	Psip1	1428071_at	3.229	1110038D17Rik
1429381_x_at	3.944	Igh-VJ558	1433474_at	3.541	Edil3	1448689_at	3.229	Rras2
1429049_at	3.936	1200013B22Rik	1431429_a_at	3.537	Arl4	1425066_a_at	3.228	1110061O04Rik
1435630_s_at	3.929	Acat2	1436216_s_at	3.535	2610204M08Rik	1435955_at	3.224	Siglec10
1450385_at	3.929	Kpna3	1420947_at	3.529	Atrx	1416743_at	3.221	Uap1
1419103_a_at	3.921	Abhd6	1452013_at	3.522	Atp10a	1449523_at	3.216	Bcl7c
1434957_at	3.92	Cdon	1417404_at	3.521	Elovl6	1448396_at	3.215	D1Bwg0491e
1438917_x_at	3.909	Nup62	1427077_a_at	3.517	Ap2b1	1452514_a_at	3.21	Kit
1417056_at	3.9	Psme1	1439348_at	3.5	S100a10	1450786_x_at	3.208	1110001A05Rik
1454637_at	3.896	Klhl8	1456475_s_at	3.496	Prkar2b	1421731_a_at	3.205	Fen1
1450750_a_at	3.896	Nr4a2	1430175_at	3.495	4930588G05Rik	1423643_at	3.204	Ddx39
1418151_at	3.895	Mtmr4	1447272_s_at	3.491	Atp10a	1424396_a_at	3.201	Asrgl1
1424600_at	3.895	Abp1	1416291_at	3.49	Psmc4	1437238_x_at	3.198	C87860
1452132_at	3.893	0610030G03Rik	1449175_at	3.476	Gpr65	1423489_at	3.195	Mmd
1437497_a_at	3.886	Hspca	1427212_at	3.473	Mapkap1	1416129_at	3.194	1300002F13Rik
1433674_a_at	3.881	Rnu22	1416010_a_at	3.472	Ehd1	1434240_at	3.193	4632434I11Rik
1427229_at	3.88	Hmgcr	1448710_at	3.47	Cxcr4	1440801_s_at	3.192	Adrbk2
1420743_a_at	3.878	Ppp3cc	1424942_a_at	3.469	Myc	1455051_at	3.186	Rnf31
1422650_a_at	3.869	Riok3	1416544_at	3.468	Ezh2	1419714_at	3.18	Pdcd1lg1
1419192_at	3.864	Il4i1	1451516_at	3.467	Rhebl1	1460191_at	3.18	0610042I15Rik
1428572_at	3.864	Basp1	1451544_at	3.466	Tapbpl	1429787_x_at	3.177	D10Ertd749e
1433897_at	3.834	AI597468	1460697_s_at	3.465	2610209M04Rik	1435415_x_at	3.177	Mlp
1452223_s_at	3.828	2900054P12Rik	1434080_at	3.463	Aebp2	1457759_at	3.176	A630081D01Rik
1424617_at	3.823	Ifi35	1417541_at	3.463	Hells	1424971_at	3.175	2600001J17Rik
1422893_at	3.82	Sfmbt1	1434180_at	3.46	Plekhc1	1426994_at	3.174	Plekhe1
1451986_s_at	3.809	Lrrk1	1436808_x_at	3.446	Mcm5	1460741_x_at	3.165	D17Wsu92e
1423401_at	3.808	Etv6	1426744_at	3.437	Srebf2	1420028_s_at	3.163	Mcm3
1418280_at	3.803	Copeb	1434437_x_at	3.437	Rrm2	1418854_at	3.162	Birc2

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1451208_at	3.161	Etf1	1456022_at	2.92	B230339E18Rik	1423878_at	2.752	Gypc
1416251_at	3.157	Mcm6	1418183_a_at	2.914	Pscd1	1416130_at	2.751	Prnp
1455902_x_at	3.15	Rhof	1423584_at	2.913	Igfbp7	1451233_at	2.749	Traf2
1451475_at	3.147	Plxnd1	1434184_s_at	2.913	9430080K19Rik	1435800_a_at	2.748	Csda
1417189_at	3.146	Psme2	1453102_at	2.912	Flrt3	1441033_at	2.747	D330034A10Rik
1451082_at	3.145	1300018I05Rik	1424800_at	2.91	Enah	1428585_at	2.746	Actn1
1441855_x_at	3.141	Cxcl1	1417138_s_at	2.91	Polr2e	1420437_at	2.746	Indo
1426712_at	3.14	Slc6a15	1448382_at	2.91	Ehhadh	1415917_at	2.744	Mthfd1
1451065_a_at	3.139	Ddx39	1448350_at	2.906	Asl	1436194_at	2.742	C330008K14Rik
1442586_at	3.129	D130043N08Rik	1448879_at	2.899	Ube2l3	1436656_at	2.74	BC062109
1417406_at	3.126	Sertad1	1422660_at	2.896	Rbm3	1428315_at	2.74	Ebna1bp2
1419069_at	3.12	Rabgef1	1448663_s_at	2.892	Mvd	1452880_at	2.74	Myohd1
1441089_at	3.119	Eif2c3	1420840_at	2.89	Plekha3	1441943_x_at	2.738	2810413N20Rik
1460710_at	3.119	Adora2a	1416844_at	2.89	Hrmt1l1	1448899_s_at	2.738	Rad51ap1
1453129_a_at	3.119	Rgs12	1424541_at	2.889	1110020A09Rik	1417094_at	2.737	Bach
1419030_at	3.118	Ero1l	1427190_at	2.888	2900057D21Rik	1423234_at	2.736	Psmd5
1454161_s_at	3.117	0610007P14Rik	1448184_at	2.886	Fkbp1a	1436805_at	2.735	2810457I06Rik
1429527_a_at	3.114	Plscr1	1449119_at	2.885	Arih2	1439352_at	2.735	Trim7
1426670_at	3.113	Agrn	1426425_at	2.884	Sugt1	1434646_s_at	2.732	Sap18
1426837_at	3.111	Metap1	1451540_at	2.884	Mpi1	1428696_at	2.73	2310015N21Rik
1447818_x_at	3.11	Rhebl1	1448802_at	2.883	Nufip1	1448657_a_at	2.728	Dnajb10
1451379_at	3.11	Rab22a	1439805_at	2.881	Nfat5	1428914_at	2.727	2310014D11Rik
1433781_a_at	3.107	Cldn12	1438682_at	2.881	Pik3r1	1422894_at	2.726	Sfmbt1
1419445_s_at	3.103	Sap18	1423053_at	2.874	Arf4	1453734_at	2.725	4833408C14Rik
1417081_a_at	3.096	Syngr2	1437458_x_at	2.873	Clu	1416981_at	2.723	Foxo1
1425855_a_at	3.094	Crk	1450981_at	2.869	Cnn2	1448261_at	2.721	Cdh1
1415926_at	3.094	Nup62	1416071_at	2.868	Ddx18	1453725_a_at	2.721	Mrps7
1452996_a_at	3.092	Aven	1429100_at	2.867	2010109K11Rik	1452777_a_at	2.721	6330412F12Rik
1450406_a_at	3.087	Siat6	1421957_a_at	2.866	Pcyt1a	1423877_at	2.72	Chaf1b
1433444_at	3.087	Hmgcs1	1415975_at	2.852	Carhsp1	1416641_at	2.72	Lig1
1430404_at	3.085	4833416J08Rik	1419838_s_at	2.849	Plk4	1439399_a_at	2.718	Rnu22
1429149_at	3.083	2700085M18Rik	1460231_at	2.848	Irf5	1428684_at	2.718	1500001M20Rik
1454232_at	3.082	9430027B09Rik	1454812_at	2.847	5730601F06Rik	1421947_at	2.713	Gng12
1418088_a_at	3.078	Stx8	1425255_s_at	2.844	2810036L13Rik	1452254_at	2.711	Mtmr9
1454884_at	3.076	Btbd4	1451346_at	2.841	Mtap	1429193_at	2.711	4631416I11Rik
1424759_at	3.073	Arrdc4	1438168_x_at	2.838	Ddx39	1426208_x_at	2.71	Plagl1
1449855_s_at	3.067	Uchl3	1428378_at	2.838	Zc3hav1	1421861_at	2.706	Clstn1
1438763_at	3.064	D330014H01Rik	1419281_a_at	2.833	Zfp259	1436325_at	2.705	Rora
1420776_a_at	3.062	Auh	1433883_at	2.832	Tpm4	1460691_at	2.704	Zfp598
1428061_at	3.059	Hat1	1456791_at	2.831	AA407452	1433874_at	2.702	AW551225
1433811_at	3.055	AI315037	1428261_at	2.831	2310042L06Rik	1439038_at	2.702	9130227C08Rik
1419036_at	3.054	Csnk2a1	1455320_at	2.829	AI480535	1416595_at	2.699	Mrps22
1417357_at	3.053	Emd	1418889_a_at	2.829	Csnk1d	1449333_at	2.698	Sf3a1
1431050_at	3.052	Rps6ka5	1426904_s_at	2.828	Dnajc10	1448956_at	2.694	Stard10
1448333_at	3.05	Adprh	1427578_a_at	2.827	Itgb4bp	1418242_at	2.692	Faf1
1430307_a_at	3.05	Mod1	1433588_at	2.826	D6Wsu116e	1417426_at	2.69	Prg
1416085_s_at	3.047	Zfp216	1416531_at	2.826	Gsto1	1438011_at	2.689	Pcyt1a
1438716_at	3.047	LOC209387	1426757_at	2.826	Ampd2	1428843_at	2.688	1810015H18Rik
1460711_at	3.045	9930116P15Rik	1433936_at	2.826	0610010E21Rik	1429776_a_at	2.684	Dnajb6
1455082_at	3.043	Cblb	1425157_x_at	2.825	1300010A20Rik	1430536_a_at	2.684	Erh
1417374_at	3.041	Tuba4	1450862_at	2.82	Rad54l	1425027_s_at	2.681	2010005O13Rik
1421118_a_at	3.04	Gpr56	1415800_at	2.819	Gja1	1424318_at	2.68	1110067D22Rik
1434062_at	3.032	8430421H08Rik	1456388_at	2.818	Atp11a	1420342_at	2.677	Gdap10
1426206_at	3.031	Robo4	1421173_at	2.818	Irf4	1437696_at	2.676	BC049807
1422608_at	3.031	Arpp19	1416653_at	2.817	Stxbp3	1438032_at	2.67	Chdc1
1433443_a_at	3.03	Hmgcs1	1424712_at	2.816	Elys	1416331_a_at	2.669	Nfe2l1
1426834_s_at	3.029	D930015E06Rik	1435552_at	2.816	AI462125	1452847_at	2.666	2410008K03Rik
1415945_at	3.029	Mcm5	1422397_a_at	2.815	Il15ra	1416222_at	2.663	Nsdhl
1433445_x_at	3.029	Hmgcs1	1420501_at	2.813	Dnajc1	1451969_s_at	2.662	Adprtl3
1421529_a_at	3.027	Txnrd1	1426533_at	2.813	Nol5a	1453372_at	2.662	4733401K02Rik
1453841_at	3.023	2310050P20Rik	1427892_at	2.81	Myo1g	1434088_at	2.66	2610028M21Rik
1428544_at	3.022	0610007L01Rik	1428579_at	2.81	Fmnl2	1429128_x_at	2.66	Nfkb2
1438606_a_at	3.019	Clic4	1423568_at	2.809	Psma7	1435627_x_at	2.659	Mlp
1423059_at	3.018	Ptk2	1434815_a_at	2.809	AI874665	1438251_x_at	2.659	Prss11
1429475_at	3.018	2810457I06Rik	1435350_at	2.804	Traf6	1429139_at	2.659	2900060B22Rik
1427172_at	3.014	Ofd1	1455821_x_at	2.802	2400006N03Rik	1416061_at	2.657	Tbc1d15
1460319_at	3.012	Fut8	1454109_a_at	2.802	Ptdsr	1435070_at	2.656	Aebp2
1448746_at	3.008	Nbn	1416379_at	2.8	Panx1	1457644_s_at	2.656	Cxcl1
1435526_at	3.007	A130072J07	1427144_at	2.799	2810036L13Rik	1429395_at	2.654	4933434L15Rik
1425263_a_at	3.002	Mbp	1451110_at	2.797	Egln1	1445888_x_at	2.654	Adprtl3
1460356_at	3.001	Esam1	1456355_s_at	2.795	Srr1	1423567_a_at	2.644	Psma7
1421399_at	2.998	Insm1	1454850_at	2.795	1810062O14Rik	1426480_at	2.644	Sbds
1417890_at	2.998	Pdxp	1429370_a_at	2.792	Psmd11	1450873_at	2.641	Gtpbp4
1453287_at	2.993	0610012A05Rik	1422648_at	2.792	Slc7a2	1417629_at	2.641	Prodh
1455904_at	2.993	Gas5	1417500_a_at	2.792	Tgm2	1431688_at	2.641	4833407H14Rik
1436649_at	2.99	Zfpn1a3	1449648_s_at	2.789	Rpo1-1	1432016_a_at	2.641	Idh3a
1426535_at	2.99	9630046K23Rik	1456126_at	2.785	Malt1	1434079_s_at	2.64	Mcm2
1448577_x_at	2.988	Syngr2	1459987_s_at	2.783	Cct3	1423804_a_at	2.638	Idi1
1427253_s_at	2.985	D11Ertd530e	1450067_a_at	2.78	1810034K20Rik	1427061_at	2.637	9930104E21Rik
1426645_at	2.984	Hspca	1436828_a_at	2.777	Tpd52l2	1434113_a_at	2.635	XPMC2H
1416230_at	2.975	Rfk	1450455_s_at	2.776	Akr1c12	1428087_at	2.634	Dnm1l
1418116_at	2.972	Ifrg15	1438948_x_at	2.774	Bzrp	1441107_at	2.634	Dmrta2
1457218_at	2.967	6430510M02Rik	1438008_at	2.771	Gga3	1428851_at	2.634	1300014I06Rik
1459791_at	2.966	Dnajc1	1427913_at	2.769	Rwdd1	1428390_at	2.632	2610318G08Rik
1448794_s_at	2.965	Zrf2	1434419_s_at	2.768	Tardbp	1454791_a_at	2.628	Rbbp4
1438215_at	2.965	Sfrs3	1416020_a_at	2.768	Atp5g1	1435647_at	2.625	Ikbkg
1452233_at	2.96	Abcc1	1452226_at	2.767	2610510H01Rik	1437494_at	2.623	AI874665
1418578_at	2.958	Dgka	1424140_at	2.766	Gale	1416070_a_at	2.621	Ddx18
1455814_x_at	2.958	Ddx39	1440275_at	2.764	Runx3	1424801_at	2.615	Enah
1434910_at	2.941	2310067E24Rik	1434252_at	2.763	C630016B22Rik	1434220_at	2.611	AI849286
1452679_at	2.936	2410129E14Rik	1423393_at	2.762	Clic4	1440770_at	2.609	Bcl2
1416563_at	2.932	Ctps	1450992_a_at	2.761	Meis1	1437869_at	2.607	3222402P14Rik
1417110_at	2.93	Man1a	1451487_at	2.76	9530020D24Rik	1418226_at	2.607	Orc2l
1437222_x_at	2.929	Rrm2b	1422707_at	2.76	Pik3cg	1434929_at	2.606	BC035044
1421459_a_at	2.928	Lrp8	1421052_a_at	2.76	Sms	1433514_at	2.604	Etnk1
1448450_at	2.921	Ak2	1452999_at	2.754	2410004J23Rik	1417679_at	2.599	Gfi1

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1417783_at	2.594	Als2	1454731_at	2.477	Myo10	1437945_x_at	2.369	Nap1l1
1452612_at	2.588	Zfp294	1426923_at	2.475	Hrb	1434482_at	2.369	D4Ertd22e
1431087_at	2.587	2410030K01Rik	1420351_at	2.474	Tnfrsf4	1452309_at	2.369	4933421H10Rik
1416724_x_at	2.585	Tcf4	1459270_at	2.466		1456586_x_at	2.368	Mvp
1416290_a_at	2.58	Psmc4	1427874_at	2.466	Zfp313	1448624_at	2.367	Cd2bp2
1449839_at	2.577	Casp3	1440192_at	2.464	1810054D07Rik	1415720_s_at	2.365	Mad2l1bp
1456494_a_at	2.577	LOC209387	1434143_at	2.463	BC060631	1428816_a_at	2.364	Gata2
1451281_at	2.575	Zfp96	1418070_at	2.463	Cdyl	1422737_at	2.363	Ncoa3
1436509_at	2.575	2410014A08Rik	1447703_x_at	2.463	3110024A21Rik	1422788_at	2.363	Slc43a3
1429005_at	2.574	Mfhas1	1433428_x_at	2.463	Tgm2	1426587_a_at	2.363	Stat3
1449324_at	2.574	Ero1l	1434954_at	2.462	Mpp5	1416585_at	2.36	Ruvbl1
1426147_s_at	2.573	Cldn10	1447837_x_at	2.46	Polh	1419444_at	2.358	Sap18
1451587_a_at	2.573	1810011K17Rik	1423038_at	2.459	Stx6	1418154_at	2.358	BC004022
1423467_at	2.572	Ms4a4b	1449209_a_at	2.456	Rdh11	1423976_at	2.358	4930453N24Rik
1448135_at	2.57	Atf4	1424296_at	2.456	Gclc	1420021_s_at	2.358	D11Ertd530e
1439120_at	2.569	BC010304	1419068_at	2.456	Rabgef1	1419253_at	2.357	Mthfd2
1431020_a_at	2.565	Fgfr1op2	1418962_at	2.455	1110005F07Rik	1451509_at	2.354	4921516M08Rik
1426495_at	2.564	2410042D21Rik	1452111_at	2.454	Mrps35	1449404_at	2.353	Pip5k2a
1427914_a_at	2.56	Tceb1	1439975_at	2.451	BC062109	1416345_at	2.353	Timm8a
1426942_at	2.559	Aim1	1436617_at	2.449	Cetn4	1426187_a_at	2.352	Hs1bp1
1417948_s_at	2.559	Ilf2	1416554_at	2.449	Pdlim1	1425142_a_at	2.351	Hnrpd
1426222_s_at	2.558	5830475I06Rik	1418828_at	2.447	3110010F15Rik	1423884_at	2.349	Tex292
1452162_at	2.556	8430408H12Rik	1428491_at	2.446	Commd10	1428745_a_at	2.348	2310003L22Rik
1453636_at	2.555	5830406C17Rik	1420330_at	2.444	Clecsf9	1451313_a_at	2.348	1110067D22Rik
1449585_at	2.555	Il1rap	1418711_at	2.443	Pdgfa	1423431_a_at	2.347	Mybbp1a
1415894_at	2.55	Enpp2	1460739_at	2.44	D11Bwg0280e	1416076_at	2.345	Ccnb1
1420196_s_at	2.549	D5Ertd110e	1416897_at	2.437	BC003281	1415670_at	2.343	Copg
1433940_at	2.547	Spag7	1454814_s_at	2.435	Prps1	1427448_at	2.342	Rabep1
1428903_at	2.545	3110037I16Rik	1429859_a_at	2.435	Arl2bp	1417035_at	2.342	2410004C24Rik
1418637_at	2.543	Etv3	1422708_at	2.434	Pik3cg	1426300_at	2.341	Alcam
1455840_at	2.542	Rapgef5	1434928_at	2.433	4930500E24Rik	1454817_at	2.339	6230425C22Rik
1435280_at	2.542	AI452195	1417700_at	2.432	Rab38	1451164_a_at	2.338	Mrps18b
1429227_x_at	2.541	Nap1l1	1455000_at	2.432	Gpr68	1427152_at	2.336	4732486I23Rik
1430303_at	2.54	4921537D05Rik	1424681_a_at	2.43	Psma5	1415739_at	2.335	3100004P22Rik
1423596_at	2.54	Nek6	1453589_a_at	2.429	2610005L07Rik	1441814_s_at	2.334	2400006N03Rik
1460556_at	2.538	D15Mit260	1448377_at	2.428	Slpi	1416506_at	2.334	Psma6
1415916_a_at	2.537	Mthfd1	1418627_at	2.425	Gclm	1438369_x_at	2.332	2610318K02Rik
1418025_at	2.536	Bhlhb2	1423212_at	2.424	Phc1	1419368_a_at	2.332	Rnf138
1434159_at	2.535	Stk4	1419280_at	2.424	Pip5k2a	1416606_s_at	2.33	Nola2
1448441_at	2.534	Cks1	1452601_a_at	2.421	Acbd6	1424075_at	2.329	9430016H08Rik
1419639_at	2.534	Efnb2	1438115_a_at	2.42	Slc9a3r1	1421880_at	2.328	Mtmr1
1449089_at	2.534	Nrip1	1416092_a_at	2.419	Mtap4	1431947_at	2.326	Ldlr
1419471_a_at	2.532	Nudc	1452585_at	2.419	Mrps28	1415859_at	2.326	Eif3s8
1450722_at	2.53	Nup50	1417140_a_at	2.419	Ptpn2	1428418_s_at	2.324	3110050N22Rik
1420583_a_at	2.53	Rora	1428300_at	2.417	4932439K10Rik	1435904_at	2.324	Eif2c3
1437277_x_at	2.528	Tgm2	1449446_at	2.417	D10Ertd718e	1433894_at	2.323	AI591476
1426084_a_at	2.528	MGC6357	1422764_at	2.417	Mapre1	1422730_at	2.319	Limd1
1455616_at	2.528	AI586002	1424883_s_at	2.416	Sfrs7	1425550_a_at	2.319	Prkar1a
1420851_at	2.528	Pard6g	1425507_at	2.416	Arfrp1	1417037_at	2.317	Orc6l
1431411_a_at	2.527	Rai12	1454942_at	2.416	AU019833	1415882_at	2.317	Ghitm
1452230_at	2.526	Dnajc10	1417767_at	2.413	1810044O22Rik	1423870_at	2.316	2610208M17Rik
1426210_x_at	2.525	Adprtl3	1419548_at	2.412	Kpna1	1424448_at	2.316	Trim6
1423039_a_at	2.524	Bzw1	1424705_at	2.407	2810411A03Rik	1422429_at	2.316	Rnf14
1427325_s_at	2.524	AI597013	1426716_at	2.406	Tdrd7	1433951_at	2.315	2810410P22Rik
1450028_a_at	2.524	Lancl2	1423306_at	2.405	2010002N04Rik	1456393_at	2.315	Pdcd4
1419385_a_at	2.523	Ubqln1	1428979_at	2.405	Mtf1	1446737_a_at	2.313	Hook3
1435085_at	2.523	AI046348	1427539_a_at	2.404	D10Ertd749e	1438902_a_at	2.311	Hspca
1426256_at	2.523	Timm17a	1426885_a_at	2.402	Cdk2ap1	1438999_a_at	2.31	Nfat5
1419641_at	2.519	Purb	1423810_at	2.401	2700017M01Rik	1456795_at	2.306	D330027G24Rik
1422547_at	2.518	Ranbp1	1437742_at	2.4	Rab21	1426875_s_at	2.305	Npn3
1433789_at	2.517	Rnu17d	1424413_at	2.399	Ogfrl1	1451206_s_at	2.304	Pscdbp
1423773_at	2.513	1700034P14Rik	1418403_at	2.397	Adam19	1428760_at	2.303	Snapc3
1426279_at	2.513	5830415L20Rik	1449362_a_at	2.397	Map4k6	1416723_at	2.302	Tcf4
1448953_at	2.513	Blm	1455039_a_at	2.396	Sin3b	1435901_at	2.302	Dgkd
1436754_at	2.509	AI839735	1425462_at	2.393	Fbxw1b	1454703_x_at	2.301	Rnu22
1452411_at	2.507	Lrrc1	1429328_at	2.393	p47	1452168_x_at	2.301	Gspt1
1436812_at	2.507	Fkrp	1448957_at	2.392	Rbpsuh	1459902_at	2.299	2700007P21Rik
1457376_at	2.506	Itga4	1424169_at	2.392	1300011C24Rik	1423386_at	2.298	Psmd9
1433656_a_at	2.504	C77032	1452833_at	2.392	Rapgef2	1439269_x_at	2.296	Mcm7
1434355_at	2.501	Zfp617	1450735_at	2.391	1810003N24Rik	1418905_at	2.294	Nubp1
1448435_at	2.5	Pcqap	1415817_s_at	2.391	Cct7	1451658_a_at	2.294	Polr3c
1424316_at	2.498	Slc25a19	1424418_at	2.391	BC010801	1448419_at	2.294	Pop4
1417592_at	2.497	Frap1	1451272_a_at	2.391	2510010F15Rik	1426736_at	2.291	Gspt1
1433782_at	2.496	Cldn12	1433451_at	2.389	Cdk5r	1427197_at	2.289	Atr
1416380_at	2.494	Mov10	1437711_x_at	2.389	Odc	1452778_x_at	2.288	Nap1l1
1448536_at	2.494	Lsm3	1426579_at	2.388	BC003262	1438095_x_at	2.288	AI326906
1416979_at	2.494	2510048O06Rik	1448586_at	2.386	Hspa14	1434035_at	2.288	Dnajb6
1427247_at	2.493	D3Bwg0562e	1449705_x_at	2.386	Mcm3	1425331_at	2.288	Zfp106
1420476_a_at	2.493	Nap1l1	1417126_a_at	2.385	3110001N18Rik	1431506_s_at	2.286	Ppih
1422535_at	2.493	Ccne2	1428648_at	2.385	D10Ertd516e	1448430_a_at	2.286	Naca
1450844_at	2.493	Stx6	1434400_at	2.385	Tgif2	1426580_at	2.286	Plk4
1442497_at	2.489	4931400A14Rik	1426503_a_at	2.385	Rnf121	1418730_at	2.285	Rnf12
1454018_at	2.487	Tlk2	1418825_at	2.383	Ifi1	1460360_at	2.283	Asrgl1
1421066_at	2.486	Jak2	1448772_at	2.383	Ube2a	1417190_at	2.283	Pbef1
1417041_at	2.486	Rpo1-1	1423519_at	2.381	2210412D01Rik	1448500_a_at	2.282	2810038K19Rik
1428195_at	2.486	4631427C17Rik	1424095_at	2.379	Rtcd1	1448677_at	2.28	Noc4
1446938_at	2.485	AA408213	1417637_a_at	2.375	Hmg20b	1452759_s_at	2.279	Ppfibp1
1416973_at	2.485	Ssfa1	1424698_s_at	2.375	Gca	1448160_at	2.279	Lcp1
1424710_a_at	2.484	Gorasp2	1449039_a_at	2.374	Hnrpdl	1427944_at	2.276	C1qdc1
1420368_at	2.484	Denr	1419649_s_at	2.374	Myo1c	1450275_x_at	2.275	2510010F15Rik
1454729_at	2.482	B130017P16Rik	1451649_a_at	2.373	2410118I19Rik	1452367_at	2.275	Coro2a
1416214_at	2.481	Mcm4	1416726_s_at	2.373	Ube2s	1449256_a_at	2.275	Rab11a
1446769_at	2.48	2810439F02Rik	1423355_at	2.373	Snap29	1456778_at	2.273	Nrp2
1418384_at	2.48	9430083G14Rik	1454888_at	2.372	Pfdn4	1425572_a_at	2.272	Ddef1
1417517_at	2.48	Plagl2	1428498_at	2.371	2610206B13Rik	1423511_at	2.272	Asf1a
1434666_at	2.478	1110054A01Rik	1425599_a_at	2.371	Odag	1455162_at	2.27	4922503N01Rik

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1454650_at	2.27	Trim35	1435396_at	2.195	C85317	1418961_at	2.104	1110005F07Rik
1416341_at	2.27	Polr2c	1447649_x_at	2.194	Dnajc1	1436947_a_at	2.103	Txnl1
1455137_at	2.269	Rapgef5	1416526_a_at	2.194	Park7	1417192_at	2.101	D16Wsu109e
1422609_at	2.269	Arpp19	1415961_at	2.193	Itm2c	1421914_s_at	2.101	Mrpl19
1421937_at	2.269	Dapp1	1417288_at	2.192	Plekha2	1442793_s_at	2.1	Tbrg4
1452393_at	2.269	AI597013	1426276_at	2.191	9130009C22Rik	1417712_at	2.1	Eif2s2
1452828_at	2.268	Fbxo21	1423661_s_at	2.19	Ctdsp2	1438161_s_at	2.1	Rfc4
1424034_at	2.268	Rora	1424136_a_at	2.189	Ppih	1419886_at	2.098	DXErtd223e
1424597_at	2.268	ORF19	1421821_at	2.189	Ldlr	1434404_at	2.098	C030011O14Rik
1428293_at	2.267	2310022M17Rik	1448122_at	2.188	Tcp1	1434619_at	2.098	4932417H02Rik
1432646_a_at	2.265	2900097C17Rik	1452662_a_at	2.188	Eif2s1	1419447_s_at	2.098	Tbc1d1
1424089_a_at	2.265	Tcf4	1452458_s_at	2.187	Ppil5	1427258_at	2.097	Trim24
1419430_at	2.264	Cyp26a1	1418527_a_at	2.187	Fusip1	1451114_at	2.097	Cklfsf6
1452912_at	2.262	2600005O03Rik	1448172_at	2.186	Mdh1	1423517_at	2.094	Cct6a
1423949_at	2.261	D11Moh35	1448101_s_at	2.181	Trim27	1426918_at	2.094	Itgb1
1449645_s_at	2.261	Cct3	1453256_at	2.179	Polr3c	1415782_at	2.093	Smt3h2
1456056_a_at	2.261	D6Wsu116e	1417622_at	2.178	Slc12a2	1428674_at	2.092	1110021E09Rik
1434273_at	2.26	A830073O21Rik	1418443_at	2.178	Xpo1	1435402_at	2.09	A930008A22Rik
1428132_at	2.256	Spec1	1434880_at	2.177	Etv6	1426436_at	2.089	8430420C20Rik
1416485_at	2.254	Timm23	1454214_a_at	2.177	2410019A14Rik	1424523_at	2.089	Elmo1
1455680_at	2.253	9630025H16Rik	1434790_a_at	2.177	Lta4h	1418369_at	2.089	Prim1
1420661_a_at	2.253	4933439F18Rik	1437468_x_at	2.177	Fbxw1b	1421751_a_at	2.089	Psmd14
1416073_a_at	2.253	Pcnt2	1452884_at	2.177	2610510E10Rik	1459289_at	2.088	
1452753_at	2.252	1110054H05Rik	1430594_at	2.17	4833414G05Rik	1427006_at	2.085	Rapgef1
1438562_a_at	2.251	Ptpn2	1423220_at	2.17	Eif4e	1419062_at	2.084	Epb4.1l3
1416742_at	2.25	Cfdp	1448757_at	2.169	Pml	1424452_at	2.084	5730555F13Rik
1427889_at	2.249	Spna2	1452241_at	2.169	2810429C13Rik	1417352_s_at	2.084	Snrpa1
1451339_at	2.248	Suox	1419558_at	2.166	Mdm4	1452257_at	2.083	Bdh
1451296_x_at	2.248	Pabpc4	1417387_at	2.165	3110004H13Rik	1428226_at	2.083	1500001L15Rik
1460498_a_at	2.248	Dnajc5	1422509_at	2.164	U2af1	1456196_x_at	2.081	Fkbp1a
1457687_at	2.248	Bcl2	1422712_a_at	2.161	Ube2i	1450815_s_at	2.079	Chchd2
1438938_x_at	2.246	Bcap37	1417547_at	2.158	Sart3	1424514_at	2.079	Rnf126
1453045_at	2.246	4921537D05Rik	1426486_at	2.158	Ubxd2	1420820_at	2.079	2900073G15Rik
1439972_at	2.243	D6Ertd3e	1449546_a_at	2.155	Zfp617	1460323_at	2.078	Tars
1422430_at	2.243	Fignl1	1415737_at	2.155	Rfk	1437131_x_at	2.078	Mrpl11
1454712_at	2.242	D130005A03Rik	1434708_at	2.154	Vhlh	1424161_at	2.075	Ddx27
1452352_at	2.238	Ctla2b	1424837_at	2.154	2810428C21Rik	1426529_a_at	2.074	Tagln2
1420826_at	2.238	Letm1	1455164_at	2.154	Cdgap	1427992_a_at	2.074	Rab12
1424244_at	2.238	BC016198	1426523_a_at	2.153	Gnpda2	1416084_at	2.073	Zfp216
1423588_at	2.237	Arpc4	1452151_at	2.151	BC021523	1437061_at	2.073	Mbd1
1432416_a_at	2.237	Npm1	1435317_x_at	2.15	Psma6	1428086_at	2.072	Dnm1l
1448178_a_at	2.237	Cct3	1416433_at	2.149	Rpa2	1450013_at	2.072	2900073G15Rik
1416240_at	2.236	Psmb7	1422731_at	2.146	Limd1	1415985_at	2.072	D8Ertd633e
1417200_at	2.236	1300007B12Rik	1431972_a_at	2.146	2900054P12Rik	1428080_at	2.071	2610528A17Rik
1423848_at	2.236	Mphosph6	1416489_at	2.145	Pi4k2b	1417657_s_at	2.07	Zrf2
1434450_s_at	2.235	Adrbk2	1416212_at	2.145	Magoh	1438992_x_at	2.069	Atf4
1426483_at	2.233	Prkrir	1437402_x_at	2.144	D7Ertd743e	1436171_at	2.068	6030405P05Rik
1433722_at	2.233	5730522G15Rik	1429054_at	2.143	4833424P18Rik	1424662_at	2.068	4933435E07Rik
1434488_at	2.232	Arfrp1	1429568_x_at	2.142	2510010F15Rik	1451217_a_at	2.067	1500034J20Rik
1460193_at	2.231	St13	1455643_s_at	2.142	AW550801	1418884_x_at	2.066	Tuba1
1420486_at	2.231	Nol7	1423890_x_at	2.142	Atp1b1	1448246_at	2.065	Hdac1
1426719_at	2.231	Apbb2	1426676_s_at	2.142	D16Wsu109e	1427228_at	2.064	2410003B16Rik
1429129_at	2.23	1200008A14Rik	1417493_at	2.141	Bmi1	1417254_at	2.063	Spata5
1428529_at	2.23	2810026P18Rik	1426739_at	2.141	Donson	1459906_at	2.063	Dgkh
1416024_x_at	2.228	Cct3	1450915_at	2.14	Ap3b1	1417353_x_at	2.062	Snrpa1
1456236_s_at	2.227	Commd10	1417938_at	2.139	Rad51ap1	1422495_a_at	2.062	Hmgn1
1435675_at	2.226	BC033574	1455409_at	2.138	SPIRE1	1423616_at	2.062	Tarbp2
1452179_at	2.225	Phf17	1423373_at	2.137	Rpp30	1439407_x_at	2.059	Tagln2
1444992_at	2.225	AI120166	1450668_s_at	2.137	Hspe1	1452128_a_at	2.059	C6.1A
1452063_at	2.225	2410081M15Rik	1428232_at	2.137	4733401N12Rik	1416336_s_at	2.059	Snrpd1
1438116_x_at	2.224	Slc9a3r1	1417252_at	2.137	Nt5c	1417509_at	2.058	Rnf19
1428915_at	2.224	Sirt5	1449007_at	2.136	Btg3	1421413_a_at	2.056	1110001A05Rik
1429749_at	2.221	9330180L21Rik	1448230_at	2.136	Usp10	1417458_s_at	2.055	Cks2
1437734_at	2.22	Ppp1r12a	1451496_at	2.136	Mtss1	1451359_at	2.054	BC005662
1448259_at	2.22	Fstl1	1422570_at	2.135	Yy1	1416549_at	2.054	AB041549
1429617_at	2.22	Cyld	1428311_at	2.134	2810012H18Rik	1422693_a_at	2.054	Rpo2tc1
1436953_at	2.219	Waspip	1451574_at	2.133	Bcl9	1437859_x_at	2.052	Eif5a
1420024_s_at	2.218	Etf1	1448597_at	2.133	Cstf1	1422627_a_at	2.051	Mkks
1426324_at	2.218	H2-Q10	1436512_at	2.132	A630084M22Rik	1423957_at	2.05	2700083B06Rik
1422959_s_at	2.217	Zfp313	1433766_at	2.13	C330023M02Rik	1426674_at	2.05	Eif3s9
1429849_at	2.216	4632411B12Rik	1416075_at	2.13	Sav1	1417774_at	2.049	Nans
1418946_at	2.215	Siat4a	1423241_a_at	2.128	Tfdp1	1455244_at	2.048	Daam1
1417303_at	2.212	Mvd	1450016_at	2.128	Ccng1	1429307_s_at	2.047	Lzic
1424562_a_at	2.212	Slc25a4	1428806_at	2.127	Csnk1g1	1434058_at	2.047	Pip3ap
1417183_at	2.211	Dnaja2	1428645_at	2.126	Gnai3	1426456_a_at	2.047	Miz1
1454042_a_at	2.21	Srpk1	1436508_at	2.125	2410014A08Rik	1448206_at	2.046	Psma2
1458438_at	2.21	4933415L06Rik	1415740_at	2.123	Psmc5	1419279_at	2.046	Pip5k2a
1454706_at	2.208	Uvrag1	1447909_s_at	2.121	Selk	1436510_a_at	2.046	Lrrfip2
1417985_at	2.208	Nrarp	1448543_at	2.121	2310042G06Rik	1432271_a_at	2.045	4833420K19Rik
1416106_at	2.207	1110001A12Rik	1430271_x_at	2.121	4930553M18Rik	1438955_x_at	2.045	Ppif
1417527_at	2.207	Ap3m2	1460004_x_at	2.117	Stx6	1417096_at	2.044	2810430M08Rik
1419263_a_at	2.206	Adrm1	1451401_a_at	2.115	0610009K11Rik	1455069_x_at	2.043	Slc25a4
1424468_s_at	2.206	D330037A14Rik	1450629_at	2.115	D15Ertd366e	1438977_x_at	2.043	Ran
1420774_a_at	2.206	4930583H14Rik	1422884_at	2.114	Snrpd3	1422443_at	2.042	Xpnpep1
1453993_a_at	2.204	Bnip2	1449406_at	2.113	Cyhr1	1426854_a_at	2.04	Set
1425687_at	2.204	Cflar	1418284_at	2.113	Tcfl1	1444478_at	2.04	2900057D21Rik
1417718_at	2.203	Eif3s4	1422000_at	2.112	Akr1c12	1456308_x_at	2.039	Trim28
1448670_at	2.203	Ube2e3	1450943_at	2.112	2010012C16Rik	1433571_at	2.039	A130038L21Rik
1428873_a_at	2.202	4121402D02Rik	1453554_a_at	2.111	1110001N06Rik	1418372_at	2.038	Adsl
1433970_at	2.199	1810056O20Rik	1453050_at	2.111	2700085M18Rik	1460460_a_at	2.038	Gorasp2
1433696_at	2.199	D17Ertd441e	1438835_a_at	2.11	Snrp116	1422848_a_at	2.038	Pabpn1
1451144_at	2.197	1110064N10Rik	1424344_s_at	2.109	Eif1a	1424305_at	2.038	Igj
1454804_at	2.197	AK129375	1451657_a_at	2.108	Cova1	1428440_at	2.038	Slc25a12
1425030_at	2.196	Zfp622	1417186_at	2.107	Hip2	1448509_at	2.034	3110001A13Rik
1417392_a_at	2.196	Slc7a7	1422103_a_at	2.106	Stat5b	1450650_at	2.034	Myo10
1416026_a_at	2.196	Rpl12	1426846_at	2.106	G630055P03Rik	1417165_at	2.034	Mbd2

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1417865_at	2.034	Tnfaip1	1418524_at	1.958	Pcm1	1415703_at	1.841	Ureb1
1418071_s_at	2.031	Cdyl	1423133_at	1.957	0610040D20Rik	1447016_at	1.837	Tbc1d1
1460339_at	2.03	Psma4	1416612_at	1.956	Cyp1b1	1423969_at	1.836	Nup37
1448234_at	2.029	Dnajb6	1433606_at	1.955	Mitc1	1419258_at	1.833	Tcea1
1434020_at	2.029	Pdap1	1430053_a_at	1.953	2810409H07Rik	1420399_at	1.83	Gfi1b
1427412_s_at	2.028	A530068K01	1437839_x_at	1.953	Mrpl11	1420873_at	1.83	Ptk9
1455084_x_at	2.028	Shmt2	1424343_a_at	1.951	Eif1a	1442761_at	1.826	Tpd52l2
1436040_at	2.028	2310005L22Rik	1451665_a_at	1.951	Ap4s1	1429476_s_at	1.826	Dnaja2
1428468_at	2.028	3110043O21Rik	1453362_x_at	1.95	Rps24	1449968_s_at	1.826	Acate2
1448317_at	2.027	2810021O14Rik	1448896_at	1.95	Pigf	1417311_at	1.826	Crip2
1429528_at	2.026	Rae1	1426471_at	1.949	Zfp52	1426626_at	1.819	Gtf2f2
1453001_at	2.025	2610207P08Rik	1436559_a_at	1.948	Psmd10	1440255_at	1.818	AA589481
1421640_a_at	2.025	Tank	1418010_a_at	1.946	Sh3glb1	1450679_at	1.815	5730470L24Rik
1435975_at	2.024	AI115600	1427439_s_at	1.944	Skb1	1455067_at	1.814	BC046518
1434335_at	2.023	AI317237	1434087_at	1.944	Mthfr	1441843_s_at	1.813	5230400M03Rik
1424132_at	2.022	Hras1	1460396_at	1.944	Ddx54	1431339_a_at	1.812	Efhd2
1415869_a_at	2.021	Trim28	1448884_at	1.94	Gtf2e2	1447752_x_at	1.811	Drg1
1417125_at	2.02	Ahcy	1435863_at	1.939	Commd6	1439030_at	1.805	Gmppb
1435890_at	2.02	AW228836	1424211_at	1.938	5730438N18Rik	1432848_a_at	1.804	1200004M23Rik
1418073_at	2.019	Acate2	1434994_at	1.937	Nit1	1455547_at	1.802	Scrg3
1429188_at	2.017	2010004I09Rik	1460614_at	1.935	D130064H19Rik	1456571_at	1.798	1700001E16Rik
1417026_at	2.016	Pfdn1	1438250_s_at	1.934	Taf9	1420478_at	1.798	Nap1l1
1422688_a_at	2.016	Nras	1426836_s_at	1.934	Metap1	1455341_at	1.798	2010003J03Rik
1448324_at	2.014	Rnps1	1418844_at	1.934	Dibd1	1422836_at	1.797	Mbnl3
1429618_at	2.014	Cyld	1455252_at	1.934	Tsc1	1435343_at	1.795	Dock10
1417974_at	2.013	Kpna4	1416235_at	1.934	AA959742	1456003_a_at	1.793	Slc1a4
1428113_at	2.013	4930403J22Rik	1430515_s_at	1.933	Aasdhppt	1438963_s_at	1.793	Tfpt
1415752_at	2.012	BC031181	1419208_at	1.933	Map3k8	1419452_at	1.792	Uchl5
1424751_at	2.012	Abt1	1419266_at	1.933	Nfyb	1455727_at	1.792	U2af1-rs2
1433547_s_at	2.011	4921532K09Rik	1453257_at	1.932	D8Ertd319e	1460351_at	1.789	S100a11
1424269_a_at	2.01	Myl6	1424164_at	1.931	Mrpl50	1416208_at	1.788	Usp14
1434230_at	2.01	Polb	1453206_at	1.928	Acad9	1438458_a_at	1.788	Sfpq
1416197_at	2.01	Snrp1c	1422624_at	1.927	Rev1l	1437082_at	1.785	Akap9
1453865_a_at	2.009	DXImx46e	1455897_x_at	1.925	Hmgn1	1424897_at	1.781	2900026B03Rik
1455287_at	2.009	5830411I20	1417019_a_at	1.923	Cdc6	1429710_at	1.781	Styx
1423799_at	2.008	Sui1-rs1	1455043_at	1.922	Tnpo1	1430798_x_at	1.78	Mrpl15
1418491_a_at	2.008	2610020J05Rik	1443159_at	1.918	9130221J17Rik	1424850_at	1.779	Map3k1
1418827_at	2.008	3110010F15Rik	1450982_at	1.917	Slc9a3r1	1433768_at	1.778	2410003B16Rik
1455102_at	2.008	D330037H05Rik	1460428_at	1.917	1100001D10Rik	1416209_at	1.771	Glud
1433565_at	2.006	2410002M20Rik	1435697_a_at	1.916	Pscdbp	1439421_x_at	1.767	Cbx3
1427718_a_at	2.005	Mdm2	1452172_at	1.915	2810421I24Rik	1437356_at	1.766	Ebi2
1421985_a_at	2.005	Eif4el3	1435535_at	1.913	Depdc5	1423577_at	1.757	C730024G01Rik
1427332_at	2.005	2410018M14Rik	1448938_at	1.913	Rpa3	1423072_at	1.756	6720475J19Rik
1454975_at	2.004	BC033596	1423545_a_at	1.912	Zfp207	1429769_at	1.749	Pggt1b
1429737_a_at	2.004	0610009I22Rik	1434173_s_at	1.911	D19Bwg1357e	1450425_a_at	1.748	2700062C07Rik
1417840_at	2.004	1500031L02Rik	1448633_at	1.911	1500019O16Rik	1425319_s_at	1.747	6530403A03Rik
1451083_s_at	2.002	Aars	1433590_at	1.91	Herc3	1417111_at	1.741	Man1a
1437749_s_at	2.001	Mrpl9	1420817_at	1.908	Ywhag	1416920_at	1.732	Rbm4
1423781_at	2.001	Appbp1	1427938_at	1.908	Mycbp	1428744_s_at	1.732	Bri3bp
1416874_a_at	2	5730511K23Rik	1444158_at	1.908	Jarid1c	1428953_at	1.723	4930463P07Rik
1419257_at	2	Tcea1	1455436_at	1.907	2900052J15Rik	1456541_x_at	1.718	Atad3a
1415710_at	2	BC038311	1424983_a_at	1.906	2700078E11Rik	1429056_at	1.718	1300019C06Rik
1434432_at	2	1700051E09Rik	1436885_a_at	1.903	Cherp	1456626_a_at	1.717	1110005A23Rik
1436222_at	2	Gas5	1418760_at	1.903	Rdh11	1455801_x_at	1.717	Tbcd
1429384_at	1.999	Csnk1g3	1424206_at	1.902	Smarca5	1445440_at	1.715	A430106J12Rik
1426914_at	1.997	Mrvldc2	1428291_at	1.902	Exosc8	1428880_at	1.715	0610038F07Rik
1451231_a_at	1.995	Cul2	1421326_at	1.9	Csf2rb2	1416354_at	1.711	Rbmx
1460684_at	1.994	Tm7sf2	1428251_at	1.9	4931400A14Rik	1417947_at	1.706	Pcna
1433668_at	1.994	Pnrc1	1429711_at	1.9	Styx	1428729_at	1.705	Ccm1
1455988_a_at	1.992	Cct6a	1428154_s_at	1.899	2310022A04Rik	1453631_at	1.695	Stx8
1423089_at	1.99	Tmod3	1415730_at	1.899	5730453I16Rik	1421046_a_at	1.693	Pabpc4
1453545_at	1.989	5730492I20Rik	1435779_at	1.897	Cep1	1440972_at	1.689	Nsd1
1436955_at	1.988	D12Ertd604e	1428213_at	1.896	2410003A14Rik	1452198_at	1.677	Fbxl10
1456738_s_at	1.987	Brp16	1434434_s_at	1.896	Tcerg1	1423898_a_at	1.676	Gtl6
1428294_at	1.987	Zfp259	1429183_at	1.892	1200008D14Rik	1429080_at	1.674	Mphosph10
1441415_at	1.986	Spred2	1416415_a_at	1.892	H2afz	1416037_a_at	1.672	Cct2
1428959_at	1.986	5830405N20Rik	1437432_a_at	1.89	Trim12	1451457_at	1.671	Sc5d
1428207_at	1.985	Bcl7a	1430778_a_at	1.889	Nubp1	1423492_at	1.664	Mrpl45
1460590_s_at	1.984	Ywhaq	1439901_at	1.889	2610208M17Rik	1425373_a_at	1.662	Tnfsf5ip1
1452681_at	1.982	Dtymk	1430134_a_at	1.887	2210023C10Rik	1434743_x_at	1.649	Rusc1
1423642_at	1.981	4930542G03Rik	1433537_at	1.886	4833408C14Rik	1428527_at	1.645	Snx7
1430292_a_at	1.981	1810030N24Rik	1432842_s_at	1.885	Ywhaq	1451076_s_at	1.643	Ga17
1415775_at	1.98	Rbbp7	1429897_a_at	1.884	D16Ertd472e	1423916_s_at	1.639	Mlf2
1448511_at	1.979	Ptprcap	1434892_x_at	1.883	Rbbp4	1436037_at	1.635	Cerkl
1455914_at	1.978	AI987944	1436419_a_at	1.879	1700097N02Rik	1416881_at	1.633	Mcl1
1433850_at	1.978	Ppp4r2	1424621_at	1.879	AA792894	1450649_at	0.624	Gng10
1435342_at	1.978	Kcnk6	1438267_x_at	1.878	Rnps1	1428490_at	0.622	2210410E06Rik
1422491_a_at	1.977	Bnip2	1426787_at	1.878	2310047I15Rik	1422449_s_at	0.617	Rcn2
1417801_a_at	1.977	Ppfibp2	1460113_at	1.878	B930093H17Rik	1419499_at	0.615	Gpam
1418790_at	1.976	Zfp312	1415934_at	1.873	Cops8	1424215_at	0.609	1500005J14Rik
1438958_x_at	1.976	Fkbp1a	1435446_a_at	1.873	Chpt1	1428327_at	0.609	2310001H13Rik
1423235_at	1.974	Ap3b1	1415965_at	1.869	Scd1	1423955_a_at	0.608	Lass2
1417367_at	1.973	Ppp2ca	1450386_at	1.868	Kpna3	1416253_at	0.607	Cdkn2d
1459842_x_at	1.972	Nubp2	1423815_at	1.867	Ddx56	1436900_x_at	0.606	Obrgrp
1429491_s_at	1.971	D2Ertd145e	1416748_a_at	1.866	Mre11a	1416668_at	0.601	4921531G14Rik
1428858_at	1.971	4833422F06Rik	1435661_at	1.864	AI853305	1454941_at	0.597	AW536594
1448575_at	1.968	Il7r	1425140_at	1.856	Lactb2	1418842_at	0.597	Hcls1
1439256_x_at	1.968	Tm7sf1	1454756_at	1.853	2210409B11Rik	1418366_at	0.597	Hist2h2aa1
1448527_at	1.967	Pdcd10	1426485_at	1.852	Ubxd2	1456177_x_at	0.597	3110006P09Rik
1422186_s_at	1.967	Dia1	1426366_at	1.851	Eif2c2	1451071_a_at	0.595	Atp1a1
1420089_at	1.965	Nfkbia	1453095_at	1.851	Rab10	1455312_at	0.594	Phc3
1449670_x_at	1.962	Tm7sf1	1418397_at	1.848	Zfp275	1447784_x_at	0.59	9130023D20Rik
1439270_x_at	1.962	Ran	1419374_at	1.845	Wbp4	1426244_at	0.587	Mapre2
1424237_at	1.962	6230400O18Rik	1421043_s_at	1.845	Arhgef2	1455486_at	0.586	Pias1
1425508_s_at	1.959	Arfrp1	1427876_at	1.845	2610312B22Rik	1448892_at	0.581	Dock7
1426351_at	1.959	Hspd1	1437317_at	1.844	Ube1l	1455266_at	0.581	Kif5c

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1449841_at	0.58	Kif3a	1456039_at	0.519	Snx14	1448434_at	0.481	Rnf103
1454606_at	0.579	4933426M11Rik	1438673_at	0.518	Slc4a7	1439502_at	0.481	4930573I19Rik
1440559_at	0.577	Hmga2-ps1	1440880_at	0.518	Mppe1	1428194_at	0.48	Usp9x
1428484_at	0.577	Osbpl3	1438396_at	0.518	9530014D17Rik	1420384_at	0.48	Col4a3bp
1434205_at	0.575	Ppp2r5c	1424887_at	0.518	BC012312	1433743_at	0.479	Dach1
1451123_at	0.574	C330016O10Rik	1433747_at	0.516	2010309L07Rik	1443896_at	0.479	Tbc1d5
1436714_at	0.574	Lpp	1420621_a_at	0.516	App	1448111_at	0.479	Ctps2
1429108_at	0.574	Msl2	1423083_at	0.515	Rab33b	1417315_at	0.478	DXImx47e
1428681_at	0.571	5530400K22Rik	1437117_at	0.515	Centb1	1447150_at	0.478	Phr1
1429642_at	0.57	Anubl1	1423759_a_at	0.515	1190006A08Rik	1450955_s_at	0.477	Sort1
1428710_at	0.57	Rit1	1436316_at	0.514	9430029L20Rik	1434767_at	0.477	C79407
1437287_at	0.57	1110020G09Rik	1452446_a_at	0.514	2010008E23Rik	1429116_at	0.477	Slc17a5
1449668_s_at	0.57	A730024A03Rik	1418894_s_at	0.514	Pbx2	1420649_at	0.476	Atbf1
1448812_at	0.57	2410018L13Rik	1460363_at	0.514	9930033H14Rik	1446444_at	0.475	D930043C02Rik
1434855_at	0.569	5730457F11Rik	1446636_at	0.514	F830010I22Rik	1419971_s_at	0.475	Slc35a5
1455031_at	0.569	2700084L06Rik	1429429_s_at	0.513	A030012M09Rik	1448534_at	0.474	Ptpns1
1439094_at	0.568	Cltc	1428147_at	0.513	0610011B16Rik	1424350_s_at	0.474	BC013667
1435616_at	0.567	Cyp20a1	1435397_at	0.512	BC038156	1460672_at	0.474	2410002F23Rik
1423126_at	0.566	Atp1b3	1419931_at	0.512	Abcb7	1430774_at	0.474	A430106A12Rik
1435637_at	0.565	D8Wsu49e	1425769_x_at	0.511	1700001C14Rik	1452737_at	0.474	2810008M24Rik
1435754_at	0.564	D6Bwg1452e	1429094_at	0.511	1700027M01Rik	1434373_at	0.473	B930006L02Rik
1457797_at	0.564	AI605517	1425525_a_at	0.511	P2rx4	1416324_s_at	0.473	2410004N11Rik
1441937_s_at	0.564	Pink1	1455737_at	0.51	C030002B11Rik	1417405_at	0.473	Stard3
1433568_at	0.563	Papd4	1457793_a_at	0.509	Whsc1l1	1424275_s_at	0.473	Trim41
1418838_at	0.563	Abcd1	1439314_at	0.508	Clock	1450686_at	0.473	Pon2
1428829_at	0.562	6820401H01Rik	1426022_a_at	0.508	Villp	1438731_at	0.473	Sgsh
1428848_a_at	0.56	Macf1	1436614_at	0.508	AI843639	1455065_x_at	0.472	Gnpda1
1422064_a_at	0.56	Zfp288	1434860_at	0.508	Narg3	1435315_s_at	0.472	2900034E22Rik
1460224_at	0.559	Snx2	1433495_at	0.507	2810024B22Rik	1441986_at	0.472	Zcchc6
1433757_a_at	0.557	Nisch	1427991_s_at	0.507	4930550B20Rik	1417930_at	0.471	Nab2
1431189_a_at	0.556	B430104H02Rik	1449087_at	0.507	Rnf141	1440878_at	0.471	Runx1
1416252_at	0.556	Stk38	1448864_at	0.505	Snrk	1457628_at	0.471	2900091E11Rik
1446433_at	0.554	Acbd5	1452167_at	0.505	2810407C02Rik	1434548_at	0.47	Tde1
1419883_s_at	0.554	Atp6v1b2	1418096_at	0.505	AI450713	1434520_at	0.469	Sc5d
1459344_at	0.553	9630019E01Rik	1426576_at	0.504	9530058O11Rik	1453061_at	0.469	2610018O07Rik
1423574_s_at	0.55	Srd5a2l	1435869_s_at	0.504	Ap2a2	1423457_at	0.469	Slc35a5
1434166_at	0.55	9330151L19Rik	1416795_at	0.504	Cryl1	1449714_at	0.469	5730472N09Rik
1450410_a_at	0.549	4930570C03Rik	1436460_at	0.504	BC030440	1426293_at	0.469	6330581L23Rik
1426840_at	0.549	9130022A11Rik	1434828_at	0.503	1600010D10Rik	1460592_at	0.468	Epb4.1l1
1439511_at	0.546	5830436D01Rik	1455597_at	0.503	AI585793	1437244_at	0.467	8430435B07Rik
1440214_at	0.546	A630001G21Rik	1436215_at	0.503	Impk	1439323_a_at	0.467	Map4k1
1436301_at	0.544	A930019K20Rik	1423702_at	0.503	H1f0	1435173_at	0.465	Ate1
1416735_at	0.543	Asah1	1416935_at	0.502	Trpv2	1436640_x_at	0.465	Agpat4
1447708_x_at	0.542	Pde2a	1449275_at	0.501	2310038H17Rik	1422414_a_at	0.465	Calm2
1420374_at	0.542	Foxj2	1421873_s_at	0.501	Rab24	1460733_at	0.465	AA407659
1428580_at	0.541	Blvra	1426589_at	0.501	Gab3	1435143_at	0.465	Elk3
1433614_at	0.54	Snx27	1455729_at	0.501	Gnaq	1436883_at	0.465	AI662535
1455261_at	0.54	Luc7l	1428667_at	0.501	Maoa	1454816_at	0.463	Rp2h
1420895_at	0.54	Tgfbr1	1454685_at	0.5	BC003323	1434218_at	0.463	C330019G07Rik
1448549_a_at	0.539	Dpagt1	1422896_at	0.5	Vamp4	1454655_at	0.462	Dgkd
1438045_at	0.539	Eea1	1428010_at	0.5	2810011L15Rik	1422637_at	0.462	Rassf5
1422881_s_at	0.539	Sypl	1430077_at	0.5	2610019N13Rik	1436200_at	0.462	A830039N02Rik
1453145_at	0.539	4933439C20Rik	1442576_at	0.499	Creb5	1442824_at	0.462	8030497I03Rik
1430672_at	0.539	5033418A18Rik	1454836_at	0.499	BC024093	1426368_at	0.462	Rin2
1452820_at	0.539	Wdr11	1451074_at	0.498	Rnf13	1455405_at	0.461	Pstpip2
1418434_at	0.539	Mkrn1	1426940_at	0.498	BC023957	1451190_a_at	0.461	Sbk
1448225_at	0.538	Gpaa1	1436088_at	0.497	0910001A06Rik	1418847_at	0.461	Arg2
1445827_at	0.535	3632413B07Rik	1436705_at	0.497	BC032271	1422880_at	0.461	Sypl
1419221_a_at	0.535	Rgs14	1424129_at	0.496	1200003O06Rik	1417878_at	0.459	E2f1
1424022_at	0.535	1700012B18Rik	1452866_at	0.496	Nars	1442182_at	0.459	1810055D05Rik
1455196_s_at	0.534	AA987161	1439181_at	0.494	BC043301	1418809_at	0.459	Pira1
1455534_s_at	0.534	Osbpl11	1454982_at	0.493	Arfgef2	1454182_at	0.459	5430417C01Rik
1456006_at	0.533	Bcl2l11	1460203_at	0.492	Itpr1	1451980_at	0.459	Cast1
1452664_a_at	0.532	Tm7sf3	1440267_at	0.492	E330005K07Rik	1434241_at	0.458	D330013L20Rik
1427097_at	0.532	8030445B08Rik	1451558_at	0.492	Fbxw7	1436841_at	0.458	B230380D07Rik
1422653_at	0.531	C030018L16Rik	1416279_at	0.492	Ap1b1	1417978_at	0.457	1300018P11Rik
1424604_s_at	0.531	AI463102	1436890_at	0.492	5730445F03Rik	1452053_a_at	0.457	1600019D15Rik
1421023_at	0.531	Pik3c2a	1428662_a_at	0.491	Hod	1460656_a_at	0.456	5630401J11Rik
1424438_a_at	0.53	Obrgrp	1448210_at	0.49	Rab1	1447537_at	0.456	1500032P08Rik
1421014_a_at	0.53	Clybl	1454741_s_at	0.49	AW547186	1449353_at	0.455	Wig1
1436756_x_at	0.53	Hadhsc	1427990_at	0.49	4930550B20Rik	1444009_at	0.455	Rassf4
1451154_a_at	0.529	Cugbp2	1452341_at	0.49	Echs1	1437200_at	0.455	5832424M12
1435157_at	0.529	5830454D03Rik	1448853_at	0.489	Synj2bp	1419228_at	0.455	Elac1
1424637_s_at	0.528	2610204L23Rik	1448020_at	0.489	Rap1a	1434714_at	0.455	1300013B24Rik
1424893_at	0.528	Ndel1	1459868_x_at	0.489	Il11ra1	1421977_at	0.454	Mmp19
1431158_at	0.528	8430406H22Rik	1439406_x_at	0.488	Fars1	1424743_at	0.454	2610003J06Rik
1417104_at	0.527	Emp3	1435672_at	0.488	3830612M24	1438259_at	0.454	Strn3
1455972_x_at	0.526	Hadhsc	1428412_at	0.488	Smbp	1428280_at	0.454	Fip1l1
1435982_at	0.526	Stx12	1448402_at	0.487	Tln	1460390_at	0.454	Sorl1
1451099_at	0.526	Mbc2	1434839_s_at	0.487	8030499H02Rik	1435666_at	0.454	BC024265
1457582_at	0.525	Uty	1428963_at	0.487	Rwdd2	1442714_at	0.454	D130037M23Rik
1440260_at	0.525	A930007B11Rik	1460024_at	0.487	D230019K20Rik	1424024_at	0.454	Mcfd2
1437121_at	0.525	4432409M07Rik	1455308_at	0.487	Tmem16f	1455206_at	0.453	C130006E23
1424124_at	0.524	Mospd2	1452922_at	0.487	2610024M03Rik	1435353_a_at	0.453	4933439C20Rik
1440847_at	0.523	Mtss1	1417857_at	0.486	Mmaa	1423963_at	0.452	Wdr26
1457709_a_at	0.523	A930005H10Rik	1421252_a_at	0.486	Mef2a	1454609_x_at	0.452	6430527G18Rik
1417044_at	0.522	Lcmt1	1428769_at	0.485	1500010M24Rik	1448294_at	0.451	Litaf
1452733_at	0.522	2810473M14Rik	1455333_at	0.485	BC023928	1459560_at	0.451	4921517N04Rik
1430546_at	0.521	Cryzl1	1460631_at	0.485	Ogt	1430700_a_at	0.45	Pla2g7
1460596_at	0.521	Agtrap	1437119_at	0.485	Ern1	1433854_at	0.449	AW547186
1420611_at	0.521	Prkacb	1416440_at	0.483	Cd164	1418774_a_at	0.448	Atp7a
1434399_at	0.52	Galnt6	1424707_at	0.483	1110014C03Rik	1436027_at	0.448	Osbpl11
1453864_at	0.52	Rdh14	1447813_x_at	0.482	Sla	1436387_at	0.448	C330006P03Rik
1422814_at	0.519	Calmbp1	1416904_at	0.482	Mbnl1	1423760_at	0.447	Cd44
			1433618_at	0.482	C330006A16Rik	1450701_a_at	0.447	Gtf2h2
			1430195_at	0.481	2810043O03Rik	1425678_a_at	0.447	Snrk

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1434515_at	0.447	Ncoa1	1427368_x_at	0.416	Fes	1455876_at	0.388	Slc4a7
1424654_at	0.447	Acp2	1452410_a_at	0.415	Fes	1447757_x_at	0.388	Inpp5f
1426584_a_at	0.447	Sdh1	1449455_at	0.415	Hck	1448106_at	0.388	1200016B17Rik
1437642_at	0.447	Hrbl	1434402_at	0.415	1110053F04Rik	1429088_at	0.387	Lbh
1451350_a_at	0.446	Obrgrp	1419181_at	0.414	Zfp326	1422106_a_at	0.387	Grcc9
1437357_at	0.446	BC037178	1452347_at	0.414	Mef2a	1429265_a_at	0.387	Rnf130
1418594_a_at	0.444	Ncoa1	1450095_a_at	0.414	Acyp1	1438328_at	0.387	1700129L13Rik
1451738_at	0.444	Ogt	1416840_at	0.414	3110038L01Rik	1417706_at	0.386	Naglu
1438809_at	0.443	Atp5c1	1456338_at	0.414	9130023D20Rik	1431038_at	0.386	Rassf4
1436786_at	0.442	1110069O07Rik	1452169_a_at	0.413	Dgkz	1434150_a_at	0.386	3300001H21Rik
1448538_a_at	0.442	D4Wsu53e	1423066_at	0.412	Dnmt3a	1428479_at	0.386	Nfatc1
1452994_at	0.442	5230400C17Rik	1425529_s_at	0.412	D19Wsu162e	1418658_at	0.385	2410005O16Rik
1424862_s_at	0.442	2210008I11Rik	1438788_at	0.412	D5Wsu152e	1428517_at	0.385	Wdfy3
1428508_at	0.442	1810061M12Rik	1452288_at	0.412	BB128963	1434017_at	0.383	Znrf2
1425406_at	0.442	Clecsf6	1430030_at	0.411	5330426P16Rik	1453054_at	0.383	Scamp1
1444726_at	0.442	Hrb2	1453795_at	0.411	B430104H02Rik	1416094_at	0.383	Adam9
1450688_at	0.442	Rab2l	1417087_at	0.411	Glg1	1423076_at	0.382	Snx9
1426051_a_at	0.441	Cenpb	1433501_at	0.411	Ctso	1455990_at	0.382	Kif23
1423297_at	0.441	Add3	1417669_at	0.411	6330583M11Rik	1436702_at	0.382	BC034068
1420699_at	0.441	Clecsf12	1426123_a_at	0.41	Rrbp1	1420375_at	0.382	Kif3a
1428725_at	0.441	Miz1	1449622_s_at	0.41	Atp6ap1	1418907_at	0.381	F5
1447947_at	0.44	4930405M20Rik	1426220_at	0.41	4930471M23Rik	1416655_at	0.381	1500002I11Rik
1450660_at	0.439	Pts	1455482_at	0.409	Ap2a2	1417395_at	0.381	Klf4
1427075_s_at	0.439	5330414D10Rik	1416703_at	0.409	Mapk14	1452217_at	0.38	1110004P15Rik
1424378_at	0.439	AA691260	1457266_at	0.409	AW322671	1441145_at	0.38	D030065N23Rik
1435543_at	0.439	Apc	1452271_at	0.409	Xpr1	1452209_at	0.38	Pkp4
1422628_at	0.439	4632417K18Rik	1428822_a_at	0.409	Snx24	1454746_at	0.379	Plekhm1
1438755_at	0.438	C80068	1448188_at	0.408	Ucp2	1460456_at	0.379	2010316F05Rik
1416206_at	0.438	Sipa1	1435008_at	0.408	Slc9a6	1423871_at	0.378	BC014795
1415937_s_at	0.438	Pdcd6ip	1441830_x_at	0.408	Akap10	1428847_a_at	0.378	Macf1
1443773_at	0.437	Zap3	1419296_at	0.408	Arhgap4	1417565_at	0.377	Abhd5
1419193_a_at	0.437	Gmfg	1436499_at	0.407	9530058O11Rik	1451247_at	0.377	1200003O06Rik
1417750_a_at	0.437	Mscp	1421022_x_at	0.407	Acyp1	1428117_x_at	0.377	Tctex1
1417073_a_at	0.436	Qk	1429337_at	0.407	2810431I02Rik	1431218_at	0.376	B230110O18Rik
1444103_at	0.436	2310046K10Rik	1455711_at	0.406	Dtx4	1426052_at	0.376	Mlh3
1420928_at	0.436	St6gal1	1455549_at	0.405	Sestd1	1435485_at	0.376	C230096C10Rik
1452867_at	0.436	Col4a3bp	1438962_s_at	0.405	Ddx31	1436590_at	0.375	Ppp1r3b
1427313_at	0.436	Ptgir	1428680_at	0.405	Cds1	1459433_at	0.375	C130051F05Rik
1419272_at	0.436	Myd88	1429237_at	0.404	6330500A18Rik	1433925_at	0.374	AA409702
1429652_at	0.435	3110038B19Rik	1435536_at	0.404	1700027M01Rik	1439948_at	0.374	BC046401
1429019_s_at	0.435	Pon2	1417648_s_at	0.404	Snx5	1423282_at	0.373	Pitpn
1445729_at	0.435	Sirt3	1422433_s_at	0.404	Idh1	1431143_x_at	0.373	Amid
1428094_at	0.434	Lamp2	1434571_at	0.404	Coh1	1453384_at	0.373	4632404N19Rik
1428723_at	0.433	2310047M10Rik	1458491_at	0.403	4930422I07Rik	1449141_at	0.373	2410043F08Rik
1452767_at	0.433	Rrbp1	1451288_s_at	0.403	1810043G02Rik	1425628_a_at	0.372	Gtf2i
1428236_at	0.433	Acbd5	1427186_a_at	0.403	Mef2a	1428833_at	0.371	4930406D14Rik
1423573_at	0.433	Srd5a2l	1421604_a_at	0.403	Klf3	1424242_at	0.371	Bphl
1447853_x_at	0.433	Kif13a	1423722_at	0.403	4930579A11Rik	1456567_x_at	0.37	Grn
1456510_x_at	0.433	3300001H21Rik	1425311_at	0.402	4930432F04Rik	1417135_at	0.37	Srpk2
1454299_at	0.433	4833422B07Rik	1451006_at	0.401	Xdh	1439272_at	0.369	Mlr1
1421857_at	0.431	Adam17	1460329_at	0.401	B4galt6	1436616_at	0.369	R74740
1416958_at	0.43	Nr1d2	1436858_at	0.401	Mbnl2	1422467_at	0.369	Ppt1
1436697_at	0.43	Dscr3	1424639_a_at	0.401	Hmgcl	1436189_at	0.369	Serpinb6a
1416614_at	0.43	Cri1	1439980_at	0.401	D14Ertd725e	1435274_at	0.368	E430033K04Rik
1440187_at	0.43	Taf3	1433632_at	0.401	Irf2bp2	1418546_a_at	0.368	1700095N21Rik
1428655_at	0.43	1110018J12Rik	1429881_at	0.4	Arhgap15	1418888_a_at	0.368	Sepr
1458347_s_at	0.429	Tmprss2	1448405_a_at	0.4	Cri1	1426833_at	0.367	Eif4g3
1418296_at	0.428	Fxyd5	1433503_at	0.4	1810016I24Rik	1439773_at	0.367	Ly6e
1417676_a_at	0.428	Ptpro	1417605_s_at	0.4	Camk1	1450734_at	0.367	Rgpr
1433505_a_at	0.428	Lrrc5	1455113_at	0.4	1200015K23Rik	1453512_at	0.366	5430437C10Rik
1438443_at	0.428	Zfp288	1441879_x_at	0.399	Mkrn1	1453412_a_at	0.366	Sec14l1
1426376_at	0.428	Dp1	1435836_at	0.398	D530020C15Rik	1439293_at	0.365	C130047D21Rik
1425156_at	0.427	9830147J24Rik	1417848_at	0.398	Gig1	1425956_a_at	0.365	Cdadc1
1455980_a_at	0.427	8430435B07Rik	1451141_at	0.398	BC004636	1434151_at	0.364	3300001H21Rik
1452969_at	0.427	2810442I22Rik	1419235_s_at	0.398	Helb	1433639_at	0.364	5730593F17Rik
1422932_a_at	0.427	Vav1	1455551_at	0.397	Attp	1439830_at	0.364	Map3k5
1437041_at	0.427	5730406M06Rik	1417134_at	0.397	Srpk2	1435326_at	0.363	AW112037
1423835_at	0.426	Zfp503	1416035_at	0.396	Hif1a	1435006_s_at	0.362	Abcb7
1440219_at	0.426	4930413O22Rik	1426258_at	0.396	Sorl1	1417604_at	0.362	Camk1
1434328_at	0.426	Rpl15	1439102_at	0.396	Falz	1438519_at	0.362	4930429H24Rik
1434021_at	0.426	C230096C10Rik	1450112_a_at	0.395	Gas2	1450626_at	0.361	Manba
1422818_at	0.425	Nedd9	1447707_s_at	0.395	Pde2a	1415743_at	0.361	Hdac5
1452277_at	0.425	6330406P08Rik	1456261_at	0.395	Sh3kbp1	1448318_at	0.361	Adfp
1416382_at	0.424	Ctsc	1449852_a_at	0.394	Ehd4	1439263_at	0.361	Fin15
1417538_at	0.423	Slc35a1	1450694_at	0.394	Fkbp2	1450647_at	0.361	Hps3
1442256_at	0.423	Prkcd	1435475_at	0.393	Lman2l	1432543_a_at	0.361	Klf13
1452832_s_at	0.423	Cds2	1456898_at	0.393	Pura	1450868_at	0.361	D8Ertd354e
1435926_at	0.423	E030003F13Rik	1451791_at	0.393	Tfpi	1447284_at	0.36	Trem1
1438971_x_at	0.422	Ube2h	1440195_at	0.393	9330147J08Rik	1420822_s_at	0.36	Sgpp1
1436982_at	0.422	AI848765	1424988_at	0.392	Mylip	1423924_s_at	0.36	D14Ertd226e
1415827_a_at	0.421	D3Ucla1	1424077_at	0.392	2610020H15Rik	1437132_x_at	0.36	Nedd9
1434286_at	0.421	Trps1	1440417_at	0.392	D19Ertd409e	1417647_at	0.36	Snx5
1452527_a_at	0.42	P2rx4	1437797_at	0.392	D5Wsu150e	1428325_at	0.36	2610019P18Rik
1428410_at	0.42	Mak3	1420974_at	0.391	1810034K20Rik	1422645_at	0.359	Hfe
1434517_at	0.42	Wdfy2	1458951_at	0.391	Vrk1	1454858_x_at	0.359	3300001H21Rik
1453416_at	0.419	8430435B07Rik	1438151_x_at	0.391	Zdhhc14	1434236_at	0.359	AI448102
1434561_at	0.419	Asxl1	1425814_a_at	0.391	Calcrl	1434407_at	0.359	9930124L22Rik
1433999_at	0.419	Stk2	1420464_s_at	0.391	Pirb	1436033_at	0.358	BC031353
1433745_at	0.418	Trio	1417884_at	0.39	Slc16a6	1435478_at	0.358	AU044014
1416654_at	0.418	Slc31a2	1454711_at	0.39	Trio	1452202_at	0.357	Pde2a
1434235_at	0.418	Slc20a2	1429336_at	0.39	2610301K12Rik	1415948_at	0.357	Creg
1425185_at	0.418	5830417C01Rik	1430681_at	0.39	Cryl1	1436395_at	0.357	Card6
1415677_at	0.418	Dhrs1	1429943_at	0.389	Ctbs	1430776_s_at	0.357	D10Bur2e
1451326_at	0.418	1810013B01Rik	1421410_a_at	0.389	Pstpip2	1451507_at	0.356	5430401D19Rik
1448313_at	0.418	Cln2	1426574_a_at	0.389	Add3	1459740_s_at	0.356	Ucp2
1436767_at	0.416	Luc7l2	1418996_a_at	0.388	4930469P12Rik	1436842_at	0.356	B230380D07Rik

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1441548_at	0.356	6030440G05Rik	1417291_at	0.327	Tnfrsf1a	1418569_at	0.297	2410043F08Rik
1430561_at	0.355	5730496F10Rik	1437113_s_at	0.327	Pld1	1458501_at	0.297	Vapb
1425516_at	0.355	Ogt	1441558_at	0.327	D230044B12Rik	1417136_s_at	0.296	Srpk2
1434700_at	0.355	6030408C04Rik	1451318_a_at	0.327	Lyn	1437124_at	0.296	A630052C17Rik
1417630_at	0.355	Mknk1	1428859_at	0.326	Paox	1425598_a_at	0.296	Lyn
1453156_s_at	0.355	1810016I24Rik	1454973_at	0.326	2610204M12Rik	1418398_a_at	0.296	Phemx
1449229_a_at	0.354	Cdkl2	1423086_at	0.326	Npc1	1430037_at	0.295	Snx27
1420967_at	0.354	Slc25a15	1455731_at	0.326	Slc29a3	1424048_a_at	0.293	1500005G05Rik
1451254_at	0.354	Ikbkap	1439622_at	0.325	Rassf4	1418553_at	0.292	Arhgef18
1415986_at	0.354	Clcn4-2	1450129_a_at	0.325	Socs6	1428937_at	0.292	2810442I22Rik
1422444_at	0.354	Itga6	1448995_at	0.325	Cxcl4	1434112_at	0.292	Lphn2
1425245_a_at	0.354	Rgs11	1449062_at	0.325	Khk	1421811_at	0.291	Thbs1
1419768_at	0.354	Cd22	1434315_at	0.325	9130020G22Rik	1449183_at	0.291	Comt
1425517_s_at	0.353	Ogt	1429728_at	0.324	4930429M06Rik	1420635_a_at	0.291	Tcirg1
1420824_at	0.353	Sema4d	1433488_x_at	0.324	Gns	1448328_at	0.291	Sh3bp2
1419394_s_at	0.352	S100a8	1454773_at	0.323	Rxra	1417399_at	0.291	Gas6
1416866_at	0.351	Bet1	1429360_at	0.323	Klf3	1429468_at	0.29	1110018F16Rik
1419194_s_at	0.351	Gmfg	1424524_at	0.323	1200002N14Rik	1434089_at	0.29	Synpo
1442098_at	0.35	AU022434	1422859_a_at	0.323	Rpl23	1422821_s_at	0.29	Stard5
1435445_at	0.35	Ccnt2	1423824_at	0.322	5031439A09Rik	1416013_at	0.29	Pld3
1434768_at	0.35	Cln2	1424249_a_at	0.322	Arhgap9	1437279_x_at	0.29	Sdc1
1448079_at	0.35	1110031E24Rik	1434472_at	0.32	5031436O03Rik	1453836_a_at	0.29	Mgll
1451555_at	0.35	Nln	1440778_x_at	0.32	4921504N20Rik	1419315_at	0.289	Slamf9
1425733_a_at	0.349	Eps8	1452016_at	0.32	Alox5ap	1448919_at	0.289	1110055L24Rik
1440885_at	0.349	Evl	1451867_x_at	0.32	Arhgap6	1430388_a_at	0.289	Sulf2
1434386_at	0.348	Atp2c1	1428749_at	0.319	6430411K14Rik	1438214_at	0.288	Trps1
1449110_at	0.348	Rhob	1416471_at	0.318	Cept1	1428122_s_at	0.288	2610528K11Rik
1455109_at	0.348	8030499H02Rik	1427691_a_at	0.318	Ifnar2	1437551_at	0.287	4930504E06Rik
1453012_at	0.348	5530402M19Rik	1423063_at	0.318	Dnmt3a	1450702_at	0.286	Hfe
1416046_a_at	0.347	0610025O11Rik	1423425_at	0.317	1300012G16Rik	1428780_at	0.286	1300017K07Rik
1425496_at	0.347	Abca3	1460688_s_at	0.315	AA407659	1418540_a_at	0.285	Ptpre
1448124_at	0.347	Gus	1417811_at	0.315	Slc24a6	1428622_at	0.285	Depdc6
1426952_at	0.347	Arhgap18	1452299_at	0.315	8030445B08Rik	1423597_at	0.284	Atp8a1
1448200_at	0.347	Tcn2	1453731_a_at	0.314	2610318G18Rik	1455112_at	0.283	Amid
1438052_at	0.345	A130071D04Rik	1415901_at	0.314	Plod3	1438234_at	0.283	Wdr26
1460283_at	0.345	Mefv	1421899_a_at	0.314	Mr1	1448161_a_at	0.283	Clcn4-2
1440268_at	0.345	Trim41	1459741_x_at	0.314	Ucp2	1460674_at	0.282	Mpra
1429321_at	0.344	Rnf149	1429093_at	0.314	1700027M01Rik	1448620_at	0.282	Fcgr3
1419494_a_at	0.344	Tpd52	1433542_at	0.313	Inpp5f	1445647_at	0.282	AI447881
1445438_at	0.344	4921528E07Rik	1435786_at	0.313	Klhl12	1454728_s_at	0.281	Atp8a1
1428612_at	0.344	Apg7l	1453244_at	0.313	5830416P10Rik	1438814_at	0.281	1700056O17Rik
1419493_a_at	0.344	Tpd52	1417779_at	0.313	2310079N02Rik	1420671_x_at	0.281	Ms4a4b
1426794_at	0.344	Ptprs	1433657_at	0.313	A130092J06Rik	1434754_at	0.281	Garnl4
1439571_at	0.344	E230008J23Rik	1436906_at	0.313	1110031E24Rik	1433968_a_at	0.28	Egfl5
1436097_x_at	0.344	Arhgap9	1426623_a_at	0.312	Arhgap17	1416050_a_at	0.28	Scarb1
1416381_a_at	0.343	Prdx5	1433827_at	0.312	Atp8a1	1455538_at	0.28	6330403M23Rik
1447880_x_at	0.342	Edg5	1427098_at	0.312	8030445B08Rik	1422468_at	0.28	Ppt1
1433964_s_at	0.342	BC032204	1417513_at	0.311	Evi5	1438021_at	0.28	BC013481
1437055_x_at	0.342	1200003O06Rik	1416165_at	0.311	1700093E07Rik	1435746_at	0.279	Srpk2
1435313_at	0.342	F630107N04Rik	1449269_at	0.311	F5	1427002_s_at	0.279	6330406P08Rik
1457445_at	0.342	Trps1	1444670_at	0.311	A130004G11Rik	1460036_at	0.278	Ap1s2
1434406_at	0.342	Fnbp2	1424259_at	0.311	2400010G15Rik	1450476_at	0.278	Cnr2
1435171_at	0.341	2810416G20Rik	1419481_at	0.311	Sell	1433905_at	0.278	Akap7
1440586_at	0.341	B430203I24Rik	1433751_at	0.31	Slc39a10	1417490_at	0.278	Ctsb
1431067_at	0.341	6330404A07Rik	1418497_at	0.309	Fgf13	1441516_a_at	0.277	C130050O18Rik
1422806_x_at	0.34	Ing3	1438156_x_at	0.309	Cpt1a	1449024_a_at	0.276	Hexa
1424655_at	0.34	Acp2	1425294_at	0.308	Slamf8	1424163_at	0.276	0610039K22Rik
1456153_at	0.34	BC030940	1436323_at	0.308	2810001A02Rik	1460409_at	0.276	Cpt1a
1431960_at	0.34	Wwox	1422438_at	0.308	Ephx1	1429083_at	0.275	Agl
1442590_at	0.339	Tnfrsf22	1453289_at	0.308	Eif2c4	1433159_at	0.275	5330429L19Rik
1455033_at	0.339	B430201A12Rik	1453851_a_at	0.308	Gadd45g	1434930_at	0.274	Tpcn1
1433930_at	0.339	Hpse	1428657_at	0.307	1110037N09Rik	1455487_at	0.274	2600014M03Rik
1417777_at	0.339	Ltb4dh	1434261_at	0.307	Sipa1l2	1460359_at	0.274	Armcx3
1450332_s_at	0.337	Fmo5	1428064_at	0.306	Centd2	1427318_s_at	0.274	Fer1l3
1424560_at	0.337	Pstpip1	1453367_a_at	0.306	6330583M11Rik	1434303_at	0.273	9430025M21Rik
1451674_at	0.336	Slc12a5	1430808_at	0.306	Tbc1d5	1448456_at	0.273	Cln8
1436478_at	0.336	3010002C02Rik	1428758_at	0.305	1810054O13Rik	1435357_at	0.273	D4Wsu53e
1434408_at	0.335	AI463012	1456479_at	0.305	4732481H14Rik	1437874_s_at	0.273	Hexb
1424336_at	0.335	8430432M10Rik	1433559_at	0.304	9330175B01Rik	1435972_at	0.273	Cast
1435194_at	0.335	Hspa4	1441850_x_at	0.304	Tcn2	1449619_s_at	0.272	Arhgap9
1457539_at	0.335	D10Ertd709e	1453386_at	0.304	2200001D17Rik	1440384_at	0.272	3632431M01Rik
1455286_at	0.335	Btbd1	1460180_at	0.304	Hexb	1421525_a_at	0.271	Birc1e
1454843_at	0.334	Prps2	1420919_at	0.304	Sgk3	1417551_at	0.271	Cln3
1428025_s_at	0.334	1110020B03Rik	1451621_at	0.304	5830417C01Rik	1460006_at	0.271	Atbf1
1436766_at	0.334	Luc7l2	1427351_s_at	0.303	Igh-6	1457063_at	0.27	Phospho1
1416333_at	0.334	Dok2	1423298_at	0.303	Add3	1435680_a_at	0.27	Dpp7
1419455_at	0.333	Il10rb	1435066_at	0.303	1110020B03Rik	1428615_at	0.27	P2y5
1417741_at	0.333	Pygl	1438321_x_at	0.302	4930504E06Rik	1455712_at	0.27	Hist3h2a
1416551_at	0.333	Atp2a2	1430000_at	0.302	B230117O15Rik	1458142_at	0.27	Zdhhc9
1434378_a_at	0.332	2810410A03Rik	1435560_at	0.301	Itgal	1433963_a_at	0.269	BC032204
1424420_at	0.332	D9Ertd392e	1420641_a_at	0.301	Sqrdl	1455291_s_at	0.267	D6Ertd365e
1435251_at	0.332	Snx13	1442855_at	0.301	B130005I07Rik	1451486_at	0.267	1200006F02Rik
1434461_at	0.332	2610041B18Rik	1448303_at	0.3	Gpnmb	1422822_at	0.267	Stard5
1427434_at	0.331	Birc1f	1454837_at	0.3	Cln6	1426505_at	0.266	Evi2b
1418162_at	0.33	Tlr4	1418539_a_at	0.3	Ptpre	1433500_at	0.266	B930096L08Rik
1456533_at	0.33	1100001I19Rik	1428651_at	0.299	1110046J11Rik	1417663_a_at	0.266	Ndrg3
1428917_at	0.329	9030425C21Rik	1429503_at	0.299	2900024C23Rik	1431355_s_at	0.265	Trpm7
1449221_a_at	0.329	Rrbp1	1418505_at	0.299	Nudt4	1415977_at	0.265	Isyna1
1436678_at	0.329	Sgcb	1429771_at	0.299	3110073H01Rik	1425362_at	0.265	Hrbl
1440831_at	0.329	6230421P05Rik	1439168_at	0.298	Camk2d	1454086_a_at	0.263	Lmo2
1436844_at	0.329	AW046287	1434487_at	0.298	2210408E11Rik	1433242_at	0.263	5830415B17Rik
1417481_at	0.329	Ramp1	1418163_at	0.297	Tlr4	1415936_at	0.262	Bcar3
1434414_at	0.329	D15Bwg0759e	1434014_at	0.297	Apg4c	1430125_s_at	0.262	2310009N05Rik
1431316_at	0.328	Itch	1455321_at	0.297	4921528E07Rik	1433546_at	0.261	Gns
1432006_at	0.328	Ap2a2	1436008_at	0.297	Tpd52	1450939_at	0.261	Entpd1
1419537_at	0.328	Tcfec	1435741_at	0.297	B230331L10Rik	1438614_x_at	0.26	Osbpl9

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1434674_at	0.26	Lyst	1423233_at	0.229	Cebpd	1433740_at	0.198	2610301K12Rik
1429359_s_at	0.26	Rbpms	1419219_at	0.229	Cyp4f18	1451161_a_at	0.198	Emr1
1452117_a_at	0.259	Fyb	1454286_at	0.229	1110004M10Rik	1429959_at	0.197	6620401D04Rik
1448143_at	0.259	Aldh2	1426389_at	0.229	Camk1d	1437072_at	0.197	Arhgap25
1419810_x_at	0.258	Arhgap9	1455659_at	0.229	3632431M01Rik	1424250_a_at	0.195	Arhgef3
1444254_at	0.258	9930017A07Rik	1420965_a_at	0.228	Enc1	1446861_at	0.195	Gns
1434866_x_at	0.258	Cpt1a	1420747_at	0.228	Ppnr	1436026_at	0.195	1110032O19Rik
1415850_at	0.258	Rasa3	1435704_at	0.228	C920006O11Rik	1459897_a_at	0.195	Sbsn
1435959_at	0.257	Arhgap15	1455073_at	0.227	Cdadc1	1417394_at	0.194	Klf4
1429831_at	0.257	Pik3ap1	1425298_a_at	0.227	Birc1a	1418049_at	0.193	Ltbp3
1427243_at	0.256	AA536743	1455612_at	0.226	AI848218	1426785_s_at	0.193	Mgll
1452841_at	0.256	Pgm2l1	1451731_at	0.226	Abca3	1451716_at	0.193	Mafb
1425530_a_at	0.256	Stx3	1416875_at	0.225	Parvg	1417696_at	0.192	Soat1
1454984_at	0.256	AW061234	1436212_at	0.224	AI661017	1459744_at	0.192	Cd53
1449124_at	0.256	Rgl1	1449027_at	0.224	Rhou	1422573_at	0.192	Ampd3
1455594_at	0.255	Sec6l1	1423363_at	0.224	Sort1	1435584_at	0.192	AI662791
1417704_a_at	0.254	Arhgap6	1417512_at	0.223	Evi5	1435748_at	0.192	Gda
1434311_at	0.254	4932442K20Rik	1453109_at	0.223	2810429K17Rik	1435749_at	0.192	Gda
1444735_at	0.254	Znrf2	1427912_at	0.223	Cbr3	1426603_at	0.191	Rnasel
1422259_a_at	0.254	Ccr5	1417070_at	0.223	Cyp4v3	1424433_at	0.191	Mrsb
1448686_at	0.253	Il16	1417932_at	0.222	Il18	1426775_s_at	0.191	Scamp1
1419234_at	0.252	Helb	1434302_at	0.221	9430025M21Rik	1442798_x_at	0.19	Unc5a
1454666_at	0.252	Klf3	1436322_a_at	0.221	2810001A02Rik	1433655_at	0.19	Rnf141
1417813_at	0.251	Ikbke	1426992_at	0.221	Xpr1	1455796_x_at	0.19	Olfm1
1423768_at	0.251	Unc93b	1455221_at	0.22	Abcg1	1433986_at	0.187	BC024659
1450678_at	0.251	Itgb2	1423586_at	0.219	Axl	1441727_s_at	0.187	Zfp467
1415904_at	0.251	Lpl	1430612_at	0.219	1810033B17Rik	1425037_at	0.186	Fgd4
1453003_at	0.251	Sorl1	1420703_at	0.219	Csf2ra	1419480_at	0.186	Sell
1441229_at	0.251	D230019N24Rik	1455331_at	0.219	A230102O09Rik	1433711_s_at	0.185	Sesn1
1424920_at	0.25	Slc37a3	1450291_s_at	0.219	Ms4a4c	1438312_s_at	0.185	Ltbp3
1448409_at	0.25	Lrmp	1433543_at	0.219	Anln	1432946_at	0.184	5230400M06Rik
1456500_at	0.25	4632417K02	1435477_s_at	0.218	Fcgr2b	1435903_at	0.184	B230315M08Rik
1436272_at	0.25	1110059F07Rik	1437776_at	0.218	3632431M01Rik	1434499_a_at	0.184	Ldh2
1437833_at	0.25	Ltbp3	1448118_a_at	0.218	Ctsd	1426917_s_at	0.184	4833415E20Rik
1419097_a_at	0.249	Epb7.2	1433965_at	0.218	AI853962	1438910_a_at	0.184	Epb7.2
1460063_at	0.249	D5Ertd798e	1418123_at	0.216	Unc119	1452592_at	0.183	Mgst2
1444119_at	0.249	B930006L02Rik	1450061_at	0.216	Enc1	1443894_at	0.183	Evi2a
1455332_x_at	0.248	Fcgr2b	1452948_at	0.216	1810019A08Rik	1417471_s_at	0.183	D1Ertd622e
1450070_s_at	0.248	Pak1	1423465_at	0.215	Sdfr2	1432007_s_at	0.182	Ap2a2
1428936_at	0.248	2810442I22Rik	1434945_at	0.215	A330042H22	1449383_at	0.182	Adss
1455820_x_at	0.248	Scarb1	1437885_at	0.213	D030029J20Rik	1438931_s_at	0.182	Sesn1
1433750_at	0.247	Slc31a1	1426743_at	0.213	Dip3b	1422662_at	0.182	Lgals8
1422411_s_at	0.246	Ear2	1451563_at	0.213	Emr4	1456888_at	0.182	C230090D14
1430264_at	0.246	2610030P05Rik	1422782_s_at	0.213	Tlr3	1455095_at	0.181	Hist2h2bb
1459900_at	0.246	C79468	1434034_at	0.213	Cerk	1448647_at	0.181	Man2a1
1450391_a_at	0.246	Mgll	1428431_at	0.212	2310047A01Rik	1436618_at	0.181	Sfxn5
1449878_a_at	0.245	Slc12a6	1454646_at	0.212	E430026E19Rik	1430367_at	0.181	8230401J17Rik
1437378_x_at	0.245	Scarb1	1439153_at	0.212	Ibrdc2	1417160_s_at	0.181	Expi
1429324_at	0.245	1700012A16Rik	1456328_at	0.212	A530094C12Rik	1417071_s_at	0.181	Cyp4v3
1454670_at	0.244	Rere	1435937_at	0.211	Sptlc2	1457163_at	0.18	D730035F11Rik
1452359_at	0.244	AA536743	1436781_at	0.211	Man2b1	1449049_at	0.18	Tlr1
1427076_at	0.243	Mpeg1	1416441_at	0.211	Pgcp	1426397_at	0.18	Tgfbr2
1431296_at	0.243	4933439K08Rik	1445545_at	0.211	2610318G18Rik	1455251_at	0.18	E130012M19Rik
1447602_x_at	0.242	Sulf2	1429742_at	0.211	Chc1l	1453007_at	0.18	3110082I17Rik
1456307_s_at	0.242	Adcy7	1440370_at	0.21	Abca13	1424714_at	0.179	Aldo3
1417590_at	0.242	Cyp27a1	1419627_s_at	0.21	Clecsf10	1426440_at	0.179	Dhrs7
1428577_at	0.242	Ppfia4	1440684_at	0.21	A330042H22	1434557_at	0.178	Hip1
1416700_at	0.242	Rhoe	1427050_at	0.21	5730420B22Rik	1448390_a_at	0.178	Dhrs3
1427041_at	0.241	BC013712	1425736_at	0.209	Cd37	1429673_at	0.178	5830407E08Rik
1435822_at	0.241	D830012I24Rik	1435144_at	0.209	Fyb	1451413_at	0.178	Cast
1457528_at	0.241	Slc4a7	1451782_a_at	0.208	Slc29a1	1449846_at	0.177	Ear2
1455204_at	0.241	1110020B03Rik	1455795_at	0.208	Sart2	1428391_at	0.177	Rab3il1
1459735_at	0.241	C430014M02Rik	1450941_at	0.208	Sdcbp	1429058_at	0.177	1110004B13Rik
1457806_at	0.24	Dock1	1434575_at	0.208	Epb4.1l1	1415855_at	0.176	Kitl
1420821_at	0.24	Sgpp1	1438975_x_at	0.208	Zdhhc14	1453915_a_at	0.176	Slc37a3
1460016_at	0.239	AW547186	1448316_at	0.207	Cklfsf3	1419132_at	0.175	Tlr2
1444097_at	0.239	BC019776	1415943_at	0.207	Sdc1	1460436_at	0.175	Ndst1
1415944_at	0.239	Sdc1	1450919_at	0.207	Mpp1	1460324_at	0.175	Dnmt3a
1455285_at	0.238	Slc31a1	1455590_at	0.207	Serpinb6a	1417697_at	0.174	Soat1
1452059_at	0.238	Slc35f5	1430097_at	0.207	8430436C05Rik	1434846_at	0.174	1700065A05Rik
1440383_at	0.238	Dclre1b	1427929_a_at	0.206	2310036D04Rik	1450065_at	0.174	Adcy7
1416607_at	0.237	4931406C07Rik	1448104_at	0.206	Aldh6a1	1418394_a_at	0.174	Cd97
1451364_at	0.237	2010003I19Rik	1448944_at	0.205	Nrp	1449153_at	0.173	Mmp12
1439616_at	0.237	AV026040	1420804_s_at	0.205	Clecsf8	1455214_at	0.173	Mitf
1425784_a_at	0.236	Olfm1	1418741_at	0.205	Itgb7	1435490_at	0.173	Hk3
1417693_a_at	0.236	Gab1	1450106_a_at	0.205	Evl	1421547_at	0.172	Ly78
1448167_at	0.236	Ifngr	1430257_at	0.205	0610008L17Rik	1456918_at	0.172	9430025M21Rik
1438702_at	0.235	Flrt2	1433885_at	0.204	A630053O10	1424998_at	0.172	Emr4
1417995_at	0.235	Ptpn8	1449065_at	0.204	Cte1	1421694_a_at	0.171	Cspg2
1444456_at	0.235	9030425P06Rik	1416985_at	0.204	Ptpns1	1436778_at	0.171	Cybb
1433864_at	0.235	C820005L12Rik	1418998_at	0.204	Kmo	1444090_at	0.171	4632424B03Rik
1440169_x_at	0.234	Ifnar2	1416340_a_at	0.204	Man2b1	1451263_a_at	0.17	Fabp4
1419985_s_at	0.234	D11Ertd461e	1423478_at	0.203	Prkcb	1419764_at	0.17	Chi3l3
1460419_a_at	0.234	Prkcb	1418701_at	0.203	Comt	1430447_a_at	0.17	Lair1
1434967_at	0.233	Zswim6	1418826_at	0.202	Ms4a6b	1418196_at	0.169	Tep1
1420382_at	0.233	Apob48r	1417000_at	0.201	Abtb1	1458869_at	0.169	2900076A13Rik
1455290_at	0.232	D6Ertd365e	1457648_x_at	0.201	BC004044	1428790_at	0.169	4921501M20Rik
1441779_at	0.232	9530006C21Rik	1430148_at	0.201	Rab19	1451750_at	0.167	Irak4
1434283_at	0.231	Arid5b	1448721_at	0.2	D1Ertd622e	1419247_at	0.167	Rgs2
1441195_at	0.231	6030487A22Rik	1434547_at	0.2	Cpd	1418806_at	0.167	Csf3r
1451755_a_at	0.231	Apobec1	1447521_x_at	0.199	D15Wsu169e	1424782_at	0.167	2610318G18Rik
1420973_at	0.231	Arid5b	1452764_at	0.199	Socs6	1438619_x_at	0.167	Zdhhc14
1419811_at	0.23	D16Wsu65e	1423668_at	0.199	Zdhhc14	1451310_a_at	0.166	Ctsl
1417203_at	0.23	Ethe1	1436056_at	0.199	C130021D12Rik	1437614_x_at	0.166	Zdhhc14
1431142_s_at	0.23	Amid	1450769_s_at	0.199	Stard5	1424965_at	0.165	Lpxn
1451361_a_at	0.229	BC027342	1436164_at	0.199	C130040I11Rik	1431374_at	0.165	6330407A03Rik

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1417695_a_at	0.165	Soat1	1456480_at	0.125	9330186A19Rik	1434848_at	0.0877	Gpr27
1415897_a_at	0.164	Mgst1	1424186_at	0.125	2610001E17Rik	1427481_a_at	0.0874	Atp1a3
1419609_at	0.164	Ccr1	1420725_at	0.125	Tmlhe	1440840_at	0.0872	D630004K10Rik
1435375_at	0.164	BC052328	1447903_x_at	0.124	Ap1s2	1456195_x_at	0.0866	Itgb5
1435745_at	0.163	5031439G07Rik	1421352_at	0.124	Tlr6	1424304_at	0.0858	Tpcn2
1416289_at	0.163	Plod1	1440285_at	0.123	Ppp1r9a	1439067_at	0.0858	Lair1
1456437_x_at	0.163	C1r	1447854_s_at	0.122	Hist2h2bb	1422978_at	0.0853	Cybb
1430252_at	0.161	3110027N22Rik	1424111_at	0.122	Igf2r	1416619_at	0.0851	4632428N05Rik
1459894_at	0.161	A630053O10	1434129_s_at	0.121	Lhfpl2	1422542_at	0.0843	Gpr34
1418018_at	0.16	Cpd	1420127_s_at	0.121	D9Ertd392e	1430172_a_at	0.0841	Cyp4f16
1442233_at	0.16	Fyb	1454067_a_at	0.12	4931406C07Rik	1432198_at	0.0841	6330414G02Rik
1451832_at	0.16	1700001C14Rik	1448128_at	0.119	Ppgb	1459992_x_at	0.0835	Cln8
1425888_at	0.159	Klra17	1422191_at	0.119	Mox2r	1427747_a_at	0.0832	Lcn2
1434298_at	0.159	Zfhx1b	1449193_at	0.117	Cd5l	1417694_at	0.0827	Gab1
1443088_at	0.159	9930031P18Rik	1444073_at	0.117	Maf	1435492_at	0.0824	1500012M23Rik
1436996_x_at	0.159	Lyzs	1421923_at	0.117	Sh3bp5	1456046_at	0.0822	C1qr1
1417108_at	0.159	1200014P03Rik	1434881_s_at	0.116	Kctd12	1434850_at	0.0822	D030034H08
1423394_at	0.158	Pcyox1	1420726_x_at	0.115	Tmlhe	1425546_a_at	0.0813	Trf
1428176_at	0.158	Edg5	1453586_at	0.115	Entpd1	1416986_a_at	0.0806	Ptpns1
1445711_at	0.158	BB163080	1435134_at	0.115	B230106I24Rik	1419589_at	0.0805	C1qr1
1457017_at	0.158	2410005O16Rik	1448595_a_at	0.115	Rex3	1433662_s_at	0.0803	Timp2
1448881_at	0.158	Hp	1440879_at	0.114	Abca9	1450731_s_at	0.08	Tnfrsf21
1460302_at	0.158	Thbs1	1434314_s_at	0.113	D6Ertd32e	1422041_at	0.0799	Pilrb
1448148_at	0.157	Grn	1452657_at	0.113	Ap1s2	1434111_at	0.0799	Lphn2
1460694_s_at	0.156	Svil	1450377_at	0.112	Thbs1	1419610_at	0.079	Ccr1
1416978_at	0.156	Fcgrt	1436368_at	0.112	Slc16a10	1418634_at	0.0788	Notch1
1455123_at	0.155	St18	1448732_at	0.111	Ctsb	1434277_a_at	0.0787	6430570G24
1429779_at	0.154	Eif2c4	1417534_at	0.111	Itgb5	1418993_s_at	0.0787	F10
1450822_at	0.154	Lyzs	1438034_at	0.111	6430576D04Rik	1436324_at	0.0776	4831403C07Rik
1450726_at	0.154	Asah2	1436225_at	0.111	Trpm2	1415856_at	0.0774	Emb
1416188_at	0.154	Gm2a	1444040_at	0.11	Lair1	1436501_at	0.0769	Mtus1
1457753_at	0.154	Tlr13	1425714_a_at	0.11	4921501M20Rik	1424076_at	0.0766	2610020H15Rik
1458941_at	0.154	D130016B08Rik	1450876_at	0.109	Cfh	1431056_a_at	0.076	Lpl
1430534_at	0.153	Rnase6	1429524_at	0.109	Myo1f	1423326_at	0.0752	Entpd1
1445539_at	0.153	Pde7b	1435203_at	0.109	1700052O22Rik	1419519_at	0.075	Igf1
1452210_at	0.153	E130315B21Rik	1416389_a_at	0.109	Chc1l	1446354_at	0.0747	C130098B18Rik
1417133_at	0.153	Pmp22	1420498_a_at	0.109	Dab2	1456150_at	0.0747	A630082K20Rik
1425951_a_at	0.153	Clecsf10	1419482_at	0.108	C3ar1	1433741_at	0.0745	Cd38
1423362_at	0.152	Sort1	1454838_s_at	0.108	AW548124	1441440_at	0.0744	Apg4c
1435842_at	0.152	1110038O08Rik	1429466_s_at	0.108	0610008A10Rik	1454200_at	0.0742	Zfhx1b
1421385_a_at	0.15	Myo7a	1447277_s_at	0.108	Pcyox1	1422748_at	0.0741	Zfhx1b
1426734_at	0.15	BC022623	1422823_at	0.108	Eps8	1423414_at	0.0733	Ptgs1
1435386_at	0.15	Vwf	1450652_at	0.108	Ctsk	1450764_at	0.0733	Aoah
1415687_a_at	0.149	Psap	1426604_at	0.107	Rnasel	1450199_a_at	0.073	Stab1
1423140_at	0.149	Lip1	1417580_s_at	0.107	Selenbp1	1438435_at	0.0729	Phca
1456133_x_at	0.146	Itgb5	1417533_a_at	0.107	Itgb5	1430345_at	0.0726	5530402H23Rik
1415857_at	0.146	Emb	1452416_at	0.106	Il6ra	1435828_at	0.0724	2810401A20Rik
1429693_at	0.144	Dab2	1429525_s_at	0.106	Myo1f	1419298_at	0.0724	Pon3
1416635_at	0.144	Smpdl3a	1455337_at	0.106	9030023J02Rik	1415996_at	0.0721	Txnip
1439189_at	0.144	D630023B12Rik	1427405_s_at	0.106	D6Ertd32e	1427327_at	0.0708	Pilra
1450699_at	0.144	Selenbp1	1452068_at	0.105	Asahl	1426501_a_at	0.0704	T2bp
1419248_at	0.144	Rgs2	1451970_at	0.105	E330036I19Rik	1434903_s_at	0.0703	Il1rl2
1429672_at	0.143	5830407E08Rik	1425451_s_at	0.105	Chi3l3	1428157_at	0.0696	Gng2
1452301_at	0.143	Aldh3b1	1431032_at	0.105	Agl	1456812_at	0.0694	Abcd2
1420905_at	0.142	Il17r	1430878_at	0.104	2210406H18Rik	1428628_at	0.0694	1190002A17Rik
1452050_at	0.142	Camk1d	1417877_at	0.104	2310005P05Rik	1428340_s_at	0.0693	1110012E06Rik
1427994_at	0.141	F730004D16Rik	1423153_x_at	0.104	Cfh	1434362_at	0.0687	AW550831
1455915_at	0.141	AV011803	1450872_s_at	0.104	Lip1	1444987_at	0.0674	Ctsb
1444003_at	0.141	Lincr	1418345_at	0.103	Tnfsf13	1449305_at	0.067	F10
1439703_at	0.14	Mox2r	1454617_at	0.102	Arrdc3	1435135_at	0.0665	B230106I24Rik
1448890_at	0.14	Klf2	1425295_at	0.101	Ear11	1422760_at	0.0662	Padi4
1438475_at	0.14	LOC382062	1452283_at	0.1	AW123240	1452746_at	0.066	1110012E06Rik
1443145_at	0.139	Apbb1ip	1457724_at	0.1	Ctsl	1430886_at	0.0659	1700112E06Rik
1423704_at	0.139	Lypla3	1449282_at	0.0996	Cysltr1	1434484_at	0.0656	1100001G20Rik
1428367_at	0.138	Ndst1	1421840_at	0.0987	Abca1	1418992_at	0.0655	F10
1439426_x_at	0.137	Lzp-s	1443302_at	0.0984	6720403M19Rik	1422603_at	0.0654	Rnase4
1428663_at	0.136	5133401H06Rik	1456424_s_at	0.0978	Pltp	1427306_at	0.0652	Ryr1
1427256_at	0.136	Cspg2	1436448_a_at	0.0978	Ptgs1	1421408_at	0.0631	Igsf6
1450241_a_at	0.135	Evi2a	1420361_at	0.0976	Slc11a1	1435043_at	0.0625	Plcb1
1437181_at	0.134	Peli2	1444599_at	0.0975	1700056O17Rik	1456887_at	0.0617	1700030P01Rik
1416827_at	0.134	Tbxas1	1439364_a_at	0.0969	Mmp2	1442082_at	0.0612	C3ar1
1420970_at	0.134	Adcy7	1416041_at	0.0966	Sgk	1418612_at	0.0599	Slfn1
1425639_at	0.134	Centa2	1417688_at	0.0964	BC004044	1422062_at	0.0599	Msr1
1417277_at	0.134	Cyp4f16	1448407_at	0.096	4632428N05Rik	1424832_at	0.0596	4732429D16Rik
1427072_at	0.134	Stard8	1441972_at	0.0953	6230424C14Rik	1441975_at	0.0596	Acpp
1421618_at	0.133	Myo1f	1416136_at	0.0938	Mmp2	1416029_at	0.0586	Tieg1
1455009_at	0.132	Cpd	1451353_at	0.0935	Tm6sf1	1419687_at	0.0584	D930010J01Rik
1434720_at	0.132	9530033F24Rik	1448455_at	0.0935	Cln8	1437245_at	0.0583	9930117H01Rik
1421027_a_at	0.132	Mef2c	1434920_a_at	0.0934	Evl	1450967_at	0.0583	4933428I03Rik
1448550_at	0.132	Lbp	1428988_at	0.0933	Abcc3	1419483_at	0.0567	C3ar1
1422868_s_at	0.131	Gda	1439808_at	0.093	A130090K04Rik	1450234_at	0.0566	Ms4a6c
1427996_at	0.131	L259	1447830_s_at	0.0929	Rgs2	1428793_at	0.0565	5830411H19Rik
1454699_at	0.131	Sesn1	1442368_at	0.0926	Kctd12b	1422010_at	0.0564	Tlr7
1431609_a_at	0.131	Acp5	1435996_at	0.0923	Card11	1423422_at	0.0563	Asb4
1420407_at	0.131	Ltb4r1	1431033_x_at	0.0918	Agl	1424852_at	0.0556	5430401D19Rik
1421168_at	0.129	Abcg3	1422875_at	0.0917	Cd84	1442804_at	0.0529	Fgr
1436625_at	0.129	Fcgr1	1418084_at	0.091	Nrp	1439819_at	0.0528	AU015263
1436193_at	0.129	Man1c1	1434308_at	0.0909	BC042513	1455227_at	0.0528	B230106I24Rik
1436989_s_at	0.129	Slc12a6	1418944_at	0.0905	Cysltr1	1418912_at	0.0527	Plxdc2
1448943_at	0.128	Nrp	1452067_at	0.09	Asahl	1455885_at	0.0523	6530401C20Rik
1435884_at	0.128	Itsn	1434387_at	0.0892	AI429612	1444176_at	0.0511	Atp6v0d2
1429097_at	0.127	C030044C12Rik	1417074_at	0.0892	Ceacam10	1452279_at	0.0508	Pfc
1460735_at	0.127	Svil	1422824_s_at	0.0887	Eps8	1452353_at	0.0507	Gpr155
1434911_s_at	0.127	AI120128	1454901_at	0.0884	6430570G24	1432757_at	0.0504	2900011L18Rik
1446505_at	0.127	Cd84	1424443_at	0.0882	Tm6sf1	1428573_at	0.05	Chn2
1421963_a_at	0.126	Cdc25b	1452338_s_at	0.0881	Itsn	1440355_at	0.0498	Kctd12b

Affymetrix Id	Fold change	Common name	Affymetrix Id	Fold change	Common name
1423547_at	0.0494	Lyzs	1420701_at	0.0104	Klk1
1425128_at	0.0493	MGC32391	1433678_at	0.01	AI132321
1448929_at	0.0481	F13a	1453152_at	0.01	Mamdc2
1421813_a_at	0.0474	Psap	1436521_at	0.00961	Slc36a2
1423805_at	0.0467	Dab2	1456060_at	0.00921	Maf
1457270_at	0.0467	B230343A10Rik	1425144_at	0.00783	Klk21
1460437_at	0.0464	Pscd4	1449454_at	0.00771	Bst1
1438431_at	0.0461	Abcd2	1425225_at	0.0073	Fcrl3
1419599_s_at	0.0456	Ms4a6d	1417491_at	0.00716	Ctsb
1425407_s_at	0.045	Clecsf6	1448025_at	0.00696	9930027N05Rik
1423141_at	0.0448	Lip1	1417381_at	0.00656	C1qa
1454677_at	0.0442	Timp2	1425182_x_at	0.00635	Klk9
1449164_at	0.0442	Cd68	1460003_at	0.00608	AI956758
1423593_a_at	0.044	Csf1r	1434413_at	0.00573	Igf1
1419691_at	0.0438	Camp	1425214_at	0.00571	P2ry6
1422869_at	0.0436	Mertk	1432466_a_at	0.00492	Apoe
1433933_s_at	0.0425	Slco2b1	1458683_at	0.00467	9930027N05Rik
1449015_at	0.0419	Retnla	1429954_at	0.00436	3110037K17Rik
1417492_at	0.0414	Ctsb	1434366_x_at	0.00429	C1qb
1416683_at	0.0411	Plxnb2	1437726_x_at	0.00417	C1qb
1455646_at	0.0411	2010004M13Rik	1449401_at	0.00331	C1qg
1444537_at	0.0404	AI429363	1417063_at	0.00318	C1qb
1437218_at	0.0399	Fn1			
1448786_at	0.0389	1100001H23Rik			
1426642_at	0.0389	Fn1			
1419872_at	0.0379	Csf1r			
1422953_at	0.0371	Fpr-rs2			
1456072_at	0.0367	Ppp1r9a			
1436999_at	0.0364	AL024069			
1449270_at	0.0363	Plxdc2			
1419598_at	0.0358	Ms4a6d			
1428156_at	0.0358	Gng2			
1422046_at	0.0356	Itgam			
1422013_at	0.0354	Clecsf6			
1423266_at	0.0339	2810405K02Rik			
1442849_at	0.0337	Lrp1			
1456389_at	0.0336	9130203F04Rik			
1421188_at	0.0334	Ccr2			
1417061_at	0.033	Slc40a1			
1428700_at	0.0323	Gpr86			
1454824_s_at	0.0322	Mtus1			
1416298_at	0.0319	Mmp9			
1444546_at	0.0309	BC027057			
1430379_at	0.0292	5830411K21Rik			
1449976_a_at	0.0285	Gpr35			
1448566_at	0.0281	Slc40a1			
1438467_at	0.0278	Mgl2			
1438936_s_at	0.0267	Ang1			
1440209_at	0.0266	2900024D24Rik			
1448502_at	0.0263	Slc16a7			
1449106_at	0.026	Gpx3			
1431110_at	0.0257	5430431D22Rik			
1437025_at	0.0253	Cd28			
1433719_at	0.0243	Slc9a9			
1434955_at	0.0242	2900024D24Rik			
1448061_at	0.0242	Msr1			
1449310_at	0.0241	Ptger2			
1437463_x_at	0.024	Tgfbi			
1450808_at	0.0237	Fpr1			
1424987_at	0.0236	5430435G22Rik			
1428018_a_at	0.0235	Igsf7			
1417963_at	0.0232	Pltp			
1424754_at	0.023	A430103C15Rik			
1417702_a_at	0.023	Hnmt			
1417597_at	0.0229	Cd28			
1416390_at	0.0227	Chc1l			
1436397_at	0.0223	BC027057			
1424842_a_at	0.0221	Arhgap24			
1447849_s_at	0.0217	Maf			
1434798_at	0.0212	Atp6v0d2			
1419873_s_at	0.0212	Csf1r			
1449453_at	0.0209	Bst1			
1419549_at	0.0203	Arg1			
1449366_at	0.0195	Mmp8			
1419605_at	0.0193	Mgl1			
1424727_at	0.0191	Ccr5			
1448891_at	0.019	Msr2			
1456250_x_at	0.0185	Tgfbi			
1438937_x_at	0.0181	Ang1			
1421187_at	0.0179	Ccr2			
1422412_x_at	0.0167	Ear2			
1433919_at	0.0166	Asb4			
1448655_at	0.0153	Lrp1			
1452014_a_at	0.015	Igf1			
1436520_at	0.015	AI450948			
1415871_at	0.0146	Tgfbi			
1448123_s_at	0.014	Tgfbi			
1452141_a_at	0.0134	Sepp1			
1436317_at	0.0127	9030223K07Rik			
1437401_at	0.0121	Igf1			
1421186_at	0.0118	Ccr2			
1417876_at	0.0115	Fcgr1			
1419321_at	0.0112	F7			
1450430_at	0.0111	Mrc1			
1448005_at	0.0106	2500002E12Rik			
1448291_at	0.0104	Mmp9			