Influence of genetic background on human monocyte derived macrophage (MDM) gene expression in response to infection with *Mycobacterium tuberculosis* H37Rv and treatment with inhalable microparticles containing anti -TB drugs

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Certificate

This is to certify that the research work presented in this dissertation entitled **"Influence of genetic background on human monocyte derived macrophage (MDM) gene expression in response to infection with Mycobacterium tuberculosis H37Rv and treatment with inhalable microparticles containing anti -TB drugs"** submitted to Jawaharlal Nehru University, New Delhi has been carried out by Amit Kumar Singh, M.V.Sc. in Animal Biotechnology, at the Division of Pharmaceutics, Central Drug Research Institute, Lucknow, under my supervision. He has fulfilled all the requirements for the degree of Doctor of Philosophy. The work included in this thesis is original and has not been submitted so far, in part or full, for any other degree or diploma of any other university.

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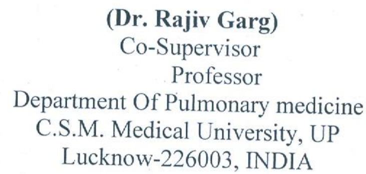

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"Life without thankfulness is devoid of love and passion. Hope without thankfulness is lacking in fine perception. Faith without thankfulness lacks strength and fortitude. Every virtue divorced from thankfulness is maimed and limps along the spiritual road"

John Henry Jowett

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ABBREVIATIONS

AIDS	- Aquired immuno deficiency syndrome
AMϕ	- Alveolar Macrophages(s)
~	- approximately
APCs	- Antigen Presenting Cells
ATP	- Adenosine tri phosphate
ARMS-PCR	-Amplification refractory mutation system-PCR
BAL	- Bronchio-alveolar lavage
BCG	- Bacille Calmette-Guerin
BMP	- Blank Microparticles
BSA	- Bovine serum albumin
CBA	- Cytokine Bead Array
CDRI	- Central Drug Research Institute
cDNA	- Complimentary Deoxyribonucleic acid
Conc.	- concentration
CFUs	- Colony forming units
CR	- Complement receptor
CTL	- Cytotoxic T Lymphocyte
^{o}C	- degree Celsius
DC-SIGN	- DC-specific intercellular adhesion molecule-3 grabbing nonintegrin
DEPC	- Diethyl Pyrocarbonate
DMSO	- Dimethyl sulphoxide
DNA	- Deoxyribonucleic acid
DNAse	- deoxyribonuclease
DOTS	- Directly Observed Shortcourse Treatment
EDTA	- Ethylene diamine tetra acetate

Abbreviation	Meaning
ELISA	- Enzyme linked immunosorbent assay
FACS	- Fluorescence-activated cell sorter
FCS	- Fetal calf serum
FITC	- Fluorescein Isothiocyanate
x g	- Relative centrifugal force
GI	-Growth index
gm	- Gram
H_2O_2	- Hydrogen peroxide
HCl	- Hydrochloric acid
HIV	- Human immunodeficiency virus
HLA	-Human leukocyte antigen
H	- Hour
HWP	-Hardy Weinberg equilibrium P value
IFN-□	- Interferon gamma
IFNGR	- Interferon gamma receptor
IL	- Interleukin
IMP	-Infected cells treated with drug containing microparticle
INH	- Isoniazid
iNOS	- Inducible nitric oxide synthase
IPA	- Iso propyl alcohol
ISD	-Infected cells treated with drugs in solution
IUATLD	- International union against tubercular and lung diseases
kg	- Kilogram
kV	- Kilo volts
L	- Litre
LAM	- lipoarabinomannan
LM	- lipomannan
LJ	- Lowenstein Jensen
M	- Milli

M	- Molar
μ	- Micro
μm	- Micrometer
mAb	- Monoclonal antibodies
MBL	Mannose binding lectins
MCP-1	- Monocyte chemoattractant protein 1
MDMs	-Monocyte derived macrophage
MDR-TB	- Multi drug resistance TB
MIC	- Minimum inhibitory concentration
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number of tandem repeats
Mg.	- milligram
MHC	-major histocompatibility antigen complex
Min	- Minute
mM	- millimolar
MOI	- Multiplicity of infection
MPs	- Microparticles
Mϕ	- Macrophage(s)
MR	- Mannose receptors
mRNA	- messenger ribonucleic acid
Mtb.	- Mycobacterium tuberculosis
MW	- Molecular weight
Nm	- nanometer
NK cells	-Natural killer cells
NO	- Nitric oxide
No.	- Number
NK	- Natural Killer
NRAMP	-Natural Resistance Associated Macrophage Protein
OD	- Optical density

PBMCs	- Peripheral Blood Mononuclear Cell
PBS	- Phosphate buffered saline
PCR-SSP	Polymerase chain reaction-sequence specific polymorphism
PDIM	-Pphthiocerol dimycocerostate
pH	- Negative logarithm of hydrogen ion concentration
PI	- Propidium iodide
PLA	- Poly(lactic acid)
PLGA	- Poly(lactide-co-glycolide)
PMN	-Polymorphic neutrophil
PPD	-Purified protein derivative
Q-RT-PCR	-Quantitative reverse transcription polymerase chain reaction
RFB	- Rifabutin
R Score	-Resistance score
RFLP	-Restriction fragment length polymorphism
RIN	- RNA integrity number
RNA	- Ribonucleic acid
RNI	- Reactive nitrogen intermediates
RNTCP	- Revised National Tuberculosis Control Programme
ROI	- Reactive oxygen intermediates
ROS	- Reactive oxygen species
RPMI	- Rosewell park memorial institute
RT-PCR	- Real time polymerase chain reaction
S score	-Susceptibility score
sec.	- second
SNP	Single nucleotide polymorphism
SOD	- Superoxide dismutase
Sp-A	- Surfactant protein A

SRs	- Scavenger receptor
TACO	- Tryptophan-Aspartate containing coat
TB	- Tuberculosis
TDW	- Triple distilled water
TDM	- Trehalose dimycolate
TGF-β	- Transforming growth factor-beta
Th	-T-helper cells
TLRs	- The Toll-like receptors
TNF-α	- Tumour necrosis factor alpha
v/v	- Volume by volume
VDR-	-Vitamin D receptor
WBCs	- White blood cells
WHO	- World Health Organization

Chapter 1

INTRODUCTION

Introduction

1.1. Background and Context

Tuberculosis (TB) is prevalent in most parts of the world, to varying extents (Fig 1.1). However, it is estimated that just about 10% of individuals infected with *Mycobacterium tuberculosis* (Mtb) and congener pathogens develop active disease (Ducati *et al*., 2006; Dye *et al*., 1999, WHO, 2007). Multiple factors are responsible for this phenomenon, such as nutrition, age, living conditions, immune status and genetic predisposition of the host on one hand, and virulence and drug resistance of infectious strains on the other (Lopez, Aguilar et al. 2003; McShane 2003).

Recent work in our lab has proposed the use of inhalable microparticles containing a combination of anti-TB drugs for use as adjunct therapy in the treatment of pulmonary TB (Misra, Hickey et al. 2011). Preclinical studies indicate that this drug delivery system possesses high efficacy against experimental TB in mice and guinea pigs (unpublished). High efficacy in animal experiments has been ascribed to the ability of inhaled microparticles to be phagocytosed by alveolar macrophages infected with Mtb. In the macrophage cytosol, microparticles not only deliver a large amount of drug within a small, enclosed space (Sharma, Saxena et al. 2001; Muttil, Kaur et al. 2007; Verma, Kaur et al. 2008) , but also apparently "stimulate the phagocyte" as recommended by Koch ahundred years ago (Young 2001). Such stimulation results in rescue of infected macrophages from alternative activation imposed by the pathogen (Gordon 2003) and elaboration of markers of a classically-activated phenotype. Thus, phagocytosed microparticles induce the production of bactericidal free radicals (Sharma, Muttil et al. 2007), Th1 cytokines (Sharma, Muttil et al. 2007; Yadav, Muttil et al. 2010) from infected macrophages, and drive the cells to apoptosis rather than necrosis (Yadav and Misra 2007). Genome wide-transcription analysis suggests that genes associated with apoptosis, proinflammatory cytokines, and innate defence responses are differentially regulated in macrophages infected in vitro with Mtb as a result of treatment with anti-TB drugs in solution, versus the same drugs in microparticles (unpublished).

Since there is a great deal of genetic variation relevant to host defense responses of humans, it is important to investigate whether observations made using a single defined strain of Mtb (H37Rv) infecting macrophages of a single defined genotype (THP-1) would hold in the context of genetic variation observed in a sample of a human population. The present investigations were undertaken to evaluate host response and bacterial survival when primary macrophages derived from human volunteers were infected in vitro with Mtb H37Rv and treated with inhalable microparticles containing isoniazid (INH) and rifabutin (RFB) reported extensively from the lab.

Salient information relevant to infection by Mtb and congeners and host-defense responses is detailed below

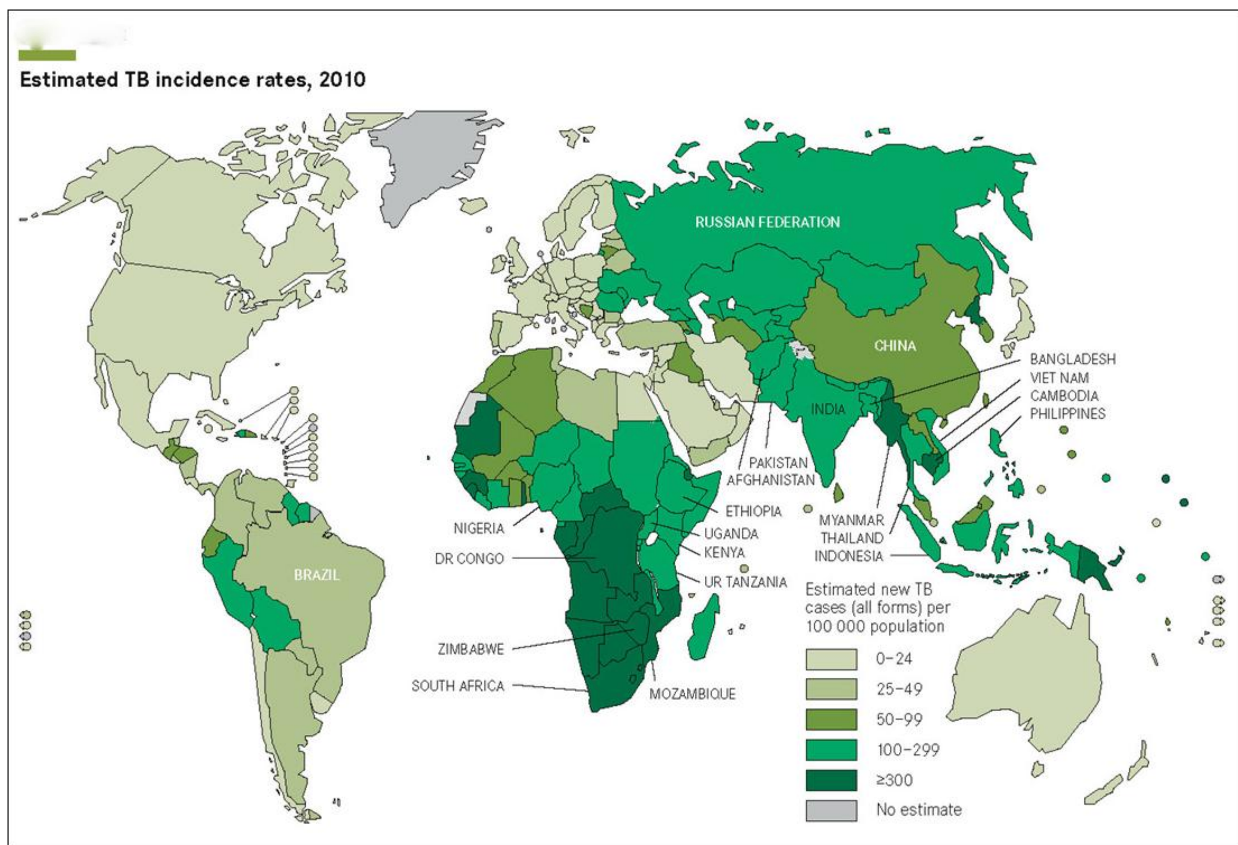


Figure1.1: Estimated TB Incidence rate 2010 (Source: WHO, 2011)

1.2. Tuberculosis

TB is a chronic granulomatous disease of humans and animals and has zoonotic importance. It is one of leading cause of death worldwide causing WHO to declare as global health emergency in 1994, the single bacterial disease so far. About 1/3rd of the world population is harboring Mtb in their body and about 10% of infected individuals progress to active TB (Ducati *et al.,* 2006; Dye *et al.*, 1999, WHO, 2007). TB incidence rates are shown in Fig 1. Infections are occurring at the rate of 1 per second (WHO 2006). There were about 8.8 million cases of TB in 2010, with Asia and Africa contributing 59% and 26% of total cases (WHO 2011). India alone accounted for an estimated 26% of all TB cases worldwide, and China and India combined accounted for 38% (WHO 2011). TB is more prevalent in low income countries and is the second largest cause of death worldwide after HIV/AIDS (WHO 2011). Rising numbers of cases in the developed world have been attributed to increase in HIV/AIDS, use of immunosuppressive drugs or substance abuse (WHO 2006). It is estimated that by year 2020, TB will be one of the world top10 global diseases with about 1 billion new cases (Murray & Salomon, 1998; Pasqualoto & Ferreira, 2001).

Mtb is usually transmitted by the aerosol route. Coughing by TB patients releases droplets that contain Mtb, and inhalation of such droplets by another person leads to infection (Kaufmann 2001). Once inside the lung, Mtb is phagocytosed by alveolar macrophages, initiating host immune response involving T cell activation, cytokine and other chemokine secretion leading either to elimination of bacteria or progression to disease (Henderson *et al.,* 1997; Roach *et al.*, 2002). On entry into macrophages, bacteria reside in an endocytic vacuole called the phagosme. Mtb and other intracellular bacteria have evolved strategies to prevent phagosome maturation, i.e phagosome- lysosome fusion which would have lead to acidic pH environment, reactive oxygen intermediate release, release of lysosomal content and bactericidal peptides inside the phagosome. Phagosome acidification is hindered due to exclusion of proton ATPases from mycobacterial phagosome. Moreover, mycobacteria inhibit Ca^{2+} signaling which would have lead to host response against infection.

Mtb infection leads to formation of tubercles in the affected part, consisting of defense cells that congregate with the objective of killing these bacilli or restricting the spread of

infection. It is the strength of the host cellular response vis-à-vis the virulence of the infecting strain that apparently decides whether infection will be contained or progress to a later stage. The contained infection is known as latent infection and may persist for the whole life without any activation in individuals capable of mounting effective cellular responses. However, at some point of time, if immunity falls because of malnutrition, immunodifficency disease/HIV, ageing or other factors the granuloma become liquefied and bacteria replicate there, followed by spread to nearby areas of the lungs and may disseminate to other parts of the body. Thus the pathogen uses phagocytic cell as its ecological niche, where it lives, replicates and persists (Henderson *et al.,* 1997; Roach *et al.*, 2002). It is well known that host genetics as well as environmental factors have important roles in the outcome of exposure and infection (Casanova & Abel, 2002). It is estimated that 10% of immunocompetent people exposed to Mtb infection develop the disease while the rest 90% never develop do so (Murray *et al*, 1990).

1.2.1 *Mycobacterium tuberculosis* complex

Six closely related organisms form the *Mycobacterium tuberculosis* complex implicated in TB: *M. tuberculosis* (mostly human tuberculosis), *M. bovis* (mostly bovine and other animals including human), *M. microti* (mostly in small mammals) *M. africanum* (mostly in humans and animals in sub Saharan African countries), *Bacille Calmette-Guerin* (BCG, attenuated strain of *M. bovis*) and *M. canetti*, (rarely encountered but can cause human disease) (Greenwood *et al.*, 1997). Mtb is an obligate aerobe, non-motile, non-sporulating, non-capsulating, has straight or slightly curved rods and may occur in clumps or individually (Casanova and Abel 2002). The importance of oxygen for Mtb can be gauged by the predominance of bacteria in oxygen rich tissues like the lung, especially its upper lobe. It has been classified into a distinct group of acid-fast organisms because it does not have characteristics of either Gram's positive or negative organisms although it contains peptidoglycan (murein) in the cell wall (Prescott *et al.*, 1996). Mtb falls in the class of slow growers, requiring 3-4 weeks to form colonies with doubling time of about 24 hr. This property is thought to contribute to a less vigorous antimicrobial response by macrophages (McKinney 1998).

1.2.2 Genome

The complete genome of Mtb strain H37Rv was deciphered in 1998 (Cole, Brosch et al. 1998), and since then substantive advances have been made in the area of bacterial genomics. The Mtb genome is highly conserved and rich in repetitive DNA sequences coding for enzymes required for lipolysis and lipogenesis (Ducati, Ruffino-Netto et al. 2006). Many other Mtb strains have been sequenced and comparative analysis shows similarity among them. However small differences in their genome serve as identification markers to differentiate one strain from another, besides conferring drug resistance/susceptibility and indicating evolutionary relationships between them (Mathema *et al.*, 2006). Some of the most common typing techniques used to study the epidemiology of TB are IS*6110* RFLP analysis, spoligotyping, MIRU-VNTR and SNP analysis (Mathema *et al.*, 2006). It has been suggested that strain fitness that refers to the heritable variation among members of a given species or phylogenetic lineage can play a major role in transmission (Cohen & Murray, 2004). However, this is still poorly understood. Certain factors that might enhance the fitness in a population may include the ability of the strains to: (1) endure and reproduce within the hostile environment of the host, (2) modulate the host response and (3) up or down regulate expression of specific genes to adjust the effectiveness of antimycobacterial agents (Mathema *et al.*, 2006).

1.2.3 Cell wall structure

The important features shared by all members of the Mtb complex include a cell wall of unique composition. Fig 1.2 depicts the major component of the Mtb cell wall and their distribution. The outer layers of the cell wall consist of large amounts of cell wall lipids and several unique components such as lipoarabinomannan (LAM), lipomannan (LM), phthiocerol dimycocerostate (PDIM), mycocerostate, mycolic acid, trehalose dimycolate (TDM) and sulpholipids (Brennan P.J. 1990; Besra 1994). These components are suggested to be responsible for mycobacterial hydrophobicity, ability to form clumps or cords, ability to survive intracellularly and it is the cell wall that gives acid-fastness, enabling it to retain basic dyes in the presence of acidic alcohol.

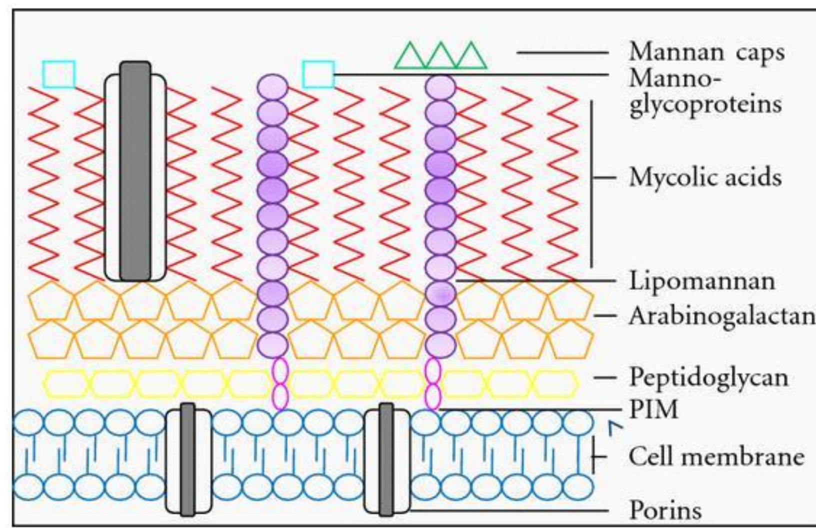


Fig 1.2: Components of the cell wall of Mtb and their distribution

The cell wall structure of Mtb is major determinant of virulence of the bacterium. More than 60% of cell wall is contributed by lipid and consist of three major factors i.e. mycolic acid, cord factor and wax-D. Mycolic acids are unique alpha branched acid and are strong hydrophobic molecules affecting permeability of cell surface. They protect the bacteria from attack by cationic proteins, lysozyme and oxygen radicals in the phagosome, and at the other end, protect extracellular bacteria from complement deposition. Cord factors are toxic to mammalian cells and inhibit polymorphic neutrophils (PMN) migration. The high content of lipids in cell wall of Mtb confers protection from many antibiotics, killing by many acidic or alkaline compounds, osmotic lysis by complement deposition and help in survival against reactive oxygen species (ROI) and reactive nitrogen species (RNI).

1.2.4 Progression of disease

Inhalation of TB bacteria may result in four different scenarios (Dannenberg 1994):

i) Innate response is sufficient to clear of the bacteria

ii) Asymptomatic, latent infection develops

iii) Active disease develops soon after the infection, known as primary infection

iv) Active disease develops many years after infection leading to reactivation of TB

Protective responses to Mtb are complex and involve both wings of immunity –innate and acquired. Innate response is not antigen specific and is triggered by bacterial cell wall components. Phagocytosis of Mtb is typically followed by increase in oxidative burst, reactive nitrogen intermediates, acidification and increased proinflammatory cytokines (Medzhitov and Janeway 1997; Beyers, van Rie et al. 1998; Fenton 1998). However, in “Susceptible” individuals, these processes are likely to be insufficient to eliminate or contain the bacteria, leading to bacterial proliferation inside the macrophage. Acquired immune responses would then be necessary for effective host defence. In most cases, acquired responses result in the containment of bacteria in well organized granuloma, marked by presence of large number of activated macrophages which infilterate the region and enclose the infected cells in tubercles. Such macrophages later differentiate into epitheloid cells, so called because of the resemblance of clustering macrophages to epithelial cells. Activated macrophages secrete large amounts of lytic enzyme and lead to formation of spheroidal regions of necrotic tissue also known as the Ghon focus (Goldsby RA 2000). Tubercle formation leads to hypoxic conditions within it and together with acidosis kills most of the bacteria. Thus granuloma formation in most cases limits the infection but does not totally eliminate it. In “Susceptible” persons, immunosuppressed individuals or HIV infected people, due to decrease in immunity, Mtb can potentially become reactivated and lead to active disease (Greenwood D 1997). Global estimate of TB is that about 1/3rd of world population is infected with tuberculosis but out of this only 10% develop active disease (Bellamy and Hill 1998; Butler 2000). In rest of the individuals, immune response is sufficient to clear the infection or arrest it to let it remain latent. In a limited number of cases, risk factors are identifiable which include HIV/AIDS, diabetes, age, alcohol, smoking and corticosteroid therapy. However, there is now increasing evidence suggesting complex interaction of environmental and genetic factors for clinical development of disease (Bellamy 2000). Clinically important differences in various strains of Mtb have been identified which may influence transmission and virulence of mycobacteria (Bloom and Small 1998).

1.3 Treatment and Prophylaxis

The current treatment regimen follows National guidelines issued by Revised National Tuberculosis Control Programme (RNTCP) and incorporates Directly Observed Therapy (Short

Course) or DOTS. DOTS consist of administration of multiple drugs under supervision of a health worker and despite being called "Short" are used for more than 6 months. TB patients are treated with four drugs which act on three forms of bacteria: i) replicating bacilli, ii) sterilization to kill semi-dormant bacteria and iii) drug resistant bacteria (Hershfield 1999). Isoniazid exhibits potent antimycobacterial activity on rapidly dividing bacteria by targeting cell wall biosynthesis (Rozwarski, Grant et al. 1998). Rifampicin and pyrazinamide kill the more slowly dividing bacterium. Rifampicin works by inhibiting DNA-dependent RNA polymerase (Hershfield 1999), while pyrazinamide acts by disrupting membrane transport in Mtb and is very effective against bacteria located in the acidic environment of phagosomes (Zhang, Wade et al. 2003). Ethambutol has bacteriostatic effect, but is used at higher dose in TB treatment and exhibits bactericidal effect. Because patients feel better in 1-2 months, they tend to leave the treatment mid way. Moreover there are increasing reports on patient non-compliance and emergence of drug resistant strains which have further complicated the treatment. Thus there is emergent need for fast-acting drugs with increased compliance among patients of all ages.

Granulomas, at first glance seem to contain bacteria in a non-replicating stage. However, recent studies have shown that some bacteria still undergo replication within granulomatous lesions (Tan, Sequeira et al. 2010). As such, there are two populations of bacteria in the granuloma i.e replicating and non-replicating. Current drugs are effective against replicating organisms, therefore non-replicating organism present in granulomas persist and show resistance to drugs which further increases the duration of drug treatment.

BCG is the only vaccine available for TB and is effective against childhood TB but does not confer significant protection against adult TB or extra pulmonary TB. Moreover, BCG has variable efficacy in different populations. The reason may be i) BCG bacteria being cultured for many years have become attenuated and with modern day vaccine preparation are too benign to generate efficacy (Behr and Small 1997) ii) exposure to environmental bacteria would have lead to development of tolerance (Brandt, Feino Cunha et al. 2002; Demangel, Garnier et al. 2005) iii) or there might be clearance of BCG bacteria prior to development of immunity.

Thus there is urgent need for development of new vaccines if we want to reduce the incidence of tuberculosis by 2050 and it should provide efficacy not only in infants but also in adults. Present vaccination strategy targets reduction of initial bacterial burden with containment of remaining bacteria which goes to latent stage and can reactivate later (Voskuil, Schnappinger et al. 2003). Therefore, post exposure vaccines are required which not only reduce initial bacterial burden but also prevent reactivation of latent mycobacterium. Current vaccine candidates can be broadly divided into 3 groups i) use of live mycobacterium which are either improvement of BCG through addition of relevant genes (Grode, Seiler et al. 2005; Tullius, Harth et al. 2008) or attenuated Mtb with deleted genes for ateenuating virulence (Martin, Williams et al. 2006; Verreck, Vervenne et al. 2009). ii) subunit or live vector based vaccines used for boosting BCG primed vaccination to complement the immune response genereated by BCG vaccination (Lingnau, Riedl et al. 2007; Aagaard, Dietrich et al. 2009; Rouanet, Debrie et al. 2009) iii) use of killed whole bacteria as adjunct to chemotherapy (Vilaplana, Montane et al. ; Dlugovitzky, Fiorenza et al. 2006; Gupta, Geetha et al. 2009) iv) use of non pathogenic Mycobacterial strains e.g. *Mycobacterium indicus pranii* which shares cross-reactive antigens with *M. tuberculosis* as a whole bacterial vaccine can be used as alternatives to *M. bovis* for vaccine use as vaccine candidate (Gupta, Geetha et al. 2009).

Apart from new drugs, there is potential in novel methods of drug delivery for more efficacious chemotherapy of TB. Thus, drug delivery systems aiming to reduce the dose and duration of treatment by targeting the lungs or more specifically infected macrophages in the lungs and airways are being investigated in the lab and elsewhere. Inhaled drug delivery systems are capable of affecting the macrophage activation state by macrophage-targeted microparticles (Sharma, Muttil et al. 2007; Yadav and Misra 2007). Inhalable microparticles induce proinflammatory immune responses with significant increase in TNF-α and IL-12 upon phagocytosis, accompanied by increase in respiratory burst, reactive nitrogen species and altered cytokine profile suggesting classical activation of macrophages (Sharma, Muttil et al. 2007; Yadav and Misra 2007; Yadav, Muttil et al. 2010). There was increase in TNF-α and IFN-γ cytokine level upon treatment of Mtb H37Ra infected mice with inhalable microparticles while oral therapy with the same drug enhanced IL-12 but decreased TNF-α level in the

bronchioalveolar lavage (BAL) fluid as well assecretion by macrophages recovered through BAL (Sharma, Yadav et al. 2011). Various groups have reported increased efficacy of inhaled microparticles containing anti-TB drug either singly or in combinations (Pandey and Khuller 2005; Yoshida, Matumoto et al. 2006; Fiegel, Garcia-Contreras et al. 2008) (Suarez, O'Hara et al. 2001; Suarez, O'Hara et al. 2001)

1.4 Macrophage response to Mtb

1.4.1 Macrophage invasion by Mtb

Alveolar macrophages (AM) and dendritic cells (DC) are the first line of defense against inhaled microorganisms that deposit in the deep lung. Despite being the first cells to encounter inhaled particles and microorganisms, AM display a phenotype of "alternative activation" with reduced oxidative radical generation (Fels and Cohn 1986), altered cytokine response and reduced microbicidal response (Gordon 2003). Though effective in removal of inhaled particles and extracellular pathogens, these responses are likely to be inadequate in "Susceptible" individuals in clearing intracellular Mtb.

Mtb binds to host cells by a variety of receptors leading to difference in immune activation, signal transduction and intracellular survival. These cells express a variety of pathogen recognition receptors such as the mannose receptor (MR) (Stephenson and Shepherd 1987), dectin receptor (β-glucan receptor) (Taylor, Brown et al. 2002) scavenger receptors (SRs) (Lohmann-Matthes, Steinmuller et al. 1994), complement receptors (CRs), mannose binding lectins (MBL), DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), CD-14 and toll like receptors (TLRs).

1.4.2 Intracellular events

Mtb preferentially and specifically uses MR and CR for uptake by macrophages. MR associated phagocytosis in "Susceptible" individuals is followed by activation of an anti inflammatory program marked by "alternative activation" of the macrophage, a typical feature of which is a lack of activation of NADPH oxidase. Subsequently, the pathogenic bacteria engage with vesicular trafficking machinery thereby causing inhibition of phagosomal maturation. Inhibiting phagosome–lysosome fusion and acidification of phagosome is

prevented by depletion of proton ATPase molecules from vacuolar membrane (Armstrong and Hart 1971; Russell 2001). The early trafficking pattern of the Mtb phagosome is normal and iron and glycospingolipids are present in the early phagosome (Russell, Dant et al. 1996; Sturgill-Koszycki, Schaible et al. 1996). The maturation arrest occurs sometime between the acquisition of Rab5 and Rab7 as marked by the absence of Rab7 in the phagosome (Deretic, Via et al. 1997). Mtb produces sulpatides and ammonia which alkalizes the phagosome environment (Gordon, Hart et al. 1980). In murine macrophage, phagosome-lysosome fusion can be blocked by retention of TACO (Tryptophan-Aspartate containing coat) and in humans, by coronin, a phagosome specific polypeptide (Anand and Kaul 2005).

Iron is important for both host and the pathogen being the cofactor in many metabolic reactions. For the host, it serves important functions in generation of reaction oxygen and nitrogen intermediates while for bacteria it is required for growth and survival (Schaible and Kaufmann 2004). Mtb exploits the host system of iron uptake, i.e. transferrin-transferring receptors (Schaible and Kaufmann 2005) besides directly taking iron from the cytoplasm (Olakanmi, Schlesinger et al. 2002). The Natural Resistance Associated Macrophage Protein (NRAMP) is found on the surface of lysosomes of macrophages and migrates to the phagosome compartment after phagocytosis and plays an important role in controlling infection by removing iron and other divalent cations from the phagosomal environment (North and Medina 1998; Canonne-Hergaux, Gruenheid et al. 1999). Polymorphism in NRAMP gene is reported to be associated with TB susceptibility in various populations (Fernando and Britton 2006).

1.4.3 Alternative activation macrophages

Activated macrophages produce cytokines and the pattern of cytokine secretion can be used to classify them into M1 and M2 phenotypes by analogy to Th1 and Th2 lymphocytes. Innate response against Mtb begins with the recognition of mycobacterial structural elements by TLRs, leading to production of IFN-γ and NO which directly kill the pathogen beside activating the macrophage and recruiting T lymphocytes. However, in "Susceptible" individuals, the macrophage displays "alternative" phenotype with diminished NO production and increased Th2 cytokines, e.g. IL-4, IL-13 and IL-10 (Gordon 2003). Such a profile of cytokine secretion inhibits Th1 responses by antagonizing IFN-γ and decreasing IL-1, IL-6 and

TNF- α (Mantovani, Sica et al. 2004). It also manipulates signal transduction pathways and suppresseses apoptosis (Verma, Singh et al. 2011). Though Mtb is intracellular, it presents non essential epitopes on major histocompatibility class II MHC II molecules for recognition by $CD4^+$ cells, leading to secretion of Th2 cytokines and thus intensifies alternative activation of macrophages. Despite inducing substantial immune response, the "Susceptible" host is unable to clear the infection, even though the growth of the pathogen may be restricted (North and Jung 2004). The inability of clearance may be related with the kinetics of immune response developed following aerosol infection. The slow development of acquired immune responses against Mtb may be due either to immunomodulatory activity of the pathogen or to low dose of infection (Medzhitov and Janeway 1997; Beyers, van Rie et al. 1998; Fenton 1998).

Several studies have demonstrated increased production of IL-4-, IL-13- and IL-4-dependent IgE secretion in TB patients (Yong, Grange et al. 1989). Microarray studies of infected macrophages primed with either IL-4 or IFN-γ showed decreased nitrosative stress and increased iron availability in "alternative phenotype" macrophages (Kahnert, Seiler et al. 2006) . Furthermore, virulent strains seem to modify MAPKs activation to impair cytokine response and interrupt the association of STAT-1 with transcriptional co-activator CREB binding protein, thus inhibiting IFN-γ signaling pathway (Stark, Kerr et al. 1998; Ehrt, Schnappinger et al. 2001).

1.5 Factors Affecting Innate Responses to Mtb Infection

1.5.1 Environmental factors

Appropriate nutrition is well-known to prevent and even reverse disease progression, and malnutrition remains one of the major contributors to development of active disease. Micronutrients are increasingly being implicated in etiology. Dietary iron overload has been shown to increase the risk of developing clinical disease (Gangaidzo, Moyo et al. 2001), and vitamin D deficiency is now established as a cause of disease rather than the effect (Sasidharan, Rajeev et al. 2002). Malnutrition-related protein deprivation results in reduced T lymphocyte number, reduced CD4+ cells, decreased NK cell activity and reduced phagocytosis. Furthermore, poor socio-economic status, overcrowding, etc are contributing factors promoting infection as well as progression.

1.5.2 Host genes

The development of clinical symptoms in 10% of infected individuals prompts consideration of non-environmental factors that may contribute to the outcome of infection with Mtb. Fig 1.3 shows some genetic components that have been studied with reference to disease development. There is evidence about the role of host genes in individual variability to TB susceptibility (Bellamy and Hill 1998; Hill 2001). The ancestors of ethnic groups which are resistant to TB tend to be derived from dense populations with higher TB incidence, while ancestors of susceptible groups tend to be derived from areas which were considered free of TB, till recently. Several studies have shown that Africans are more likely to develop clinical disease than Caucasians, even if they are living in the same conditions (Stead, Senner et al. 1990). Infection with virulent bacteria and poor socio-economic and environmental conditions is not sufficient to explain individual susceptibility to infection (Levin and Newport 2000). A number of epidemiological studies have provided compelling evidence that susceptibility to TB is determined genetically, at least in part. Several twin studies have shown that larger numbers of monozygotic twins developed TB than fraternal twins (Schurr 2007). A number of genes are involved in genetic susceptibility with each factor contributing small but significant effects on "Susceptibility" (Bellamy 2000; Marquet and Schurr 2001). Case control, family based, candidate gene and genome wide linkage studies have identified different polymorphisms associated with susceptibility to TB. Various candidates genes associated with TB susceptibility or resistance have been summarized in Tables 1-3.

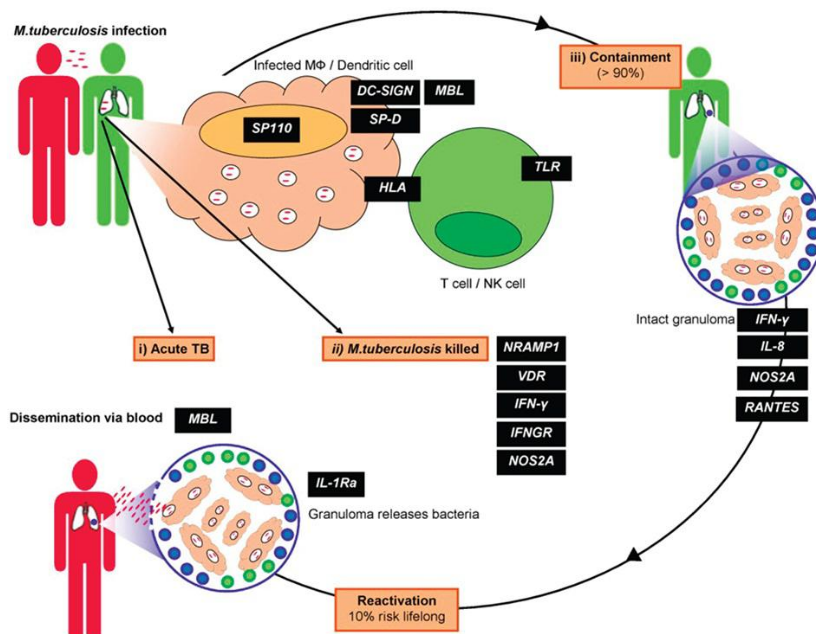


Figure 1.3: Genetic contribution in the disease susceptibility (Adapted from (Kaufmann and McMichael 2005)

Table 1.1: Selected studies showing HLA association with TB

Population	HLA antigen/allele	Nature of association	Case	Control	Reference
Canadian	B8	Susceptibility	543	46	(Selby, Barnard et al. 1978)
Indian	A2	Susceptibility	329	153	(Rajalingam, Mehra et al. 1997)
	B18	Protective			(Rajalingam, Mehra et al. 1997)
	A1-like supertype	Protective	—	235	(Balamurugan, Sharma et al. 2004)
	A3-like supertype	Susceptibility			(Balamurugan, Sharma et al. 2004)
	DR2	Susceptibility	—	25 families	(Singh, Mehra et al. 1983)
			404	204	(Brahmajothi, Pitchappan et al. 1991)
			289	153	(Rajalingam, Mehra et al. 1996)
			122	209	(Selvaraj, Uma et al. 1998)
	DRw6	Protective	109	124	(Singh, Mehra et al. 1983)
	DRB1*1501(DR2)	Susceptibility	87	126	(Ravikumar, Dheenadhayalan et al. 1999)
			36	72	(Sriram, Selvaraj et al. 2001)
	DQ1	Susceptibility	122	209	(Selvaraj, Uma et al. 1998)
	DQB1*0601(DQ1), *DR B1*1501-DQB1*0601*	Susceptibility	87	126	(Ravikumar, Dheenadhayalan et al. 1999)
	DRB1*14(DR6), DQB1*0502 and *0503[‡]	Susceptibility	—	114	(Sharma, Turaga et al. 2003)
Black American	B5 and DR5	Susceptibility	54	72	(Cox, Arnold et al. 1982)
	DR6	Protective			(Cox, Arnold et al. 1982)
Korean	DRB1*08032 and DQB1*0601[‡]	Susceptibility	200	53	(Park, Lee et al. 2002)
Italian	DR4 alone or along	Susceptibility	1089	122	(Ruggiero, Cosentini et al. 2004)

	with B14				
	A2+, B14-, DR4-	Protective			(Ruggiero, Cosentini et al. 2004)
Indonesian	DR2 and DQw1	Susceptibility	64	101	(Bothamley, Beck et al. 1989)
	DQw3	Protective			(Bothamley, Beck et al. 1989)
Mexican	DRB1*1501, DQA1*0101, and DQB1*0501	Susceptibility	95	50	(Teran-Escandon, Teran-Ortiz et al. 1999)
	DR4, DR8 and DQB1*0402	Protective			(Teran-Escandon, Teran-Ortiz et al. 1999)
Venda, South African	DRB1*1302, DQB1*0301-0304, *DRB1*1101-1121-DQB1*05*	Susceptibility	117	95	(Lombard, Dalton et al. 2006)
	*DRB1*11-DQB1*03*	Protective			
Cambodian	DQB1*0503	Susceptibility	49; 39[§]	78; 48[§]	(Goldfeld, Delgado et al. 1998)
	DQ β57 Asp/Asp	Susceptibility	107	436	(Delgado, Baena et al. 2006)
Thai	DQB1*0502	Susceptibility	160	82	(Vejbaesya, Chierakul et al. 2002)
	DQA1*0601, DQB1*0301	Protective			(Vejbaesya, Chierakul et al. 2002)
Iranian	A26 and B27	Protective	108	44	(Mahmoudzadeh-Niknam, Khalili et al. 2003)
	B17 and DR14	Susceptibility			(Mahmoudzadeh-Niknam, Khalili et al. 2003)
	DRB1*07, DQA1*0101	Susceptibility	100	40	(Amirzargar, Yalda et al. 2004)
	DQA1*0301 and *0501	Protective			(Amirzargar, Yalda et al. 2004)
Soviet Union (six ethnic groups)	DR2	Susceptibility	984	643	(Khomenko, Litvinov et al. 1990)
	DR3	Protective			(Khomenko, Litvinov et al. 1990)

Table 1.2: Selected studies showing cytokine gene polymorphism association with TB

Cytokine	Location	Association	Controls	TB	Population	Reference
IFN-γ	+874 (A/T)	Susceptibility	188	178	Pakistani	(Ansari, Talat et al. 2009)
			97	45	Sicilian	(Lio, Marino et al. 2002)
			235	313	South African	(Rossouw, Nel et al. 2003)
			451	385	Chinese	(Tso, Ip et al. 2005)
			100+82 (PPD$^-$)	113	Spanish	(Lopez-Maderuelo, Arnalich et al. 2003)
		None	50	81	Turkish	(Oral, Budak et al. 2006)
			913	514	Malawian	(Fitness, Floyd et al. 2004)
			174	240	African American	(Moran, Ma et al. 2007)
			64	161	Caucasian	(Moran, Ma et al. 2007)
			98	319	Hispanics	(Moran, Ma et al. 2007)
			594	667	West African	(Cooke, Campbell et al. 2006)
			188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
			111	183	Chinese	(Wu, Qu et al. 2008)
IL-12B	Intron 2	Susceptibility	117	106	Whites	(Ma, Xie et al. 2011)
			167	186	African American	(Ma, Xie et al. 2011)
		None	188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
IL-12BR1	−2 (C/T)	Susceptibility	78	101	Moroccan	(Remus, El Baghdadi et al. 2004)
			197	98	Japanese	(Akahoshi, Nakashima et al. 2003)
	−111 (A/T)	None	151	115	Korean	(Lee, Lee et al. 2005)
IL-1B	−511 C/T	Susceptibility	298	335	Gambian	(Awomoyi, Charurat et al. 2005)
			166	122	Colombian	(Gomez, Camargo et al.

						2006)
	+3954 T/C	Protective			Colombian	(Gomez, Camargo et al. 2006)
		None	400	400	Gambian	(Bellamy, Ruwende et al. 1998)
			106	358	Cambodian	(Delgado, Baena et al. 2002)
			114	89	Gujarati Asians	(Wilkinson, Patel et al. 1999)
IL-2	−330 (T/G)	Susceptibility	188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
	+160 (G/T)					
	330 G+160 G	Protective	123	41	Iranian	(Amirzargar, Rezaei et al. 2006)
		None	188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
IL-4	−590 (T/C)	None	123	41	Iranian	(Amirzargar, Rezaei et al. 2006)
	−1098 (G/T)		123	41	Iranian	(Amirzargar, Rezaei et al. 2006)
	−33 (C/T)					
IL-6	−174 (G/C)	None	188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
			54+81	140	Colombian	(Henao, Montes et al. 2006)
		Susceptibility	123	41	Iranian	(Amirzargar, Rezaei et al. 2006)
			61+42+91		Canadian	(Larcombe, Orr et al. 2008)
IL-10	−1082 (G/A)	Susceptibility	106	358	Cambodian	(Delgado, Baena et al. 2002)
					Sicilian	(Scola, Crivello et al. 2003)
			80	128	Turkish	(Ates, Musellim et al. 2008)
			54+ 81	140	Colombian	(Henao, Montes et al. 2006)
		None	400	400	Gambian	(Bellamy, Ruwende et al. 1998)
			871	459	Korean	(Shin, Park et al. 2005)
			100+125 (PPD^+)	113	Spanish	(Lopez-Maderuelo, Arnalich et al. 2003)
			100+ 82 (PPD^-)			
			188	166	South Indian	(Selvaraj, Alagarasu et al. 2008)
TNF-α	−592 (A/C)	None	111	183	Chinese	(Wu, Qu et al. 2008)
	−819 (C/T)					
	−308 (G/A)	None	120	210	South Indian	(Selvaraj, Sriram et al. 2001)
	−238 (G/A) & −376 (G/A)		106	358	Cambodian	(Delgado, Baena et al. 2002)
	−308 (G/A)	Protective			Sicilian	(Scola, Crivello et al. 2003)
	−308 A-238 G	Protective	430	135	Colombian	(Correa, Gomez et al. 2005)
TGF-β	Codon 10 (+869 T/C)	None	111	183	Chinese	(Wu, Qu et al. 2008)
			54+ 81	140	Colombian	(Henao, Montes et al. 2006)
	Codon 25 (+915 C/G)		110	101	Japanese	(Niimi, Sato et al. 2002)

Table 1.3: Selected studies showing VDR polymorphism association with TB

Gene	Polymorphism	Population	Cases	Controls	*P* value (P_c)	OR/RR	Reference
VDR	Various SNPs	Japan	87	265		NS	(Kusuhara, Yamamoto et al. 2007)
	ApaI	Tanzania	443	426		NS	(Soborg, Andersen et al. 2007)
		South Africa (Venda)	~85	~88		NS	(Lombard, Dalton et al. 2006)
		Guinea-Bissau	321	347		**0.03**	(Olesen, Wejse et al. 2007)

BsmI	South Africa (Venda)	~85	~88		N	(Lombard, Dalton et al. 2006)
	India	64	103		**2.2**	(Selvaraj, Kurian et al. 2004)
	Asia (Gujarati)	52	116		NS	(Wilkinson, Llewelyn et al. 2000)
FokI	Tanzania	443	426		NS	(Soborg, Andersen et al. 2007)
	South Africa (Venda)	~85	~88		NS	(Lombard, Dalton et al. 2006)
	India	64	103		**2.4**	(Selvaraj, Kurian et al. 2004)
	Asia (Gujarati)	52	116		**2.8**	(Wilkinson, Llewelyn et al. 2000)
	China (Han Chinese)	76	171		**3.67**	(Liu, Zhang et al. 2003)
	China (Han Chinese)	120	240	**0.03**	**2.35**	(Liu, Cao et al. 2004)
	Peru	103	206		**9.6**	(Roth, Soto et al. 2004)
FokI–BsmI–ApaI–TaqI	West Africa	382 trios		**0.009**		(Bornman, Campbell et al. 2004)
TaqI	Tanzania	443	426		NS	(Soborg, Andersen et al. 2007)
	South Africa (Venda)	~85	~88		NS	(Lombard, Dalton et al. 2006)
	Cambodia	358	106		NS	(Delgado, Baena et al. 2002)
	Asia (Gujarati)	52	116		NS	(Wilkinson, Llewelyn et al. 2000)
	Peru	103	206		**5.6**	(Roth, Soto et al. 2004)
	Gambia	408	414	**0.01**	**0.53**	(Bellamy, Ruwende et al. 1999)

1.5.3 Polymorphisms in cell surface receptors

1.5.3.1 Human leukocyte antigens

The human leukocyte antigen (*HLA*) region is situated on the short arm of chromosome 6 and comprises approximately 200 genes, most of which are involved in antigen processing and presentation. *HLA* genes are highly polymorphic across populations, suggesting a role of selection pressure against infectious agents (Lombard, Brune et al. 2006). Associations of HLA with susceptibility to infectious diseases like malaria, hepatitis B and C have been reported earlier (Yee 2004; Hill 2006). *HLA* could be one of the important genetic determinants responsible for different clinical outcome of the same infection in different individuals because of different allelic variants which participate in immune response to infection and determine

susceptibility or resistance to infectious disease (Singh, Agrawal et al. 1997). Mounting of efficient immune response against infectious agents involves the presentation of microbial peptides associated with HLA molecules to a repertoire of T cells. This repertoire includes clones that can be activated to proliferate and differentiate upon recognition of the peptide-HLA complex with high affinity. A person with a particular allele combination which does not bind appropriately with microbial peptides crucial for eliciting a protective immune response, or inadequately activates naïve T cells is more susceptible to infection than those without such deficiencies (Klein and Sato 2000). The locus includes two alleles, i.e., *HLA* I and II. *HLA* I consist of *A, B* and *C* alleles respectively that present pathogen-derived peptides generated in the cytosol to cytotoxic CD8+ T cells. HLA II consists of *DP*, *DQ* and *DR* respectively that present antigenic peptides generated in the phagosomal compartment, to helper CD4+ T cells. In TB, both CD4+ and CD8+ cells have important functions in the generation of a protective immune response (Flynn and Chan 2001).

HLA allele polymorphisms have been reported by various investigators to be correlated with TB susceptibility (Geluk, Taneja et al. 1998). HLA genes were investigated in several TB case-control studies and were among the first and still commonly reported genes associated with TB susceptibility. Various studies have consistently reported association of *HLA DR2* with TB. These include, for instance, studies in India (Singh, Mehra et al. 1983; Selvaraj, Uma et al. 1998; Selvaraj, Uma et al. 1998; Ravikumar, Dheenadhayalan et al. 1999), Thailand (Vejbaesya, Chierakul et al. 2002), Indonesia (Bothamley, Beck et al. 1989), and Russia (Khomenko, Litvinov et al. 1990). Mehra *et al* reported higher frequency of *DRB1*1501* (*DR2* allele) in TB patients in comparison to *DRB1*1502* in patients with tuberculous (TT) leprosy. They found significantly lower frequencies of the haplotype *DRB1*1501-DRB5*0101-DQA1*0102-DQB1*0502* in TT leprosy and a significant increase of *DRB1*1501-DRB5*0101-DQA1*0103-DQB1*0601* in pulmonary TB patients (Mehra, Rajalingam et al. 1995). Rajalingam *et al* have shown prevalence of *HLA DR2* in TB patients with severe and multibacillary form of TB as well as its association in patients in the drug failure group (Rajalingam, Mehra et al. 1996). Studies in Cambodia have reported association of *HLA DQB1*0503* with pulmonary TB (Goldfeld, Delgado et al. 1998). Studies in a Venda

population by Lombard *et al* have shown the association of *DRB1*1302* with TB susceptibility (Lombard, Dalton et al. 2006). A study in Poland found association of HLA-DR16(2) antigen with the risk of developing TB whereas HLA-DR13(6) antigen had a protective role (Dubaniewicz 2000). In India HLA, the DPB1* 04 allele was found to associate with resistance to TB (Ravikumar, Dheenadhayalan et al. 1999).

1.5.3.2 Vitamin D receptor (*VDR*)

Even before the discovery of Mtb by Koch, vitamin D was used in several forms for treatment of TB and a vitamin D rich diet plus sunlight was the basis of sanatorium treatment (Evans 1994; Zasloff 2006). The increased susceptibility of more pigmented human phenotypes to TB may be due to higher melanin content which blocks skin exposure to ultra violet light specially when living in low sunny areas (Wilkinson, Llewelyn et al. 2000). Vitamin D concentration was also low in the serum of TB patients (Davies, Brown et al. 1985; Wilkinson, Llewelyn et al. 2000). Increased expression of VDR on activated lymphocytes and monocyte shifted the focus on role of vitamin D3 as immunoregulator (Chan 2000; Hayes, Nashold et al. 2003). Vitamin D3 acts as an immunomodulatory hormone and mediates its action through VDR by activating monocytes, resulting in suppression of mycobacterium growth (Crowle, Ross et al. 1987; Chandra, Selvaraj et al. 2004; Liu, Stenger et al. 2006). The possible mechanism is either increased nitric oxide (NO) synthesis (Rockett, Brookes et al. 1998; Waters, Palmer et al. 2004), increased expression of reactive oxygen intermediate (Sly, Lopez et al. 2001) or increased expression of anti microbial peptides like cathelicidin (Liu, Stenger et al. 2006). The study by Liu *et al* has shown association of lower serum 25D level with TB susceptibility (Liu, Stenger et al. 2006). Activation of TLR 2/1 gene in humans resulted in induction of genes in the vitamin D pathway, including VDR and 25-hydroxyvitamin D3-1a-hydroxylase (CYP27b1). The level of 25D in the induction of host defense response through TLR2/1 pathway supported clinical observation of higher TB incidence rates in black people and people with lower serum 25D levels. However current studies have pointed toward pleiotrophic effect of vitamin D3 with increased localized innate immune response, decreased Th1 immunity and promotion of Th2 cells.(Gauzzi, Purificato et al. 2005).

The *VDR* gene is found in chromosome 12q13 region. Several polymorphisms on *VDR* were initially considered to be associated with bone density and osteoporosis (Uitterlinden, Fang et al. 2004). However, studies have associated these polymorphism with osteoarthritis, cardiovascular disease, diabetes, cancer (Uitterlinden, Fang et al. 2004) and TB (Bellamy, Ruwende et al. 1999). Studies from different populations have found differences in susceptibility or resistance to TB and have focused on four polymorphisms that are identified by absence or presence of restriction enzyme site by restriction fragment length polymorphism (RFLP). These sites are *TaqI, BsmI, ApaI* and *FokI* respectively so that each polymorphism has 2 possible alleles i.e. *T/t, B/b, A/a, and F/f* (Bornman, Campbell et al. 2004).

A study carried out in Guajarati Indians in London has shown the association of *ff* genotype with pulmonary TB (Wilkinson, Llewelyn et al. 2000). Studies by Selvaraj *et al* in south Indian populations have shown higher frequency of the *tt* genotype in female TB patients (Selvaraj, Narayanan et al. 2000) while higher *Bb* and *FF* genotype frequency was reported in male patients (Selvaraj P 2003). Studies in a Venda population of South Africa have shown that although *VDR* SNP phenotypes were not associated with TB, but the haplotype *F-b-A-T* significantly protected from TB (Lombard, Dalton et al. 2006). Case-control studies in West Africa did not find any association with genotype. However, transmission disequilibrium test on the family based data found associations between TB and *FokI-BsmI-ApaI-TaqI* and the *FA* haplotype. Thedse observations support the view that there is a role of *VDR* haplotype, rather than individual genotypes, in susceptibility to TB (Bornman, Campbell et al. 2004). A family based association study in a native South Americans population found protective effect of the *Fok1 F* allele against infection and the *Taq1 t* allele from active disease, but not from infection (Wilbur, Kubatko et al. 2007) . These results were similar to a Gambian study where the *Taq1 tt* genotype was found less frequently in TB patients (Bellamy, Ruwende et al. 1999). In a Peruvian community, *VDR* variants were found to be associated with sputum culture and auramine stain conversion during anti TB treatment with patients possessing the *Fok1 ff* and/or the *Taq1 Tt* allele showing faster conversion rates (Roth, Soto et al. 2004).

1.5.3.3 Cytokines and their receptors

Immune response to TB is regulated by interaction between lymphocyte and antigen presenting cells, with cytokines playing important roles in anti-TB immune responses. The Th1/Th2 cytokine paradigm has attracted immunologist all over the word with the discovery of alternative activation of macrophage in TB patients. Many studies have looked for association of TB susceptibility with polymorphisms in promoter or coding regions of cytokine genes and their receptor which are important in controlling the infection (Oh, Yang et al. 2007; Naslednikova, Urazova et al. 2009). Mutations might alter the binding site for the transcriptional factor, affecting transcriptional activation and altering cytokine levels (Bidwell, Keen et al. 1999; Pravica, Asderakis et al. 1999).

In the majority of TB patients, innate response is not able to clear the infection and adaptive immune responses involving T helper lymphocyte develop via either the Th1 or the Th2 maturation pathway. The effectors cytokines of Th1 pathway are necessary to restrict bacterial growth which include TNF-α and IFN-γ and also include IL-12 (Van Crevel, Ottenhoff et al. 2002). The importance of IL-12 is highlighted by the fact that it induces lymphocytes to secrete IFN-γ and serves as important link between innate and adaptive immunity (Medzhitov and Janeway 1997).

IFN-γ

IFN-γ is a pleiotrophic cytokine with immunomodulatory activities that are crucial for regulation of immune response (Farrar and Schreiber 1993; Huang, Hendriks et al. 1993). IFN-γ induces the production of other cytokines, increases MHC-I and II expression, Fc receptors, RNI production, and increased Vitamin D3 productions which have immunomodulatory effects and leucocyte adhesion molecules. It augments Th1 cell expansion and may be required for Th1 differentiation (Chan, Kobayashi et al. 1992; Farrar and Schreiber 1993; Murray 1994; Wu, Kirman et al. 2002). IFN-γ also up regulates expression and synthesis of TNF-α which plays a pivotal role in granuloma formation and macrophage activation (Kindler, Sappino et al. 1989). It evokes ROS and RNI, inducible nitric oxide synthetase (*iNOS*) and cytokines such as TNF-α (Nathan, Murray et al. 1983; Chan, Kobayashi et al. 1992; Farrar and Schreiber 1993; Ehrt, Schnappinger et al. 2001). The role of IFN- γ in macrophage activation during TB

infection is well established in different experimental models (Flynn, Chan et al. 1993). There is decrease in IFN- γ level during active TB and its local and systemic levels correlate with the severity of disease. The exceptional susceptibility to TB in human with Mendelian susceptibility to mycobacterial disease MSMD), wherein mutations in the α and β chains of IFN-γ receptor (*IFNGR*), *IL12 p40* and *IL12 Rb1* genes provides evidence of the role of IFN-γ in controlling Mtb infection (Altare, Jouanguy et al. 1998).

Circulating IFN- γ level is genetically controlled and previous studies have shown that any variations in either the gene or its receptor affect the cytokine level or its signaling (Ottenhoff, Kumararatne et al. 1998; Casanova and Abel 2002). Several polymorphisms have been identified in the cytokine gene or genes for α and β chains of *IFNGR*. In vitro studies have reported higher cytokine level due to polymorphism in non coding region of the first intron region due to insertion of 12 CA microsatellite allele (Pravica, Asderakis et al. 1999). The 12 CA microsatellite is in linkage disequilibrium with *T* allele at +874 position from transcription start site which lies within binding site for NF-κB transcription factor (Pravica, Perrey et al. 2000). Electrophoretic mobility assays have shown that the presence of the *T* allele at this position leads to more specific binding resulting in higher IFN-γ expression while the *A* allele is associated with lower cytokine levels (Rossouw, Nel et al. 2003). Polymorphism at the +874 *A/T* allele has been linked with TB susceptibility in studies conducted in Siciliy (Lio, Marino et al. 2002), South Africa (Rossouw, Nel et al. 2003), Spain (Lopez-Maderuelo, Arnalich et al. 2003), and Hong Kong (Tso, Ip et al. 2005). A recent study by Ansari *et al* in Pakistan showed association of IFN-γ *AA* genotype in combination with IL-10 *GG* (low secreting genotype) in advanced stage pulmonary TB (OR= 5.26; *P*=0.005) and disseminated TB (OR= 3.59; *P*= 0.045) (Ansari, Talat et al. 2009). A case control study reported significant association between the IFN-γ *+874T/A* polymorphism and susceptibility to PTB in a sample of Iranian population (Hashemi, Sharifi-Mood et al. 2011). Similar studies in Tunisia have shown that the +874 *AA* genotype (low IFN-γ producer) was significantly associated with increased risk of developing of active pulmonary TB (OR ≥2.18; 95% CI). By contrast, the *AT* genotype was found to be significantly associated with resistance to pulmonary TB (OR = 0.46; 95% CI, 0.28-0.74; Pc = 0.0018) and extrapulmonary TB development (OR = 0.46; 95% CI) (Selma, Harizi et al.

2011). A meta-analysis of several population studies showed statistically significant protective association of +874 *TT* genotype (OR= 0.75, CI=95%) (Pacheco, Cardoso et al. 2008). A study in Japan reported significantly lower rates of culture conversion during TB treatment in patients of the *AA* genotype than that in patients with *AT* or *TT* genotypes (Shibasaki, Yagi et al. 2009). However, no association was noted in reports from Turkey (Oral, Budak et al. 2006), Malawi (Fitness, Floyd et al. 2004), Texas(Moran, Ma et al. 2007) and West Africa (Cooke, Campbell et al. 2006).

Several polymorphisms in the IFN-γ receptor have been studied with respect to TB susceptibility but very few reported association (Fraser, Bulat-Kardum et al. 2003; Awomoyi, Nejentsev et al. 2004; Park, Im et al. 2004; Cooke, Campbell et al. 2006; Mirsaeidi, Houshmand et al. 2006; Sahiratmadja, Baak-Pablo et al. 2007). The polymorphism reported to be associated with development of TB was the genotype of 56*CC* in the promoter region (Cooke, Campbell et al. 2006) and the cytosine –adenine repeats polymorphism on intron 1(Ding, Li et al. 2008).

Thus it seems that lower levels of IFN-γ might affect Mtb replication and affect disease outcome (Etokebe, Bulat-Kardum et al. 2006). The association of +874*AA* genotype with TB susceptibility in various populations suggest the significant role of genetic variation at IFN-γ locus and strengthen the concept of genetic susceptibility and the disease

TNF-α

TNF-α, aTh1 cytokine, exist both in membrane bound and soluble forms, and exerts its action through its receptor p55 and p75 (Winthrop 2006). TNF-α plays important role in regulation of TB pathology (Stenger 2005). Upon infection with Mtb , there is release of TNF-α by macrophages, dendritic cells and NK cells (Flynn and Chan 2001). It helps in the recruitment of inflammatory cells at the site of infection by induction of various chemokines and vascular adhesion molecules (Ehlers 2005). TNF-α mediates killing of Mtb by both RNI dependent and independent pathways, in conjunction with IFN-γ (Bekker, Freeman et al. 2001; Ehlers 2005). It also mediates apoptosis of infected macrophages and thus indirectly reduces bacterial burden (Keane, Balcewicz-Sablinska et al. 1997). In TNF-α deficient mice, there is delay in recruitment of inflammatory cells at the infection site, and if even recruited, these are

not able to contain the infection (Roach, Bean et al. 2002). Administration of exogenous TNF-α leads to reduction in logarithmic growth of Mtb in TNF-α knockout mice (Bekker, Freeman et al. 2001; Hehlgans and Pfeffer 2005). A study by Kramp *et al* demonstrated the in vivo immunomodulatory effect of TNF-α injection in BCG vaccinated guinea pig (Kramp, McMurray et al. 2011). Treatment with recombinant guinea pig TNF-α reduced CFU count, increased skin test response to PPD and also increased PPD induced proliferation in the lymph node six week after injection. Neutralization of TNF-α using an antibody led to immune dysregulation and improper enhancement of antibody titres (Lasco, Cassone et al. 2005). Autophagy plays an important role in clearance of intracellular Mtb and is enhanced by TNF-α and IFN-γ (Ni Cheallaigh, Keane et al. 2011). Other studies have shown that TNF-α prevents reactivation of persistent TB, modulates immunogenic factors at the site of infection and limits the pathological response of host (Mohan, Scanga et al. 2001). Its importance in TB disease control is exhibited by the observation that patients treated with the anti- TNF-α antibody infliximab develop active TB (Keane 2005). Thus TNF-α play the role of a double edged sword, i.e. apart from controlling infection it also causes immunopathology and severe tissue damage.

TNF-α production by PBMC is remarkably constant in healthy humans, although level vary greatly (Jacob, Fronek et al. 1990). Two polymorphisms have been identified that influence cytokine production level-a biallelic SNP at promoter sequence -308 (*G/A*) and another *G→A* transversion in the first intron region (+252) of lymphotoxin α (*LTA*) (Meenagh, Williams et al. 2002). Higher level of TNF-α has been linked to -308*A* and +252*A* in *LTA* when compared between heterozygous and homozygous individuals (Pociot, Briant et al. 1993; Roberts, Monzon-Bordonaba et al. 1999).

However, there is conflicting evidence about the association of polymorphism in *TNF-α* gene and TB. Studies in Sicily have found a smaller number of -308*GG* genotype among TB patients (Scola, Crivello et al. 2003) while in Columbian study, the -308*A*-238*G* haplotype was protective (Correa, Gomez et al. 2005). No evidence of association however, was reported from studies in India (Sharma, Rathored et al. ; Selvaraj, Sriram et al. 2001), Cambodia (Delgado, Baena et al. 2002), Korea (Oh, Yang et al. 2007) and Turkey (Ates, Musellim et al. 2008). A

study in an Iranian population did not find significant differences between patients and controls with respect to -308 *A/G* polymorphism, though the frequency of -308*A* allele was higher in TB patients (Merza, Farnia et al. 2009). A meta-analysis by Wang *et al* showed that polymorphism at the -308 position in the promoter sequence is not associated with TB susceptibility. However significant association of -308*A* allele was found among Asian patients than Caucasians (Wang, Zhan et al. 2011).

IL-12

IL-12 is produced mainly by macrophages and its production is stimulated by phagocytosis (Fulton, Johnsen et al. 1996; Ladel, Szalay et al. 1997). The importance of IL-12 can be depicted by the fact that it is the dominant cytokine in the production and maintenance of Th1 immunity and induces IFN-γ production (O'Neill, Matthews et al. 1997). Higher IL-12 levels were found in patients of TB pleuritis, a self healing form of disease (Zhang, Gong et al. 1994). It is heterodiameric (IL-12p70) protein having proinflamatory activity and is produced by monocytes, macrophages, dendritic cells and B lymphocytes and regulates Th1 differentiation. IL-12 synergizes with other activating stimuli like TNF-α and IL-1 to induce IFN-γ production. Studies in knockout mice have shown that *IL-12*-deficient mice are more susceptible to infection with *M. bovis* bacille Calmette-Guérin (Wakeham, Wang et al. 1998) and Mtb than are wild-type mice (Cooper, Magram et al. 1997). Moreover, *IL12B*-deficient mice develop more severe mycobacterial infection than do IL12A-deficient mice (Cooper, Kipnis et al. 2002).

A study in Hong Kong showed association between polymorphism in *IL-12B* intron 2 and TB and also between a specific haplotype and TB (Tso, Lau et al. 2004). In Indian leprosy and TB patients, polymorphism in promoter region and 3'UTR region associated with higher cytokine level, was more prevalent among patients than controls (Morahan, Kaur et al. 2007). Both case control and family based association studies in Russia suggested *IL12B* 1188*A/C* polymorphism as a factor of common susceptibility to Th1-mediated infectious disorders due to intracellular bacteria. The *C* allele was more common in TB patients (Freidin, Rudko et al. 2006). In studies on the *IL-12BR1* gene, polymorphism in *IL12BRβ1* coding sequence have been reported to be associated with TB susceptibility in Japanese (Akahoshi, Nakashima et al.

2003) and Moroccan patients (Remus, El Baghdadi et al. 2004), though the associated SNP was different. However, no association was found between IL12 receptor β1 gene polymorphism and TB in Korean patients (Lee, Lee et al. 2005).

IL-6

Il-6 is a multifunctional cytokine showing proinflammatory activity along with TNF-α and IL-1 (Akira and Kishimoto 1992). It is involved in the regulation of T and B- cell responses (Kopf, Baumann et al. 1994) and also participates in hematopoeisis (Van Snick 1990). Though both IL-6 and Il-10 use the STAT3 pathway to exhibit their effects, it is the Supressor of Cytokine Signaling-3 (SOCS3) which plays a key role in the divergent action of these two cytokines (Yasukawa, Ohishi et al. 2003). The common response to bacterial infection includes increase in IL-6 leading to M1 polarization. Though infection with virulent strain of Mtb leads to increase in IFN-γ level, the macrophage is unable to kill the bacteria because of transcriptional inhibition of IFN-γ through a bystander effect involving IL-6 (Ting, Kim et al. 1999; Nagabhushanam, Solache et al. 2003). A study by Saunders *et al* in IL-6-/- mice showed early increase in Mtb burden in the lung with concomitant decrease in early upregulation of IFN-γ (Saunders, Frank et al. 2000). Blockade of IL-6 recptor in mice led to increase in intracellular burden of Mtb, though the increase was far less when compared to TNF-α receptor blockade (Okada, Kita et al. 2011).

IL-10

IL-10 is also a pleiotropic cytokine. It suppresses Th1 activity and also negatively regulates IFN-γ production, secretion of TNFα, nitric oxide production, expression of co-stimulatory molecules and MHC-II expression on macrophage (Moore, de Waal Malefyt et al. 2001). IL-10 is produced by macrophages after Mtb phagocytosis through binding of LAM. It is a major regulator of innate immunity and interferes with proinflammatory cytokine production by immune cells and increases the expression of molecules that support anti-inflammatory effects (Moore, de Waal Malefyt et al. 2001). IL-10 regulates adaptive immune response by inhibiting IL-12 and IL-18 production and decreasing MHC-II expression on antigen presenting cell (Moore, de Waal Malefyt et al. 2001). Several reports have indicated increased IL-10 level during disease (Torres, Herrera et al. 1998; Lee, Song et al. 2002). IL-10

production is higher in TB patients than in PPD+ persons (Demissie, Abebe et al. 2004). It is higher in tissue from patients with leprotomous leprosy than patients of tuberculoid leprosy suggesting Th2 polarization (Yamamura, Uyemura et al. 1991). Comparsion of cytokine profiles of Mtb infected macrophages and dendritic cells showed the biased polarization with dendritic cells displaying Th1 phenotype while macrophages failed to produce to IL-12 and produced higher IL-10 (Hickman, Chan et al. 2002). Studies by Patel *et al* showed higher IL-10 in HIV+ positive TB patients and impairment of Mtb mediated apoptosis through a *BCL-3* dependent mechanism (Patel, Swan et al. 2009). There was decreased Mtb load in the lungs of infected *IL-10*$^{-/-}$ mice and was preceded by accelerated and enhanced IFN-γ response in the lung (Redford, Boonstra et al.).

Mice over-expressing IL-10 did not display increased susceptibility to TB, but, instead showed a reactivation susceptible phenotype (Turner, Gonzalez-Juarrero et al. 2002). Studies in monozygotic twins have shown that 75% of the variability in IL-10 cytokine level can be attributed to genotype (Westendorp, Langermans et al. 1997). Three single base substitutions have been reported in the *IL-10* promoter region at positions -1082 *G-A*, -819 *T-C* and -592*A-C* from the transcription start site (Kube, Platzer et al. 1995). The -1082 base substitution site is located within the E-twenty-six specific (Ets) –like recognition site and affects binding of transcription factors. Variation in promoter strength thus occurs, affecting transcriptional activation and hence altered protein level. The allele *A* at -1082 is associated with low cytokine level, while *G* is associated with higher production by PBMC in culture (Turner, Williams et al. 1997)

A case control study in South Korean adults found over representation of IL-10 -1082 *A* among TB patients, though there was no significant difference between genotype frequencies in newly diagnosed TB versus reoccurring TB (Oh, Yang et al. 2007). However, studies in Cambodia (Delgado, Baena et al. 2002), Sicily (Shin, Park et al. 2005) and Turkey (Ates, Musellim et al. 2008) reported higher occurrence of the *G* allele at -1082 SNP in TB patients than controls. In a Columbia population, the -1082*A* allele was reported at higher frequency in pleural TB patients (Henao, Montes et al. 2006).

A case control study in China found no association between -819*C/T* SNP and TB (Ma, Xie et al. 2011). However, another study found increased frequency of both -819*T* and -592*A* alleles in pleural TB patients. SNPs at -1082*G/A*, -819*C/T* and -592*A/C* were associated with IL-10 levels in the pleural fluid of TB patients while only-1082*G/A* polymorphism was associated with the IL-10 level in the control group (Liang, Zhao et al. 2011). A meta-analysis performed to find association between IL-10 polymorphisms at -1082*G/A*,-819*C/T* and -592*A/C* and TB did not find any risk in combined analysis, though subgroup analysis by ethnicity found that -1082*G/A* could be a risk factor for TB in Europeans (Zhang, Chen et al. 2011). A study in Egyptian children with TB did not find any significant association between -1082 *GG* genotype and TB, though increased *GG* genotype frequency was observed among patients (Mosaad, Soliman et al. 2011). No association was found between -1082 *G/A* SNP and TB in studies in Gambia (Bellamy, Ruwende et al. 1998), South India (Selvaraj, Alagarasu et al. 2008), Spain (Lopez-Maderuelo, Arnalich et al. 2003) and Korea (Shin, Park et al. 2005).

IL-4

IL-4 is produced mainly by activated T cells and promotes proliferation and development of B cells (Arai, Lee et al. 1990; Abe, De Waal Malefyt et al. 1992). IL-4 is a Th2 cytokine and exerts anti-inflammatory effects by decreasing Th1 cytokine production. In Mtb-infected mice, the progression of disease and reactivation of latent infection was associated with increased IL-4 production (Hernandez-Pando, Orozcoe et al. 1996; Howard and Zwilling 1999). Mice exhibited increased host resistance to TB and improved IgA production upon IL-4 depletion using antibody to IL-4 (Buccheri, Reljic et al. 2007).

IL-4 production is partially regulated by a SNP at -589 *C/T* lying in the promoter region, leading to increased promoter strength and hence increased cytokine level (Rosenwasser, Klemm et al. 1995) and also increased level of immunoglobulin IgE (Luoni, Verra et al. 2001). Very few studies have reported positive association between IL-4 gene variants and TB (Vidyarani, Selvaraj et al. 2006), while studies in Iran reported significant negative association between TB, IL-4 *T* allele and *TT* genotype, with *CC* and *CT* genotype more common among patients (Amirzargar, Rezaei et al. 2006). A multicase TB family study

in Brazil found no association between guanine –thymine dinucleotide repeat in intron3 and 70 base pair in intron2 (Mout, Willemze et al. 1991).

1.6 SNP effects on cytokine secretion

The effect of genotype on secretion of the relevant cytokine is summarized in Table 1.4

Gene SNP	Low producing Genotype	Intermediate producing Genotype	High producing Genotype
IFN-γ +874 A/T	AA	AT	TT
TNF-α -308 G/A	GG	GA	AA
IL-10 -1082 A/G	AA	AG	GG
IL-4 -590 C/T	CC	CT	TT

1.7 Scope of the study

Infection of macrophages with Mtb leads to cell activation which results in bacterial clearance while some of the remaining bacteria enter into latency or dormancy. Their reactivation occurs when immunity goes down and leads to active clinical disease. It is the quality and magnitude of the immune response which decides whether the infected individual will remain healthy in spite of harboring the pathogen or will reactivation occur leading to disease. Further, genetic diversity not only affects the host-pathogen interaction, but also the host response to drug treatment.

Though anti-TB drugs are available in the market for last 40 years, there is increase in incidence of TB particularly in association with the HIV pandemic. In the last few years, with the emergence of drug resistant strains (MDR, XDR), there is greater urgency of developing new drugs and therapeutic strategies for TB control. Since there is a large gap between new drug discovery and release there is scope for development of efficient drug delivery systems for existing drugs, increasing drug bioavailability and efficacy while reducing drug dose and toxicity. We (Sharma, Saxena et al. 2001; Muttil, Kaur et al. 2007; Sharma, Muttil et al. 2007; Kaur, Muttil et al. 2008; Verma, Kaur et al. 2008) and others (O'Hara and Hickey 2000; Dutt

and Khuller 2001; Suarez, O'Hara et al. 2001; Sethuraman and Hickey 2002; Ul-Ain, Sharma et al. 2003; Makino, Nakajima et al. 2004; Tian, Klegerman et al. 2004; Yoshida, Matumoto et al. 2006; Hasegawa, Hirota et al. 2007; Lu, Garcia-Contreras et al. 2007) have proposed an alternative drug delivery system, namely, microparticles containing anti-TB agents for pulmonary delivery to lung and alveolar macrophages. The extremely high efficacy of this drug delivery system (Suarez, O'Hara et al. 2001; Lu, Garcia-Contreras et al. 2007; O'Sullivan, O'Leary et al. 2007) can be best understood from the point of view of (1) efficient drug targeting to the exact site of infection, and (2) classical, bactericidal activation of the infected macrophage. We have shown that microparticles containing isoniazid and rifabutin can rescue Mtb-infected human macrophages from 'alternative activation' imposed upon the host by *Mtb*. (Yadav, Muttil et al. 2010).

1.7.1. Research Problem and Objectives:

Previous studies in cell lines are limited in scope, since they investigated the response of cultured cells of a single genetic background to infection with Mtb and treatment with anti TB drugs. Human genotypes are hugely diverse. Some persons are more 'susceptible' to infection, and show variable responses to drug treatment, while others are more 'resistant' to infection and responsive to treatment. The genesis of the present project lies in the question whether macrophages obtained from individuals of various genotypes would display similar or different patterns of alternative and classical activation in response to Mtb infection and treatment with anti-TB drugs in soluble form or drug-containing microparticles. Peripheral blood mononuclear cells (PBMC) from healthy volunteers were differentiated to obtain monocyte-derived macrophages (MDM), and infected in vitro with Mtb strain H37Rv. Treatment and subsequent analysis of responses of MDM from 'susceptible' versus non susceptible volunteers was then used to ask: (a) whether the efficacy advantage displayed by inhalable microparticles in infected THP-1 cells would be still be observed in MDM of other genotypes;. (b) whether 'rescue' from alternative activation would apply to MDM of diverse genotypes, and (c) whether volunteers could be stratified in terms of 'susceptibility' and 'resistance to Mtb infection on the basis of the progress of infection and immune response markers displayed by diverse MDMs in the experimental system employed. A related objective

was to study effects on bacterial survival and cytokine secretion profile due to difference in genetic background upon infection and different forms of treatment. During the course of work, genotypes of healthy volunteers were determined, and were stratified into different groups according to an arbitrary susceptibility and resistance score.

Experiments performed to study the objectives mentioned above are listed below:

- ✓ Determination of volunteer’s genotype using PCR-SSP, RFLP, and ARMS- PCR
- ✓ Assignment of arbitrary susceptibility “S” and resistance “R” scores based upon the number of factors present or absent and segregation of based on score
- ✓ Isolation of monocytes from blood using CD14 magnetic beads from selected volunteers of representative groups
- ✓ Cell culture for 5-7 days to differentiate into macrophages, infection with *Mycobacterium tuberculosis* H37Rv at 10 MOI for 3 hrs and treatment with anti TB drugs isoniazid (INH) and rifabutin (RFB) at 3µg/ml, either in microparticulate form or as drugs in solution for 2hrs
- ✓ Isolation of RNA at different times post treatment and establishment of expression profiles of selected genes by quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)
- ✓ Genome wide transcription profiling in THP- 1 monocyte ce1l line by Illumina microarray
- ✓ Assessment of bacterial survival in terms of colony forming unit and Bactec assay
- ✓ Delineation of M1/M2 cytokine responses and any correlation with host genotype, S score, R score and magnitude of response using cell culture supernatant
- ✓ Nitric oxide estimation in cell culture supernatant using Geiss reagent

Chapter 2

Materials & Methods

Materials and methods

2.1 Study Population

The present study was carried out in 52 apparently healthy volunteers drawn from contacts of students and staff of the Department of Pulmonary Medicine, Chhattrapati Shahuji Maharaj Medical University (CSMMU, formerly, KGMC), Lucknow or CSIR-CDRI, Lucknow. Some volunteers had regular contact with patients diagnosed with active TB by CSMMU; others were not exposed either to TB patients or mycobacteria in culture. All volunteers were residents of Lucknow during the study period, and provided informed consent (see Appendix 1 for consent form). The study population can thus be divided into two groups: -

a. Those with regular exposure to Mycobacteria in the workplace,

b. Those who were not in direct contact with TB patients Mycobacteria in the workplace.

The study protocol was approved by the Institutional Ethics Committees of the CSIR-CDRI, and CSMMU, Lucknow, India. All the subjects were apparently healthy at the time of blood collection with age ranging from 25-45 years and included 7 females and 45 males.

2.2 HLA typing

HLA DR2 and the allele *DRB1*1501* were determined in volunteers by polymerase chain reaction (PCR) amplification with sequence-specific primers. The method was based on the procedure developed by Olerup and Zetterquist (Olerup and Zetterquist 1992). Each sample was typed twice and repeated in case of any discordant result. Briefly, genomic DNA was isolated from whole blood using the Qiagen DNA extraction kit according to the manufacturer's protocol and quality was checked by agarose gel electrophoresis. PCR was carried out for *HLA DR2* and *HLA DRB1*1501*. To monitor the PCR reaction for each sample, the 3rd intron of *HLA DRB1* was amplified as a positive control. The primers and annealing conditions are mentioned in Table 2.1. A 50 µl reaction was set up with 100 ng genomic DNA; 0.5µM of each primer; 200 µM dNTP (MBI Fermentas); 1.5mM $MgCl_2$ (Promega); 1X PCR buffer and 2.5 U Taq polymerase (Promega). The PCR conditions were as follows: initial denaturation at 94°C for 5

minute, followed by 35 cycles of 1 minute at 94°C, 30 sec at 63°C for annealing, and extension at 72°C for 1minute. The last step included final extension for 7 minutes at 72°C. The PCR product was identified by 1.5% agarose gel electrophoresis.

Table2.1: Primer sequence and annealing temperature for HLA primers

Gene	Forward primer	Reverse Primer	Annealing temp (°C)
DRB1* 1501	5'-CCG CGC CTG CTC CAG GAT-3'	5'-TCC TGT GGC AGC CTA AGA G -3'	63 °C
DR2	5'-TTC CTG TGG CAG CCT AAG AGG-3'	5'-CCG CTGCACTGTGAA GCTCT-3'	63 °C
DRB1	5'-CGG ATC CTT CGT GTC CCC ACA GCA CG-3′	5′-TCGCCGCTGCACTGTGAAG-3′	63 °C

2.3 VDR Typing

Genotypes for Vitamin D receptor were determined by amplification of sequences containing reported VDR restriction fragments amenable to enzymatic digestion of the amplified bases with *TaqI, BsmI, ApaI* and *FokI* restriction enzymes (RE). The primer sequences and annealing temperatures are stated in Table 2.2 and the PCR mixture composition in Table 2.3.

Table 2.2: Primer sequence and annealing temperature for VDR primers

Gene	Forward primer	Reverse Primer	Annealing Temperature	Reference
BsmI	5'– CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA –3'	5'– AAC CAG CGG GAA GAG GTC AAG GG – 3'	63°C	(Morrison, Qi et al. 1994)
TaqI and ApaI	5'– CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA – 3'	5'– CAC TTC GAG CAC AAG GGG CGT TAGC – 3'	63°C	(Sainz, Van Tornout et al. 1997)
FokI	5'– AGC TGG CCC TGG CAC TGA CTC TGC TCT – 3'	5'– ATG GAA ACA CCT TGC TTC TTC TCC CTC – 3'	60°C	(Harris, Eccleshall et al. 1997)

Table 2.3: PCR Mixture for VDR gene amplification

Stock	Ingredient	Final Concentration	Volume (µl)
5X	*Taq* Buffer	1X	5
25mM	$MgCl_2$	1mM (2 mM)	1 (2 µl for *ApaI* and *TaqI*)
10mM each	dNTP Mix	0.2mM	0.5
5 µlM	Forward Primer	0.5 µl M	2.5 l
5 µM	Forward Primer	0.5 µl M	2.5
5 U/ µl	*Taq* Polymerase		0.3

	Nuclease free water		13.2 (12.2 µl for *ApaI* & *TaqI*)
Total Reaction Volume			**25** µl

Cycling conditions for all reactions involved 35 cycles with an initial denaturation step at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension 72°C for 60 s (for *ApaI* and *TaqI*, extension time was 90 s) and finally 7 min extension at 72°C. With *TaqI, BsmI, ApaI* and *FokI*, the respective genotypes are henceforth identified as T, B, A and F (indicating the absence of the restriction site) or t, b, a, f (indicating the presence of the restriction site). The reaction conditions and resulting identification of genotype are detailed in Table 2.4

Table 2.4: RFLP details

Restriction Enzyme	Incubation Temperature	Amplified Fragment Size (Base pair)	Restriction Fragment Size (Base pair)	Assigned Genotype
TaqI	65 °C	2000	1800, 200	TT, Tt, tt
BsmI	37°C	825	650, 175	BB, Bb, bb
ApaI	30°C	2000	1700, 300	AA, Aa, aa
FokI	37°C	345	196, 69	FF, Ff, ff

Ten microlitres of the PCR product were restriction-digested with 10 units of the respective enzymes in a 20 µl reaction for 3 hours and the enzyme-digested product was electrophoretically run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide for 45–60 min at 80 V along with phiX DNA digested with *Hae*III (MBI, Fermentas) as marker.

2.4 Cytokine gene polymorphism

The base exchange polymorphism at +874 of the IFN-γ gene ,-308 of TNF-α gene,-590 of IL-4 gene and -1082 of IL-10 gene was studied by amplification refractory mutation system-PCR (ARMS-PCR). The ARMS method is a simple and rapid method of detecting point mutation, simple insertion or deletion of small sequences or restriction fragment length polymorphism (RFLP) and was first described by Newton *et al* (Newton, Graham et al. 1989). Genomic DNA was amplified using DNA polymerase in two different PCR reactions; each reaction contained one generic specific antisense primer and one of the two allele specific

primers. Each PCR reaction was carried out in 25 µl volumes containing 200 ng DNA, 0.2 mM dNTP mix, 1.5mM $MgCl_2$, 1nM of allele specific primer and 0.2 nM of generic specific antisense primer and 1 unit Taq polymerase. To monitor PCR amplification, one internal control of 426 bp of the human growth hormone gene was amplified in each reaction mixture. In the case of IL 10 -1082 polymorphism, *HLA DRB1* was used as internal control. The primers and PCR conditions are summarized in Table 2.5

Table 2.5: Primer Sequences and PCR Conditions for cytokine gene amplification

Gene Polymorphism	Primer Sequences and PCR condition	Amplicon size	Reference
IFNG +874(A/T)	Common primer: 5′-TCAACAAAGCTGATACTCCA-3′, T allele primer: 5′-TTCTTACAACACAAAATCAAATCT-3′ A allele primer:5′-TTCTTACAACACAAAATCAAATCA-3′ 1 cycle: 95°C 1 minute 10 cycles: 94°C 30 sec, 62°C 50 sec, 72°C 40 sec, and 20 cycles: 94°C 20 sec, 56°C 50 sec, 72°C 40 sec	261 base pair	(Pravica, Perrey et al. 2000)
IL10-1082(G/A)	Common primer: 5′- CAGCCCTTCCATTTTACTTTC -3′, G allele primer: 5′-TACTAAGGCTTCTTTGGGAG-3′, A allele primer: 5′-CTACTAAGGCTTCTTTGGGAA-3′, 30 cycles: 94°C 30 sec, 56°C 30 sec, 72°C 30 sec	550 base pair	(Huang, Zhou et al. 1999)
TNFα-308(G/A)	Common primer: 5′-TCTCGGTTTCTTCTCCATCG-3′, G allele primer: 5′-ATAGGTTTTGAGGGGCATGG-3′, A allele primer: 5′-ATAGGTTTTGAGGGGCATGA-3′, 30 cycles: 94°C 30 sec, 61°C 2.5 min, 72°C 1 min	184 base pair	(Verjans, Brinkman et al. 1994)
IL4-590 (C/T)	Common primer: 5’-CACTTGGGGCCAATCAGCA-3’ C allele primer: 5’-CTAAACTTGGGAGAACATTGTC-3’ T allele primer: 5’-CTAAACTTGGGAGAACATTGTT-3’ 30 cycles: 94°C 30 sec, 62.5°C 1.5 min, 72°C 1 min	447 base pair	(Borish, Mascali et al. 1994)
HGH (Control primer)	Forward primer: 5’- GCCTTCCCAACCATTCCCTTA -3’ Reverse primer: 5’- TCACGGATTTCTGTTGTGTTTC -3’	429 base pair	(Bunce, O'Neill et al. 1995)
DRB1 (Control primer)	Forward 5′-CCG GAT CCT TCG TGT CCC CAC AGC ACG-3′ Reverse 5′-TCG CCG CTG CAC TGT GAA G-3′.	292 base pair	(Rajeswari, Selvaraj et al. 2007)

2.5 PBMC isolation from blood of healthy donors and monocyte seperation

CD14^{+} monocytes were isolated from peripheral blood from healthy donors by Ficoll-Hypaque, selected with anti-CD14 MACS beads (Miltenyi Biotech, Auburn, CA), and cultured in the presence of M-CSF or phorbol myristate acetate (PMA). Briefly, peripheral blood

mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by density gradient centrifugation using Histopaque 1077 (Sigma, USA). Following the manufacturer's protocol, 5 ml of blood was gently overlaid on an equal volume of Histopaque in 15 ml centrifuge tubes and spun at 400 X g for 40 min at 25°C. Cells at the interface of plasma and Histopaque were carefully collected. Two volumes of 1X PBS were added to the cells and centrifugation carried out at 250 X g for 10 min. Ten millilitres of 1X PBS were then added and again centrifuged at same speed. Supernatant was carefully discarded and cells were suspended in 10 ml 1X PBS and cell number determined by counting using a Neubauer haemocytometer. Monocytes were isolated using CD14 magnetic beads. Briefly, cells were centrifuged again at 300 X g for 10 minutes and the supernatant was aspirated. Cells were suspended in 80 µl of buffer containing 1X PBS, 2mM EDTA and 0.5% FBS and 20 µl of CD14 microbeads were added and mixed well. The mixture was incubated for 15 minutes in the refrigerator. Two ml of buffer was then added and centrifuged for 10 minutes at 300 X g. The supernatant was aspirated and cells were suspended in 500 µl of buffer. The MS column was then placed in the magnetic separator and rinsed with 500 µl of buffer and then the cellsuspension was added. The column was then washed with 500 µl of buffer thrice and bound cells were flushed out by taking the column out of the magnetic separator. One ml of buffer was added and the plunger was pushed firmly to flush the cells. The cells were counted, suspended in RPMI medium supplemented with 20% autologous plasma and 50ng/ml MCSF (R&D systems, USA) or xnM of PMA for y hrs. One hundred thousand cells per wells were then seeded in 96 well tissue culture plates. Half of the medium was replaced after three days with complete RPMI medium and cells were cultured for 6 days to convert them to monocyte derived macrophages (mMDM).

2.5.1 THP-1 cell culture

THP-1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, USA). Cells were differentiated to macrophage phenotype using established protocol in our lab (Yadav, Muttil et al. 2010). Briefly, the cells were treated with 20 nM phorbol myristate acetate (PMA, Sigma) per mL of medium for 14 hrs and then washed three times with incomplete medium. Fresh medium without antimycotic and antibiotic was added and cells cultured for another 10 hrs. This procedure induced optimal differentiation of THP-1 monocytes

to the macrophage phenotype

2.6 Infection and treatment

M. tuberculosis strain H37 *Rv* (*M. tb.*) was grown to log phase on Sauton's liquid medium. The culture was aliquoted in 15 ml centrifuge tube and washed by centrifugation at 2000 X g for 10 minutes at room temperature. The supernatant was discarded and the bacterial pellet was re-suspended in RPMI containing no FBS (incomplete medium) and washed again. The above process was repeated twice. Finally, the bacteria were suspended in 3 ml incomplete medium and centrifuged at 100 x g for 5 minute to remove the clumps. The supernatant was transferred into a fresh tube and the optical density of an aliquot of the supernatant read at 600 nm. The OD of the supernatant was used to calculate the number of colony forming units (CFU)/ml using a standard curve routinely employed in the lab. The required volume of bacterial culture was added to RPMI containing 10% FBS (complete medium) and used for infection. Differentiated MDMs from human volunteers were exposed to *M.tb. H37Rv*, except in the case of normal controls (Group N).

The wells were washed after 3 hours and treatment initiated. Infected control wells (Group I) received fresh complete medium alone. RFB and INH in solution were added to achieve a final concentration of 3μg/ml each in duplicate wells designated as Group ISD; microparticles containing an equivalent amount of the two drugs (Group IMP). Drugs or microparticles were incubated with the infected cells for the next 2 hrs. After 2 hrs, wells were washed free of drugs or extracellular microparticles and complete medium replaced. The culture was then incubated for 48 hrs.

2.7 RNA isolation using Trizol

Differentiated MDMs from human volunteers were infected with *M. tuberculosis* H37Rv at a MOI of 10 for 3 hrs. Cultured cells were subjected to different treatments as described in section 2.6. Cells was lysed at 0 hrs post treatment using Trizol reagent (Invitrogen) and kept at -80°C till further processing. RNA was isolated from cell lysates using the manufacturer's protocol. Briefly, lysed cells were taken out from -80°C and incubated at room temperature for 15-20 minutes to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2

ml per ml of Trizol reagent) was added and the sample tubes capped securely. The tubes were shaken vigorously by hand for 15 s and incubated at room temperature for 5 min. The tubes were spun at 12000 × *g* for 15 min at 4°C. The aqueous phase was collected, 500 µl isopropanol/ml of trizol was added, mixed properly and kept at room temperature for 10 min. The tubes were centrifuged again at 12000 × *g* for 10 min at 4°C. The supernatants were removed and translucent pellets at the bottom of the tubes were washed with 75% ethanol, spun down at 7500 × *g* for 5 min at 4°C, supernatants decanted and the pellet dried at 37°C for 20 min. Pellets were redispersed in 30 µl DEPC treated water and RNA content quantified using Nano Photometer (IMPLEN, USA).

2.7.1 RNA Purification, Quality Assessment & Quantitation

RNA isolated using Trizol was further purified using Qiagen RNeasy MinElute cleanup kit (Qiagen) following the manufacturer's protocol. In brief, to a total volume of 100 µl of RNA solution (adjusted by adding nuclease free water), 350 µl RLT buffer was added and mixed properly. Ethanol (250 µl, absolute) was added, mixed and the contents of the tube transfered to two spin columns. The columns were spun down at 8000 rpm for 15 sec. The eluate was discarded and RPE buffer added before centrifuging again as above. The columns were washed two times with 500 µl of 80% ethanol by spinning at 10000 rpm for 2 min to remove all salts present in the RNA sample. The columns were centrifuged again, with open cap, at high speed (12000 rpm) for 5 min to remove traces of ethanol. Bound RNA was eluted from the columns using 14 µl of DEPC treated water. RNA was quantitated using Nano photometer (IMPLEN, USA) taking reading at 260 nm and protien contamination judged by A260/230 ratios. The quality of the RNA preparation was finally assessedusing the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies). In brief, about 100 ng of RNA was loaded in each well in the chip. In the first well a 1Kb ladder was loaded and a maximum 12 samples were loaded on each chip.

2.7.2 cRNA Preparation, Hybridization and Data analysis

The Illumina TotalPrep RNA Amplification Kit (Ambion, TX, USA) was used to generate biotinylated, amplified RNA. In brief, 500 ng of total RNA was reverse transcribed with

an oligo(dT) primer using ArrayScript enzyme and amplified overnight with T7 RNA polymerase and labeled with biotin according to the manufacturer's protocol. Labelled amplified RNA (cRNA) was hybridized at 58°C overnight to Illumina Genome-Wide Expression BeadChips (Human- HT12v4, Illumina, CA, USA) representing around 47,000 human transcripts. Arrays were incubated with Cy3 streptavidin and washed according to the manufacturer's protocol. The chip was scanned using Illumina scanner (iScan) and the analysis of the microarray data was done using Illumina Beadstudio 2.0 software. The data was average normalized and the genes which crossed the threshold of detection p value # 0.05 among all the samples and differential score p value # 0.05 among the test samples were considered to be differentially expressed genes.

2.8 Quantitative Real Time Reverse Transcriptase polymerase chain reaction (Q-RT-PCR)

Quantitative Real time RT-PCR was used to validate differential expression of selected genes. Reactions were performed using Light Cycler 480 Sybr Green I master mix kit (Roche) and Light Cycler 480 II Real time Cycler (Roche, USA). DNA contamination was removed from total RNA isolated using DNA Free kit (Ambion, USA) following the manufacturer's protocol. Briefly, 0.1 volume of 10X DNase buffer1 and 1 µl of rDNase1 was added to RNA in 50 µl reaction and incubated at 37°C for 25 minutes. The reaction was stopped by adding 0.1 volume of DNase inactivation agent, mixed properly and incubated at room temperature for 2 min, mixing occasionally. The reaction mix was centrifuged at 10,000 × *g* for 90 seconds and RNA was transferred into a fresh tube. Corresponding cDNA was synthesized from total RNA samples using reverse transcription with Superscript II cDNA synthesis kit (Invitrogen, USA) following the manufacturer's protocol. About 1056 ng of RNA was reverse transcribed using oligo $d(T)_{18}$ (Fermentas, USA) and transcribed into cDNA under conditions described in **Table 2.6A**.

Table 2.6A: Reaction Composition for Reverse Transcription

Sr. No.	Components	Volume
1	RNA	X µl (1056 ng)
2	Oligo (dT)18 primers(0.5µg/µl)	1 µl
3	dNTP Mix(10mM)	1 µl
4	DEPC Treated Water	Upto 13 µl

The above reaction mixture was incubated at 65°C for 5 minutes and immediately chilled on ice. It was then briefly centrifuged and contents mentioned in Table 2.6B were added.

Table 2.6 B: Reaction Composition for Reverse Transcription

Sr. No.	Components	Volume
1	5X First Strand buffer	4 µl
2	0.1 M DTT	2 µl
3	Superscript IIRT enzyme	1 µl (200 U)
	Total reaction volume	20 µl

The contents of the tube were mixed gently and incubated at 42°C for 50 min. The reaction was terminated by heating at 72°C for 15 min. The cDNA was kept at -20°C till further use.

Q-RT-PCR was performed as above, using 20 ng of template cDNA, and gene-specific primers. The primers were designed using NCBI primer blast or adopted from published literature (Maertzdorf, Repsilber et al. 2011). Typical reaction conditions are depicted in **Table 2.7.** Forward and reverse primers are listed in **Table 2.8** for selected genes. The annealing temperature in each case was 60°C. All reactions were performed in duplicate, and relative quantitation (**$2^{-(ddCt)}$**) (Livak and Schmittgen 2001) was used to evaluate the expression of selected genes**.** To assess the effect of Mtb infection and/or various treatments as described above on MDMs, glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) served as the control for calculation of dCt and uninfected cells served as the calibrator (calculation of ddCt). The following PCR protocol was used: 10 minutes at 95°C for 10 minutes, followed by 45 cycles of 10 seconds at 95°C, 20 seconds at 60°C and 20 seconds at 72°C and 10 seconds at 80°C for data acquisition to suppress fluorescence reading caused by generation of primer-dimers.

Table 2.7: Reaction Composition for Real Time PCR (per well)

Component	Volume (µl)	Final Concentration
Cyber green master Mix (2X)	**1 µl**	**1X**
Template	**1 µl**	**20 ng**
Forward primer (5 µM)	**1 µl**	**0.5µM**
Reverse Primer (5 µM)	**1 µl**	**0.5µM**
Water	**6 µl**	
Total volume	**10 µl**	

Table 2.8: Primer sequences and amplicon size and annealing temperatures

Gene	Forward primer	Reverse primer	Amplicon Size
CD64	AGGCCTGGTTTGCAGCTTT	CTGCCTCGCAGGGTCTTG	59
FCGR1B	GGAAGGGGTGCACCGGAAGG	CACGGGGAGCAAGTGGGCAG	98
Rab33A	AGATCCAGGTGCCCTCCAA	GAGCATGTTGTGGGCATCAG	56
GAPDH	CATGTTCGTCATGGGTGTGAA	ATGGACTGTGGTCATGAGTCCTT	147

2.9 Cytokine estimation

Validation of cytokine transcription profiles was conducted using a multiplex flow cytometric assay using a cytokine bead array (Th1/Th2 Human Cytokine, BD Biosciences, USA). Human MDMs (0.1 X 10^6 per well) were infected with H37 *Rv* and after different treatments described in Section 2.9 above, culture supernatant was collected for estimation of cytokines (TNF-α, IFN-γ, IL-10, IL-2, IL-4 & IL-6) at 0, 6& 12 hrs. The assay was performed according to manufacturer's protocol with some modifications. In brief, cytokine standards of different concentrations were prepared by serial dilution. Capture beads, each bearing antibody to one of six cytokines were mixed (10µl each per tube). Of the 60 µl thus obtained, 50 µl were

mixed with 100 µl of culture supernatant or 50 µl of standard and mixed well. Human Th1/Th2-II PE detection reagent was added and incubated overnight at 4°C. Wash buffer (1ml/tube) was added and the tubes centrifuged at 200 X g for 5 min. After spinning, the supernatant was removed and the pellet dispersed in 300 µl of wash buffer. Samples were analyzed on the flow cytometer (FACS Calibur, BD, USA). The concentration of cytokine in unknown samples was calculated using the calibration curve between concentration and mean fluorescence intensity (MFI) prepared by log-log four parameter fitting using CBA software (BD Biosciences) for data analysis.

2.10 Nitric Oxide estimation

Nitric oxide was estimated after different treatments in the supernatants of MDM cultures infected with *M tb* H37Rv. For the assay, MDMs ($0.1x10^6$/well in 96 well plates) were exposed to mycobacteria for 3 hrs, at a MOI of 10. Soluble or microparticle-incorporated drugs were administered, as described above in section 2.8. The supernatants were collected at 0 and 12 hrs and NO was measured in the cell supernatants using modified Greiss reagent (Sigma). An aliquot of 100 µl culture supernatant was incubated with equal volume of Greiss reagent for 30 min in dark at room temperature. Accumulated NO_2^-, a stable end product of NO formation in the cell supernatant reacts with Greis's reagent to form a purple azo dye and was measured colorimetrically as an indicator of NO production. The absorbance at 540 nm was measured using a microplate reader (Powerwave XS, Bioteck USA). The NO_2^- concentrations in the samples were calculated against a standard curve for sodium nitrite (0.5-20 µM).

2.11 Bacterial Survival Assay

There are various methods to estimate bacterial survival such as Almar blue assay, Bioscreen analyzer, Bactec assay and colony forming unit (CFU) counting. Bactec assay and colony counting assays were used for estimating bacterial survival in cell lysate of primary cells infected with H37Rv after different treatments.

2.11.1 Bactec Assay

Pheripheral monocytes were differentiated into macrophage phenotype, infected and treated with drugs in solution or microparticles as described in Section 2.8. Cells were lysed at 48

after removal of drugs and microparticles from the wells by 1X PBS containing 0.25% Triton X-100 and incubated at 37°C for 15 minutes. Following this, 100 µl of the cell lysate was transferred into a microfuge tube containing 100 µl of 20% BSA to stop the reaction. The mix was supplemented with 100 µl PENTA PLUS (BD biosciences) and injected into a BACTEC B12 vials and bacterial growth monitored using a BACTEC 460-TB system (BD, Biosciences, USA). Vials were sampled 24-hourly till a reading of 999 was attained.

2.11.2 Bacterial Viability by CFU counting

Primary macrophages isolated from different healthy volunteers were plated in 96 well culture plates (0.1 million cells/well). Cells were infected with H37Rv and subjected to different treatments as described in Section 2.8. After incubation of 48 hrs cells were lysed using 0.25% Triton X-100 in 1 X PBS. Bacterial colony counting was done by making 10 times serial dilutions of cell lysate in 1X PBS and 50 µl of serial dilution were plated on 7H11 agar plates supplemented with 10% of OADC and 25 µg/ml of cycloheximide. Colonies were counted after 21-28 days of incubation in a humid chamber at 37^0C.

2.11.3 Statistical analysis

The SNP genotyping frequencies were calculated by direct counting method and dividing the result by total number of genotypes. Allele frequencies were determined directly from the observed genotypes with the frequency of given SNP being equal to its homozygous frequency plus one half the heterozygous frequency. The data were tested for Hardy Weinberg equilibrium fit by calculating expected genotype frequencies and using chi square test to determine whether any genotype significantly differed from expected frequency. Pearson Chi square statistics test (*Joosse SA. 2011. Chi-square test calculator. Available from http://in-silico.net/statistics/chi2test*) was performed to compare genotype frequencies of different population. As the data were not in Gaussian approximation, non parametric t test i.e. Mann-Whitney t test was performed to compare significant differences between observed values. Principal component analysis was carried out using XLSTAT (limited version) and clustering was done using cluster 3 software and output was visualizd using Java Tree view software.

Chapter 3

Results & Discussions

Result & Discussion

3.1 'Susceptibility' and 'Resistance' Scores

This section describes the genotyping of the donors and their segregation or grouping on the basis of arbitrary 'susceptibility' (S) and 'resistance' (R) scores. As described in the Methods section, a value of 1 unit was assigned on the presence of each genetic marker, regardless of the magnitude of its contribution to net overall susceptibility. The presence or absence of *HLA DRB1*1501* and indicated SNPs in the *VDR* gene were used for such scoring.

3.1.1 HLA typing of volunteers

Several studies in and outside India have demonstrated the association of *DRB1*1501* with susceptibility to TB and leprosy (Tables 1.1-1.3). Therefore, the presence/absence of the *HLA DR2* allele *DRB1*1501* was used to assign 1 S/R score unit.

There were total 52 donors with mean age of about 30 years and included 47 males and 5 females. Blood were collected from these volunteers by phlebotomy using a heparinized vaccutainer , DNA was isolated using Qiagen DNA Amp kit and subjected to PCR-SSP for *HLA DRB1*1501* , *HLA DR2* and *HLA DRB1* (used as control) genotyping. The amplified PCR product was subjected to gel electrophoresis (Figure 3.1 & 3.2). The results of genotyping and allele freqency obtained are summarized in Table 3.1.

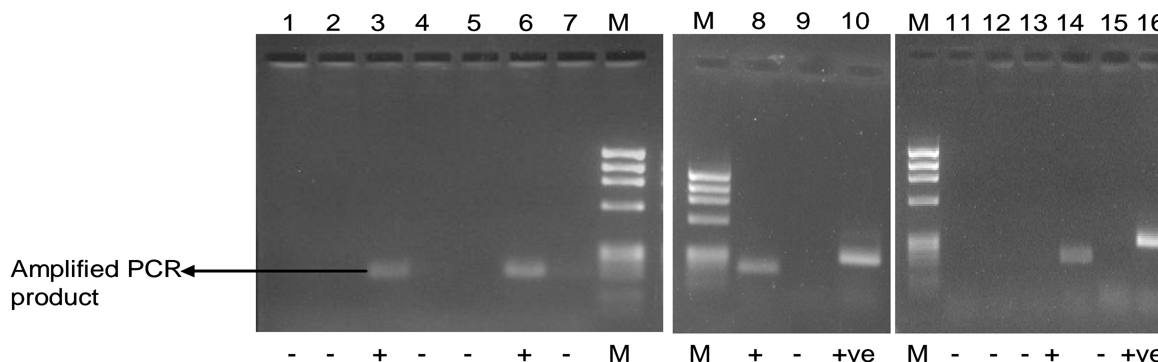


Figure 3.1: The presence or absence of *DRB1*1501* (amplified PCR product) in different volunteers. Lane M: DNA ladder; lane7, 9, 15: negative control; Lane 6, 10 and 16: positive control, lane 1-5, 8, 11-14: PCR products for different volunteer. (+ indicates presence, - denotes absence)

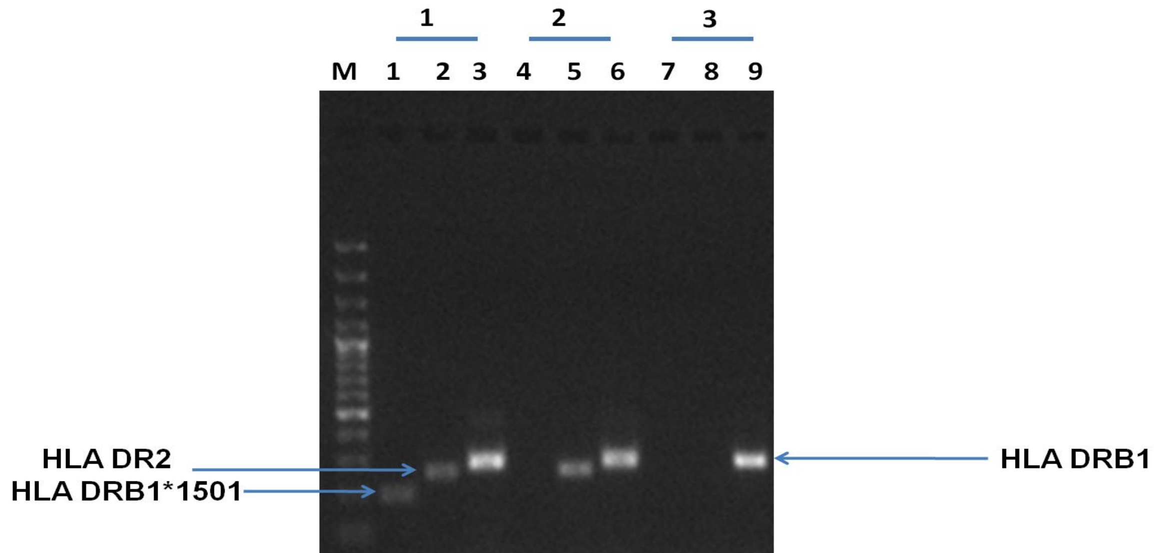


Figure 3.2: Presence or absence of *HLA DRB1*1501*, *HLA DR2* and *HLA DRB1* in three different volunteers. Lane M: DNA ladder (100 bp); lane1, 4, 7: DRB1*1501; Lane 2, 5, 8: DR2, lane 3, 6, 9: *HLA DRB1*

Table 3.1: Allele frequency distribution of *HLA DR2* and DRB1*1501 in studied population

No.of donors positive for *HLA DR2* (N=52)	No. of donors positive for *HLA DRB1*1501*	*HLA DR2* antigen frequency (%) (2n=104)	*HLA DRB1*1501* allele frequency (%)	% of *HLA DR2* positive for *HLA DRB1*1501*
29	14	27.9	13.6	48.3

Out of 52 donors, 29 (27.9%) were positive for *HLA DR2* and the *DRB1*1501* subtype was present in 14 donors (13.6%). Thus, *HLA DRB1*1501* contributed to 48.3% of *HLA DR2* subtype. *HLA DRB1*1501* was not present in all the volunteers positive for *HLA DR2* (Figure 3.2), highlighting the specificity of reaction and indicating the presence of different DR2 alleles other than *DRB1*1501*. To verify that the correct allele was amplified, the PCR product was sequenced and sequence alignment was done using CLUSTAL 2 software. The results of the alignment are as follows:

```
CLUSTAL 2.0.10 multiple sequence alignment


1         CACGTTTCCTGTGGCAGCCTAAGAGGGAGTGTCATTTCTTCAATGGGACCGAGCGGGTGC 60
2         -----------------------------------------CCATGGA--GGACGGGTGC 18
                                                   * ****** * *******

1         GGTTCCTGGACAGATACTTCTATAACCAGGAGGAGTCCGTGCGCTTCGACAGCGACGTGG 120
2         GGTTCCTGGA-AGATACTTCTATAACCAGGAGGAGTCCGTGCGCTTCGACAGCGACGTGG 77
         ********** ************************************************

1         GGGAGTTCCGGGCGGTGACGGAGCTGGGGCGGCCTGACGCTGAGTACTGGAACAGCCAGA 180
2         GGGAGTTCCGGGCGGTGACGGAGCTGGGGCGGCCTGACGCTGAGTACTGGAACAGCCAGA 137
          ************************************************************

1         AGGACATCCTGGAGCAGGCGCGGCCGCGGTGGACACCTACTGCAGACACAACTACGGGG 240
2         AGGACATCCTGGAGCAGGCACGGCCGCGGTGGACACCTACTGCAGACACAACTACGGGG 197
          ****************** ****************************************

1         TTGTGGAGAGCTTCACAGTGCAGCGGCGAG 270
2         TTGTGGAGAGCTTCACAGTGCAGCGAA--- 224
          *************************
```

At base pair 50 in the case of *DRB1*1501* there is a *c* nucleotide while in *DRB1*1502* there is a *g* nucleotide. This base could not be sequenced. At position 158 and 161 there was no variation as would be expected in case of *DR*1502*. There was no variation at the 243 and 245 positions. Finally, no variation at 257 position strongly suggests that the genotype is *HLA DRB1*1501*.

HLA DR2 is common in Asian countries and occurs with the frequency of 25-40% in Indian population (Rajalingam, Krausa et al. 2002; Singh, Balamurugan et al. 2007). *HLA DRB1*1501* accounted for around 13.8%-14.8% of total population (Rani, Mukherjee et al. 1998; Rajalingam, Krausa et al. 2002). The frequency of *HLA DRB1*1501* allele was found to vary between 50-60% in most of the studies conducted in north Indian populations (Mehra, Verduijn et al. 1991; Singh, Balamurugan et al. 2007). Our results were consistent with reported frequencies. However, allele frequencies for *HLA DR2* and its subtype differed from those reported for Maratha (13.6% vs 11.3%) (Shankarkumar, Devaraj et al. 2003), Kashmiri Brahmins (13.6% vs 0%) (Mehra 1998) and south Indian populations (13.6% vs 17.3%) (Selvaraj, Nisha Rajeswari et al. 2007) and also from studies in north Indian Hindu population (13.6% vs 9%) (Agrawal, Khan et al. 2007). The reasons for these differences in allele frequencie may lie the smaller sample size in the present investigation, or may actually represent the sample group, since volunteers were not stratified on the basis of place of habitation, religion or kin-group as in other studies (Agrawal, Srivastava et al. 2008).

3.1.2 VDR polymorphism

Polymorphisms in *VDR* were determined using PCR-RFLP (Figure 3.3). The distribution of observed *VDR* genotypes (*TaqI, BsmI, ApaI* and *FokI*) and allele frequencies are detailed in Table 3.2. A chi squared test was performed to calculate expected frequency and to determine the Hardy Weinberg equilibrium P value (HWP value). For all the VDR variants except *FokI*, genotype distribution was in agreement with Hardy Weinberg equilibrium.

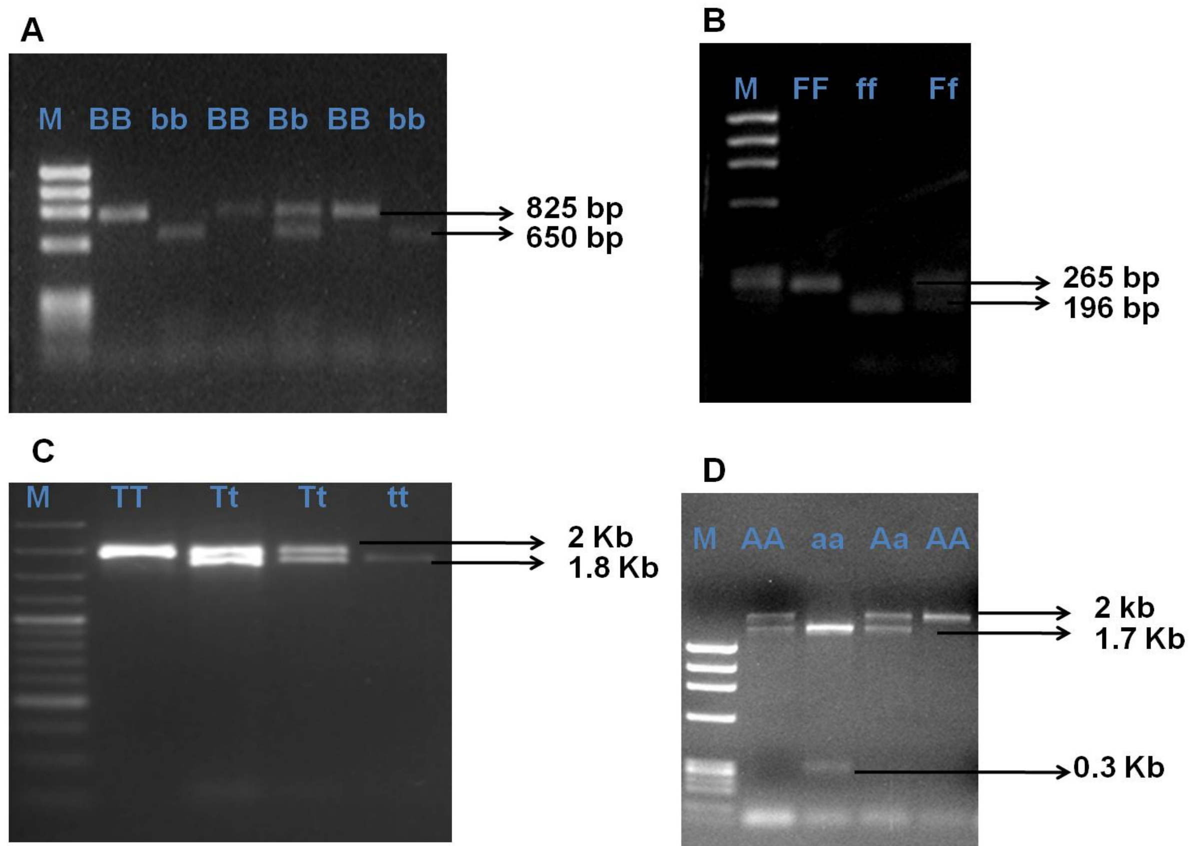


Figure 3.3: Restriction endonuclease digestion of PCR amplified *VDR* fragments. (**A**): *BsmI*; M- ϕX174 DNA/BsuRI (HaeIII) Marker, BB- absence of *BsmI* restriction enzyme (RE) site (*BB*: 825 bp), *Bb* and *bb* denote presence of RE site (*Bb*: 825, 650 and 175 bp; *bb*: 650 and175 bp); (**B**) *FokI*: M- ϕX174 DNA/BsuRI (HaeIII) Marker, FF: absence of *FokI* RE site (FF: 265 bp), *Ff* and *ff*-presence of RE site (*Ff*: 265, 196 and 69 bp; *ff*: 196 and 69 bp); (**C**) *TaqI*: M- 100 base pair DNA ladder, TT-absence of *TaqI* RE site (*TT*: 2000 bp), *Tt* and *tt*- presence of RE site (*Tt*: 2000,1800 and 200 bp; *tt*: 1800 bp and 200 bp); (**D**) *ApaI*: M- ϕX174 DNA/BsuRI (HaeIII) Marker, AA- absence of *ApaI* RE site (*AA*: 2000 bp), *Aa* and *aa* -presence of RE site (*Aa*: 2000, 1700 and 300 bp; *aa*: 1700 and 300bp)

Table3.2: Genotype, observed and expected allele frequencies of *VDR* polymorphism and Hardy Weinberg equilibrium P values (HWP)

Frequency	Genotype (% in parentheses) N=52			Allele (%)	frequency	HWP value
	TT	**Tt**	**tt**	**T**	**t**	
Observed	26 (50)	21 (40.38)	5 (9.61)	70.19	29.81	0.8012
Expected	25.62	21.76	4.62			
	BB	**Bb**	**bb**	**B**	**b**	
Observed	12 (23.07)	23 (44.23)	17 (32.69)	45.19	54.81	0.4398
Expected	10.62	25.76	15.62			
	AA	**Aa**	**aa**	**A**	**a**	
Observed	16 (30.77)	24 (46.15)	12 (23.08)	53.845	46.155	0.6065
Expected	15.08	25.85	11.08			
	FF	**Ff**	**ff**	**F**	**f**	
Observed	24 (46.2)	27 (51.92)	1 (1.92)	72.113	27.887	0.0358
Expected	27.04	20.91	4.04			

The allele frequencies of *T* vs *t*, *B* vs *b*, *A* vs *a* and *F* vs *f* were about 70.2 vs 29.8%, 45.2 vs 54.8%, 53.9 vs 46.2% and 72.1 vs 27.9% respectively in the donor population. Significant differences were observed in a comparision of genotypes and allele frequencies reported with different populations, including studies in different parts of India, using the χ^2 test (Table 1.3, 1.4, 1.5 and 1.6). *VDR p*olymorphisms differ among Asian countries like Japan (Tokita, Matsumoto et al. 1996), China (Kung, Yeung et al. 1998) and also within the Indian subcontinent. The frequency of *TaqI* and *FokI* SNPs in the present study did not significantly differ from those reported (*TT* 49%, *Tt* 43%, *tt* 8%*FF* 59%, *Ff* 36 5%, *ff* 49%,) in a study conducted in Western India (Bhanushali, Lajpal et al. 2009). There was no significant difference between *VDR* polymorphism for *TaqI, BsmI, ApaI* and *FokI* frequency between north Indian and south Indian populations (Table 3.3).

An assessment of *FokI* genotype distribution in the light of Hardy Weinberg equilibrium demonstrated that there is significant difference between observed and expected frequency in the study group (0.0358<p< 0.05) with excess of hetrozyote over homozygote genotypes. Studies

conducted in various part of the world have reported lower frequency of the *f* allele. Values reported here are similar in trend, with the lowest frequency reported as 0.004% in a native Paraguayan population (Bid, Mishra et al. 2005; Wilbur, Kubatko et al. 2007; Jafar 2009).

Table 3.3: Genotype and allele frequency distribution of *VDR TaqI* polymorphism in various populations and $\chi 2$ *P* values. Each population compared with present study

Country/ population	N	Genotype (%)				Allele frequencies (%)		Reference
		TT	Tt	tt	P value	T	t	
North India	52	50	40	10	Ref.	70	30	Present Study
Gujarati Indians in London	116	39	50	11	0.3	64	36	(Wilkinson, Llewelyn et al. 2000)
Native paraguayans (Achè)	242	45	48	7	0.59	69	31	(Wilbur, Kubatko et al. 2007)
Western India	143	49	43	8	0.74	71	29	(Bhanushali, Lajpal et al. 2009)
France	189	33	49	18	0.05*	57.5	42.5	(Garnero, Borel et al. 1995)
Japan	488	77	22	1	0.0001***	88	12	(Tokita, Matsumoto et al. 1996)
North India	569	47	40	13	0.76	67	33	(Jafar 2009)
North India	346	49	40	11	0.97	66	34	(Bid, Mishra et al. 2005)
Austria	163	12	49	39	0.0001***	36	64	(Boschitsch, Suk et al. 1996)
Soth India	103	39.8	46.6	13.6	0.41	63	37	(Selvaraj, Kurian et al. 2004)

* = p<0.05, ** = p<0.01, *** = p<0.001, at 5 % level of significance

Table 3.4: Genotype and allele frequency distribution of *VDR BsmI* polymorphism in various populations and $\chi 2$ *P* values. Each population compared with present study

Country/ population	N	Genotype (%)				Allele frequencies (%)		Reference
		BB	Bb	bb	P value	B	b	
North India	52	23	44	33	Ref.	45	55	Present study
South India	80	40	36.2	23.8	0.12	58.1	41.9	(Selvaraj P 2003)
Spain	136	11	59	30	0.0692	40	60	(Barber, Rubio et al. 2001)
Taiwan	169	0	9	91	0.0001***	5	95	(Hou, Tien et al. 2002)
Europe	460	16	47	37	0.43	40	60	(McCullough, Stevens et al. 2007)
Africa	446	11	38	51	0.01**	30	70	(Trabert, Malone et al. 2007)

* = p<0.05, ** = p<0.01, *** = p<0.001, at 5 % level of significance

Table 3.5: Genotype and allele frequency distribution of *VDR ApaI* polymorphism in various populations and $\chi 2$ *P* values. Each population compared with present study

Country/ population	Number	Genotype (%)				Allele frequencies (%)		Reference
		AA	Aa	aa	P Value	A	a	
North India	52	31	46	23	Ref.	54	46	Present study
North India	569	30	57	13	0.1	58.6	41.4	(Jafar 2009)
South India	80	37.5	46.3	16.3	0.55	61	39	(Selvaraj P 2003)
Europe	477	27	52	21	0.7	53	47	(McCullough, Stevens et al. 2007)
Taiwan	169	9	43	8	0.0001***	30	70	(Hou, Tien et al. 2002)
North India	150	36	44	20	0.7	58	42	(Bid, Mishra et al. 2005)

Australia	518	26	51	23	0.73	51	49	(Tokita, Matsumoto et al. 1996)

Table 3.6: Genotype and allele frequency distribution of *VDR Fok1* polymorphism in various populations and $\chi 2$ *P* values. Each population compared with present study

Country/ population	Number	Genotype (%)				Allele frequencies (%)		Reference
		FF	Ff	ff	P	F	f	
North India	52	46.2	51.9	1.9	Ref.	72.113	27.887	Present Study
North India	569	47.4	50	2.6	0.928	72.4	27.6	(Jafar 2009)
North India	346	44	49	7	0.38	68.5	31.5	(Bid, Mishra et al. 2005)
Achè	264	67	33	<1	0.01**	83	17	(Wilbur, Kubatko et al. 2007)
Gujarati Indians in London	116	64	34	2	0.08	81	19	(Wilkinson, Llewelyn et al. 2000)
South India	250	62.8	23.2	14	0.0001	74.4	25.6	(Annamaneni, Bindu et al. 2011)
South India	80	53.8	36.2	10	0.07	71.9	28.1	(Selvaraj P 2003)
Italy	253	37	50	13	0.05*	62	38	(Braga, Sangalli et al. 2002)
Western India	143	59	36	5	0.1	78	22	(Bhanushali, Lajpal et al. 2009)

* = p<0.05, ** = p<0.01, *** = p<0.001, at 5 % level of significance

3.1.3 Cytokine gene polymorphism

Cytokine gene polymorphisms are associated with cytokine production level and any dysregulation of cytokine biosynthesis due to polymorphism affects disease susceptibility.

In the current study, polymorphism frequencies of IFN-γ (+874A/T), TNF-α (-308A/G) IL-10 (-1082A/G) and IL-4 (-590C/T) were studied in healthy volunteers using ARMS-PCR. Genotype was assigned according to amplification (Figure 3.4), and genotype and allele frequency were evaluated by gene counting. The results are presented in Table 3.7.

Table 7: Observed versus expected genotype and allele frequencies for each cytokine SNP and χ^2 test for deviation from HWP in the study sample

SNP	Genotype	Observed Genotype frequency (%)	Expected Genotype frequency	HWP value	Allele	% Allele frequency
IFN-γ +874A/T	AA	27 (52)	25.62	0.3604	A	70.2
	AT	19 (36.5)	21.76		T	29.8
	TT	6 (11.5)	4.62			
TNF-α -308 A/G	AA	1 (1.9)	0.81	0.8121	A	12.5
	AG	11 (21.2)	11.38		G	87.5
	GG	40 (76.9)	39.81			
IL-10 -1082 A/G	AA	29 (55.8)	25.62	0.0251	A	70.2
	AG	15 (28.8)	21.76		G	29.8
	GG	8 (15.4)	4.62			
IL-4 -590 C/T	CC	42 (80.8)	40.69	0.0756	C	88.5
	CT	8 (15.4)	10.62		T	11.5
	TT	2 (3.8)	0.69			

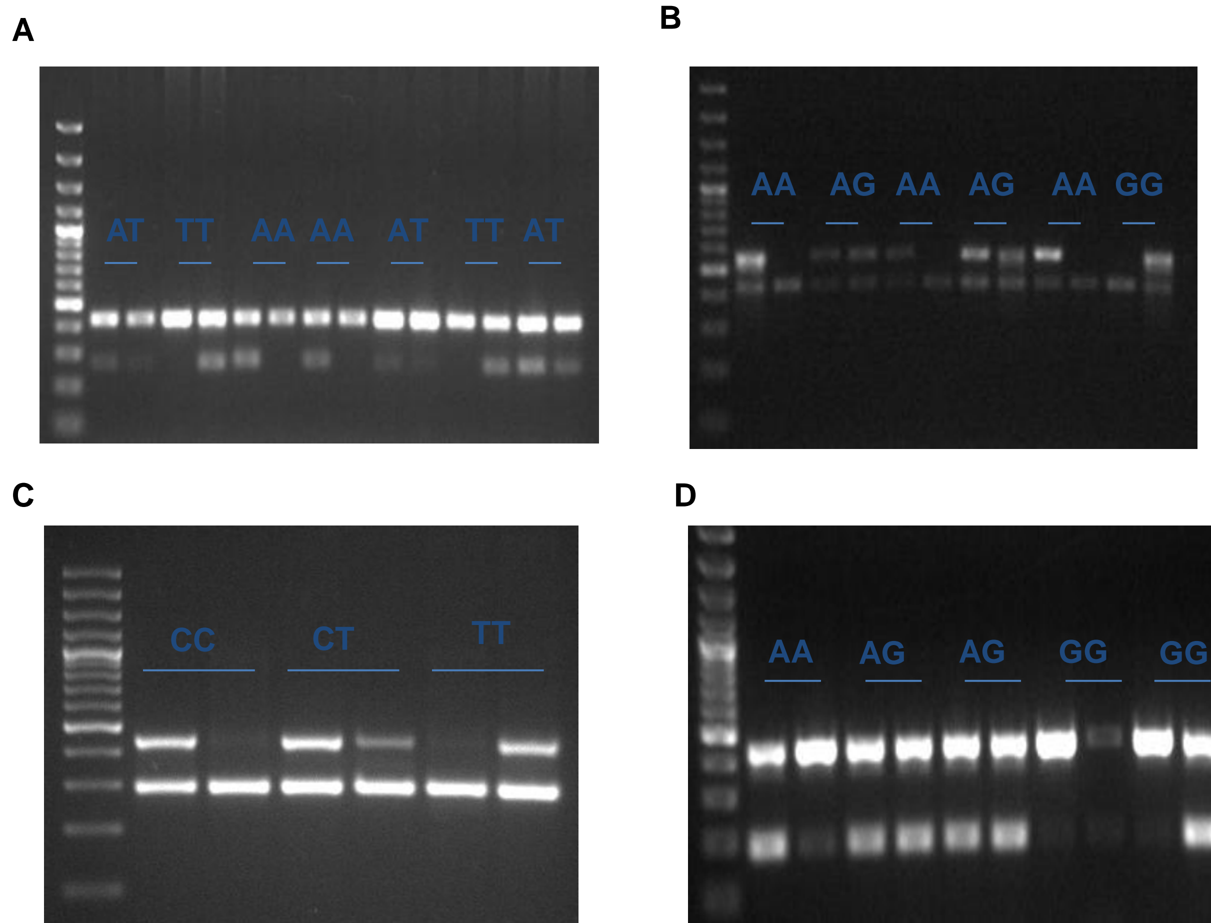


Figure 3.4: Typical PCR amplification of sequences using SNP-specific reverse primer and a common forward primer. (**A**): amplification of IFN-γ sequence using reverse primer specific for +874A/T and common forward primer. (**B**): IL-10 -1082 A/G subsititution. (**C**) IL-4 -590C/T subsititution. (**D**): TNF-α -308 A/G subsititution.

The observed and expected frequencies of various cytokine genotypes in the Indian population and their Hardy-Weinberg equilibrium P value (HWP) are shown in Table 3.7. Most genotypes fitted Hardy-Weinberg equilibrium ($p>0.05$, χ^2=0.836, 0.057, 3.156 for IFN-γ, TNF-α, and IL-4 respectively). However, IL-10-1082 SNP was not in HW equilibrium ($p<0.05$, χ2= 5.018). The basis of this observation may be the modest sample size, or it might be population specific. Technical error during genotyping is unlikely since all precautions were followed and retyping was done in case of ambiguous results. When allele frequencies observed in the present study were compared with a study on a North Indian population located in Delhi and surrounding area using contingency chi square test, there was no significant difference between these (P value=0.90, 0.3365, 0.1054, 0.1316 for IFN-γ, TNF-α, IL-10 and IL-4 respectively)

(Kaur, Rapthap et al. 2007). Moreover, there was no significant difference between the present data for the SNP at TNF-α -308 A/G and studies from Punjab region of North India (P value=0.3979) (Mohindru and Changotra 2004).

There was no significant difference between SNP frequencies in the IFN-γ, TNF-α and IL-4 loci estimated in this study versus South Indian populations (P value=0.541, 0.154, 0.9586 respectively for IFN-γ, TNF-α and IL-4) (Selvaraj, Sriram et al. 2001; Vidyarani, Selvaraj et al. 2006), but there was a significant difference in the case of IL-10-1082A/G (P value= 0.0137) (Prabhu Anand, Selvaraj et al. 2007).

3.1.4 Arbitrary Score of Susceptibility and Resistance

Studies conducted in India and particularly in the northern region have reported association between *HLA DRB1*1501* and *VDR* (*TT, Bb, FF*) with susceptibility to TB and leprosy. All SNPs associated with disease susceptibility have very similar OR, ranging around 2.5-3. Therefore equal weightage was assigned to each SNP, and its presence or absence in the donor's genome was assigned an arbitrary value of 1 on the 'Susceptibility' or 'Resistance' Score, as applicable. The net S and R Scores of each volunteer was the sum of the number of markers found in the genome. Thus, if 4 factors associated with "susceptibility" and none associated with 'resistance' were present, the S Score was taken as +4. **Table 3.8** summarizes *HLA* and *VDR* genotypes of each donor and the corresponding arbitrary 'S' and 'R' scores.

Donors were ranked in descending order of 'resistance' to infection, on the basis of lowest S Score and highest R Score, downwards. Donors showing the highest and lowest S and R scores were selected along with a few volunteers with intermediate scores were randomly selected from each score group for further studies. The S and R scores of these donors ranged from zero to three. These donors agreed to donate 20 ml more blood, and this was used for further experiments, namely estimation of spontaneous cytokine secretion in culture, and upon infection and/or treatment with drugs or microparticles, and survival of intracellular bacilli following infection and/or treatment.

Table 3.8: Donors and their arbitrary 'S' and 'R' scores

Donor ID	HLA DR 2	HLA DRB1*1501	Vitamin D Receptors gene variants				S Score	R Score	Net S Score (S-R)
			TaqI	*BsmI*	*ApaI*	*FokI*			
D1	Y	N	Tt	BB	AA	Ff	0	3	-3
D2	N	N	tt	BB	AA	Ff	0	3	-3
D3	N	N	tt	BB	Aa	Ff	0	2	-2
D4	N	N	Tt	Bb	Aa	Ff	0	1	-1
D5	Y	N	TT	Bb	aa	FF	0	2	-2
D6	N	N	tt	BB	AA	Ff	0	2	-2
D7	N	N	TT	BB	aa	FF	0	2	-2
D8	N	N	TT	Bb	Aa	FF	0	2	-2
D9	N	N	Tt	Bb	Aa	FF	0	1	-1
D10	N	N	TT	Bb	Aa	Ff	0	1	-1
D11	N	N	Tt	Bb	Aa	Ff	1	1	-1
D12	N	N	Tt	Bb	AA	Ff	1	1	-1
D13	N	N	Tt	Bb	Aa	Ff	1	1	-1
D14	Y	N	TT	bb	Aa	Ff	1	1	-1
D15	Y	N	Tt	Bb	Aa	Ff	1	1	0
D16	N	N	Tt	Bb	Aa	Ff	1	1	0
D17	Y	N	Tt	Bb	AA	Ff	1	2	-1
D18	Y	N	Tt	BB	AA	FF	1	3	-2
D19	N	N	TT	bb	aa	Ff	1	1	0
D20	N	N	TT	bb	AA	Ff	1	2	-1
D21	Y	N	Tt	Bb	aa	Ff	1	1	0
D22	Y	Y	Tt	bb	AA	Ff	1	1	0
D23	N	N	TT	bb	aa	Ff	1	1	0
D24	Y	N	Tt	Bb	Aa	Ff	1	1	0
D25	Y	N	TT	bb	AA	Ff	1	2	-2
D26	Y	N	Tt	Bb	Aa	Ff	1	1	0

D27	N	N	tt	BB	AA	Ff	1	1	0
D28	N	N	TT	Bb	aa	Ff	1	1	0
D29	Y	N	Tt	Bb	Aa	FF	2	1	0
D30	N	N	TT	Bb	AA	Ff	2	2	0
D31	Y	Y	Tt	BB	Aa	FF	2	1	1
D32	Y	N	TT	Bb	aa	FF	2	1	1
D33	Y	Y	tt	BB	AA	FF	2	0	2
D34	Y	Y	TT	Bb	aa	Ff	2	0	2
D35	Y	Y	TT	BB	AA	ff	2	2	0
D36	N	N	TT	bb	aa	FF	2	1	1
D37	Y	Y	Tt	Bb	Aa	Ff	2	0	2
D38	N	N	TT	Bb	AA	Ff	2	2	0
D39	Y	N	TT	bb	aa	FF	2	1	1
D40	Y	Y	Tt	BB	AA	FF	2	2	0
D41	Y	Y	Tt	BB	AA	FF	2	2	0
D42	Y	N	Tt	Bb	Aa	FF	2	1	1
D43	N	N	TT	bb	aa	FF	2	1	1
D44	Y	Y	TT	bb	aa	FF	3	0	3
D45	Y	Y	Tt	Bb	Aa	FF	3	0	3
D46	N	N	TT	Bb	Aa	FF	3	1	2
D47	Y	Y	TT	bb	Aa	FF	3	0	3
D48	Y	Y	TT	Bb	Aa	FF	3	1	2
D49	N	N	TT	Bb	Aa	FF	3	1	2
D50	Y	Y	TT	bb	Aa	FF	3	0	3
D51	Y	Y	TT	Bb	Aa	FF	4	0	4
D52	Y	Y	TT	Bb	Aa	FF	4	0	4
THP-1	Y	Y	TT	bb	Aa	FF	3	0	3

3.2 Cytokine assay in culture supernatant

Cytokine produced at the site of infection after interaction of T lymphocyte with infected macrophage are important in pathogenesis of TB. The course of Mtb infection is regulated by two distinct cytokine patterns Th1 cytokines includes IFN-γ, TNF-α, IL-2 and IL-6 and are associated with protection to TB. The Th2 cytokine includes IL-10 and IL-4 and is associated with disease progression. In present study, cytokine determination was done in culture supernatant derived from primary MDMs infected with Mtb H37Rv followed by different treatments. The supernatant was collected at 0, 6 and 12 hrs post treatment and analysed using using cytometric bead assay (CBA, BD Biosciences). IFN-γ and IL-2 was detected in very few samples and therefore excluded from analysis. Since individual's response varied widely, therefore values were represented as index, obtained by dividing the value of control i.e. the uninfected and untreated cells at each time point with cytokine level at that time in other groups.

3.2.1. Spontaneous and stimulated cytokine secretion by MDMs of polymorphic genotype

TNF-α

Blood levels of TNF-α remain remarkably constant in a healthy person, yet its level varies widely between individuals (Jacob, Fronek et al. 1990). This variation has been ascribed to polymorphism in the promoter region of the gene and results in differential production of the cytokine upon inflammatory stimulus and may have clinical significance (Wilson, di Giovine et al. 1995). Several authors have found that the presence of an *A* nucleotide at the -308 position in the promoter region of TNF-α is associated with higher cytokine level (Braun, Michel et al. 1996; Wilson, Symons et al. 1997; Wu and McClain 1997). Other studies have not found any correlation between -308 G/A polymorphism and cytokine level (Pociot, Briant et al. 1993; Uglialoro, Turbay et al. 1998). It is to be remembered that the predominant sources of circulating TNF-α are Natural Killer (NK) cells and activated cytotoxic T cells (Tc).

Spontaneous TNF-α seceretion by macrophages isolated from the blood of identified donors was studied, and the results are shown in Figure 3.5 panel 1A to 1C. Since there was only a single donor in the sampled population corresponding to the high secretor genotype

(*AA*), macrophages from intermediate (*AG*) and low secretors (*GG*) were compared. The median TNF-α level was not significantly ($p<0.05$) different between variant genotypes of TNF-α polymorphism in non infected cells at 0 hrs post treatment, despite larger median values. No correlation between phenotype and genotype was found in samples at 6 hr and 12 hr either. Progressively lower median values in the 6 and 12 hr samples were due to dilution with fresh culture medium.

Our results are similar to those observed in a study performed on North Indian population, where no significant differences beteen spontaneous cytokine secretion and the *GG* versus GA genotype were observed in healthy controls. Differences were significant ($p<0.01$) only between the high secreting *AA* genotype and other genotypes (Abhimanyu, Mangangcha et al. 2011). There are conflicting reports on association of TNF-α genotype and TB. Studies carried out in some populations have shown association of TNF-308 $G^{low}\rightarrow A^{high}$ with increased TNF-α production and presence of the *A* allele in healthy controls (Scola, Crivello et al. 2003; Correa, Gomez et al. 2005). However, this finding was not supported by studies in other parts of the world including India (Selvaraj, Sriram et al. 2001; Wu, Qu et al. 2008). Differences between these reports can be explained by the facts that TNF polymorphisms are different in each ethnic population. Moreover, the present study has been done in modest sample size and therefore might have been unable to demonstrate the effect of genotype on cytokine level. Furthermore, TNF- α -308 G/A polymorphism lies within MHC region on chromosome 6. Therefore, lack of association between -308 polymorphism and TNF- α level in the above studies can be due to role of HLA and other closely linked genes in influencing the cytokine level. Studies by Knight and collegues have also ruled out the regulation of TNF- α level with -308 SNP, and it is not likely to be a functionally important SNP as previously hypothesized (Knight, Keating et al. 2003).

Thus, it was concluded that TNF-α secretion by resting host macrophages from the sampled population was not significantly associated with the SNPs tested.

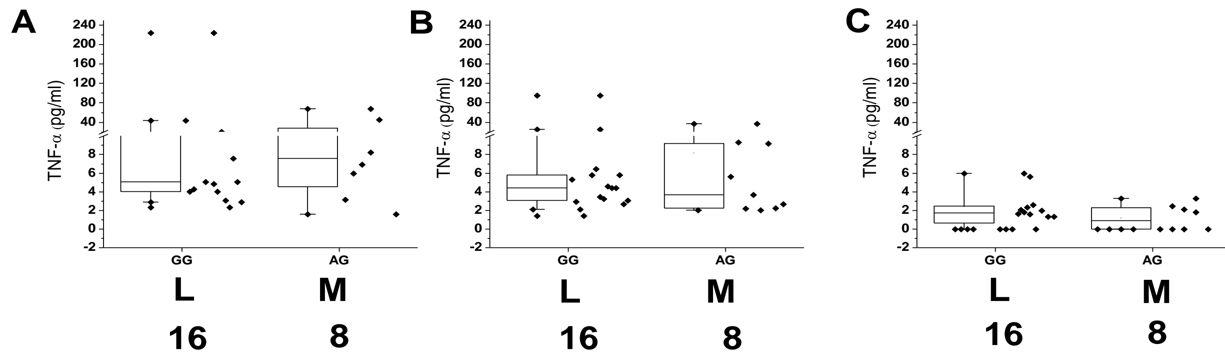


Figure 3.5: Influence of genotype (-308 *G/A*) on spontaneous TNF-α cytokine production level. Results are expressed as median cytokine level at 0 h **(A)**, 6 h **(B)** and 12 h **(C)** post treatment. Numbers represent the number of individuals studied for that particular genotype and **L**, **M** and **H** stand for low, medium and high secreting genotypes respectively. The boxes include the 25th and 75th percentile. The horizontal line in the box indicates the median, and the whiskers indicate the 10th and 90th percentile of the cytokine level. Black diamonds on right represent the individual values of donors and above and below boxes represent outlier

No statistically significant differences using a Mann-Whitney one tailed P test were found following infection and treatment with either the drugs in solution or by microparticles. The results are shown in Figure 3.6.

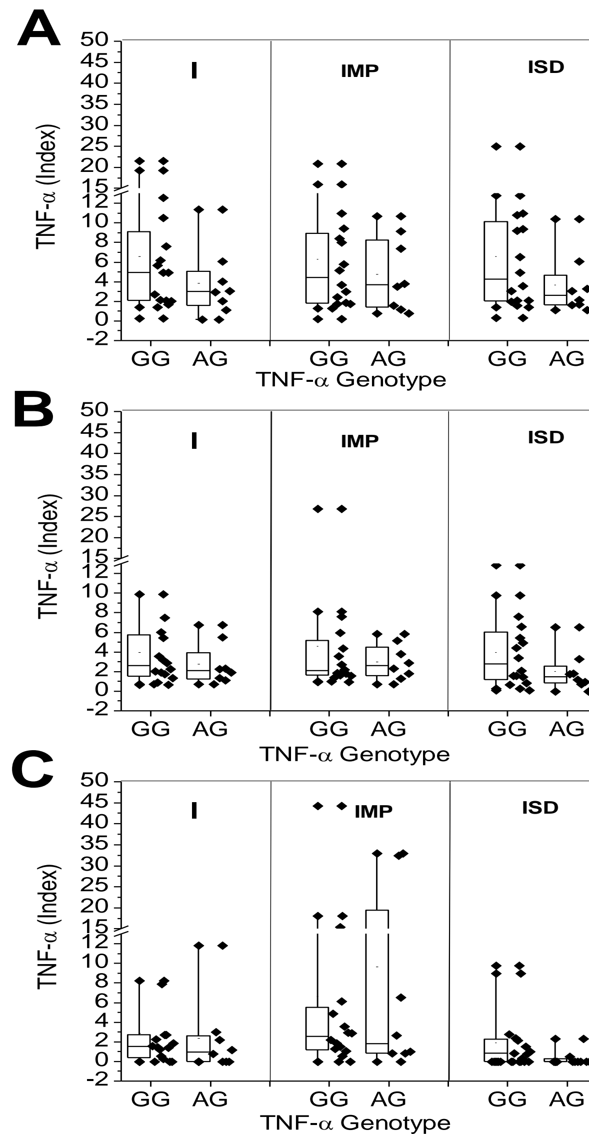


Figure 3.6: Association between TNF-α SNP (-308 *G/A*) and production of cytokine by MDMs of healthy human volunteers upon infection (**I**), and treatment with drug containing microparticles (**IMP**) and soluble drug (**ISD**). Results are expressed as median cytokine indices. Indices were calculated by considering the value of uninfected, untreated cells at each time point as 1.Panel **A**, **B** and **C** represent median indices for cytokine at 0 h, 6 h and 12 h post treatment.

IL-10

Studies have demonstrated a higher level of circulating IL-10 in individuals of the *AA/AG* genotype than the *GG* genotype in respect of the SNP at the -1082 position. Studies in monozygotic twins have ascribed 75% of the variation in IL-10 production to genotype (Westendorp, Langermans et al. 1997) and the -1082 *A* allele correlates with lower cytokine

level after T cell stimulation *in vitro* (Kim, Brannan et al. 1992). However, there was no correlation between genotype and IL-10 secretion by macrophages isolated from individuals sampled for the present study. Though uninfected cells of intermediate secreting *AG* genotype secreted higher levels of IL-10 than those of the *AA* genotype, the differences were statistically non significant (p>0.05), as shown in Figure 3.7; panel A to C.

Studies conducted in South India also did not find association between spontaneous cytokine level and IL-10 SNP (Selvaraj, Alagarasu et al. 2008). These results are similar to those reported by Abhimanyu *et al* in a North Indian population, wherein no association was observed between IL-10 levels and genotype in healthy individuals. These authors also observed that the spontaneous secretion of IL-10 by PBMC of different genotypes was in the order *GA >AA> GG* though differences were statistically (p>0.05) non significant (Abhimanyu, Mangangcha et al. 2011). There is evidence that more distal polymorphisms in the IL-10 promotors regions are more important in controlling cytokine level than the proximal polymorphisms investigated here. Furthermore, one haplotype of distal polymorphisms is reported to be associated with increased IL-10 production and is linked to the common proximal haplotypes, perhaps explaining the variable results of the studies of proximal polymorphisms (Gibson, Edberg et al. 2001).

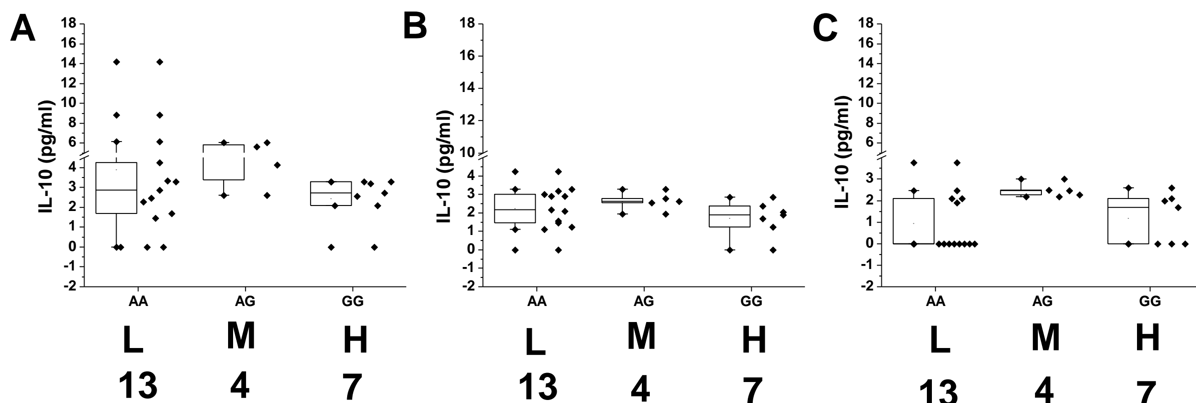


Figure 3.7: Influence of genotype (-1082 *A/G*) on spontaneous IL-10 cytokine production level. Results are expressed as median cytokine level at 0 h (**A**), 6 h (**B**) and 12 h (**C**) post treatment. Numbers represent the number of individuals studied for that particular genotype and L, M and H stand for low, medium and high secreting genotype respectively.

Differences in secretion of IL-10 between genotypes were not significantly different after infection and treatment at the 0.05 level of confidence (Figure 3.8). Microparticles

reduced IL-10 median indices in *AA* genotype compared to drugs in solution at 12 h post treatment (p<0.1).

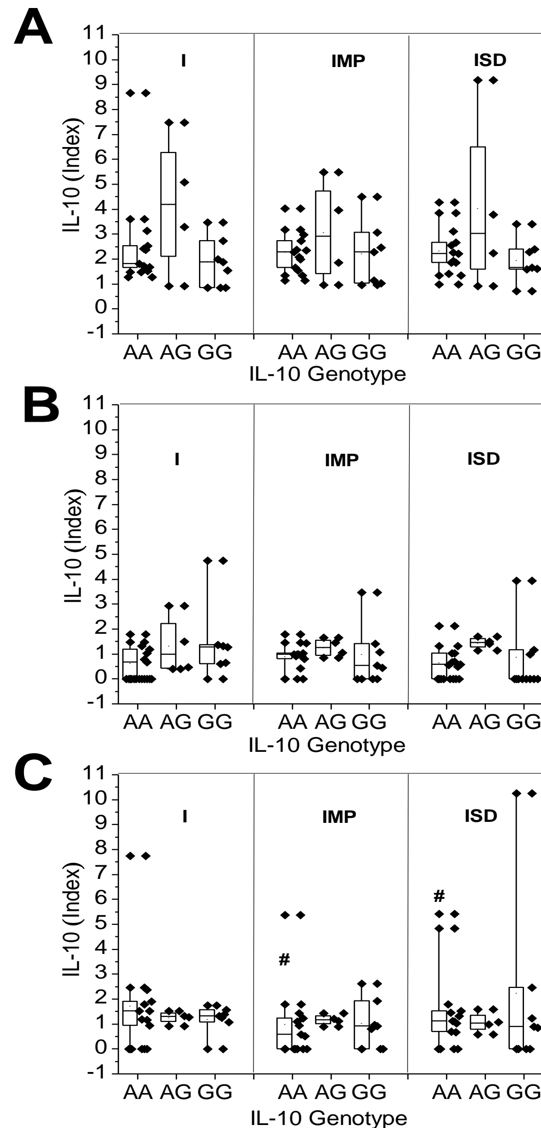


Figure 3.8: Association between IL-10 SNP (-1082 *A/G*) and production of cytokine by MDMs upon infection (**I**), followed by treatment with drug containing microparticles (**IMP**) and drugs in solution (**ISD**) from healthy human volunteers. Results are expressed as median cytokine indices. Panels **A**, **B** and **C** represent median indices for cytokine at 0 h, 6 h and 12 h post treatment. (Panel **C:** # *AA* IMP vs *AA* ISD; p<0.1)

IL-4

The SNP-specific genotype (*C* or *T*) at -590 in the promoter region of the *IL-4* gene is associated with cytokine production, as this polymorphism generates a stronger binding site for

nuclear factor for activated T cells (NF-AT1) with transition from *GGAGAA* to *GGAAAA*. Stronger binding of NF-AT1 mediates upregulation of IL-4 and can influence disease susceptibility (Nguyen, Genc et al. 2004). A reporter gene assay demonstrated 1.5 fold difference between expression of IL-4 for *C* to *T* transition (Go 2011). Figure 3.9 represents the sponatenous median values of IL-4 secretion by MDMs from different volunteers arranged according to genotype. No correlation between genotype and phenotype was observed at any time point during the course of study in uninfected cells. The possible reason may be the small number of volunteers of the higher secreting genotype limiting the comparision. The cytokine level ranged from 0-6 pg/ml in the *CC* genotype while for the *CT* and *TT* genotype it ranged from 1.76-3.12 pg/ml and from 0-3.12 pg/ml respectively. Similar to these results, a study in South Indian healthy contacts of TB patients by Vidyarani *et al* did not find any correlation between cytokine level and genotype. Neither was correlation observed in the level of IgG, which is regulated by IL-4 (Vidyarani, Selvaraj et al. 2006). The possible explanation may be that though IL-4-590 *C/T* is the only known SNP in the IL-4 promotor region; there might be some distal polymorphisms which are yet to be discovered, also regulating IL-4 production.

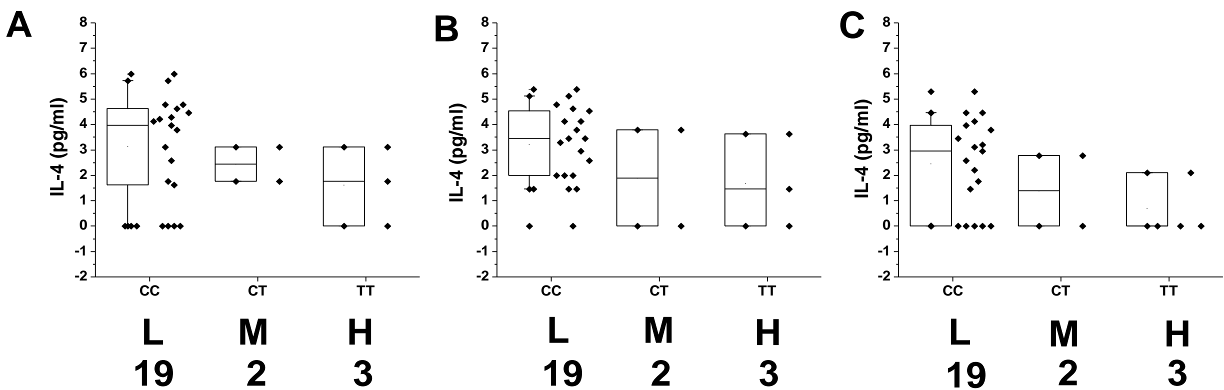


Figure 3.9: Influence of -590 *C/T* on spontaneous IL-4 production. Results are expressed as median cytokine level at 0 h (**A**), 6 h (**B**) and 12 h (**C**) post treatment. Numbers represent the number of individuals studied for that particular genotype and L, M and H stand for low, medium and high secreting genotype respectively.

Treatment with both microparticles and drugs in solution significantly ($p<0.001$) reduced IL-4 production by MDMs of the *CT* genotype compared to infection alone at 6 h post

treatment. At 12 h in the *TT* genotype, IL-4 levels were significantly ($p<0.05$) downregulated upon treatment with microparticles, but not dissolved drugs, compared to infection (Figure 3.10).

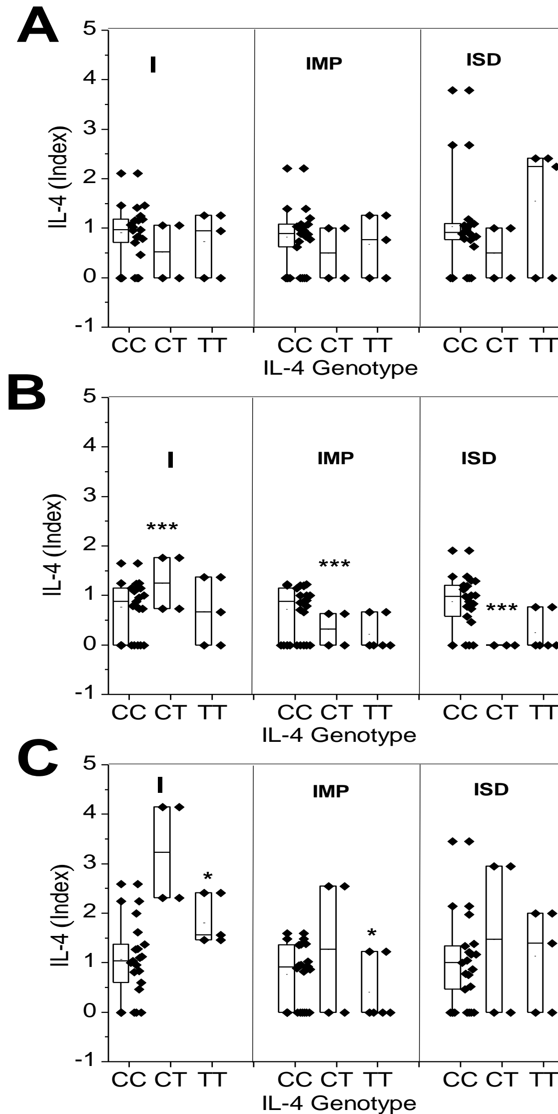


Figure 3.10: Association between IL-4 SNP (-590 *C/T*) and production of cytokine by MDMs from healthy human volunteers upon infection (**I**), and treatment with drug containing microparticles (**IMP**) or soluble drugs (**ISD**). Results are expressed as median cytokine indices. Panel **A**, **B** and **C** represent median indices at 0 h, 6 h and 12 h post treatment. (Panel **B**: *** *CT* **I** vs **IMP**; $p<0.0001$, *CT* **I** vs **ISD**; $p<0.0001$, Panel **C**, * *TT* **I** vs **IMP**; $p<0.05$)

3.2.2 Cytokine secretion in context of *HLA DRB1*1501* allele

The *HLA DR* gene/ gene products are major players in regulating immune response against mycobacterial antigens and in resistance and susceptibility to various infectious agents. *HLA DRB1*1501* has been identified as prominent in the Indian context in determining susceptibility against Mtb. Variation in *HLA DR* has been shown to affect Th1 or Th2 CD4+ T cells responses to *M leparae* heat shock protein (HSP), and increased production of Th1 cytokines in the presence of *HLA DRB1*1501*allele have been reported (Mitra, Rajalingam et al. 1997). A decisive role of MHC polymorphism on the type of helper cell responses has been demonstrated in mouse models (Murray, Madri et al. 1989). The influence of HLA genotype on cytokine production in response to infection with Mtb and effect of drug modalities in modifying the response was investigated.

TNF-α

Figure 3.11 shows the effect of *HLA DRB1*1501* on TNF-α secretion by MDMs upon infection and treatment with different forms of the drugs. At 0 h post treatment, primary MDMs of *HLA DRB1*1501* positive donors showed higher magnitude of cytokine response upon infection. The median value of the cytokine secretion index was 5.9 while the median values in the case of *HLA DRB1*1501* negative donors was 2.9; though the difference was not significant at the 95% confidence level. A similar study by Mitra *et al* using CD4+T cells from leprosy patients and healthy human subjects of genotype *DR 15* and other non-*DR1* alleles reported higher Th1 responses upon challenging with *M leprae* HSP. However, the cited study reported IFN-γ, IL-4 and IL-2 and not TNF-α (Mitra, Rajalingam et al. 1997).

Treatment with drug containing microparticles increased the median cytokine indices in both *DRB1*1501* positive and negative groups and the increase was higher in comparision to treatment with soluble drugs for the *DRB1*1501*positive group. At 6 h, median cytokine indices for *DRB1*1501* positive and negative groups fell to 1.9 and 3.4 respectively upon infection. Treatment with either microparticles or soluble drugs led to marginal increase. At 12 h, median TNF secretion fell to 1.5 and 0.99 if infection was left untreated. Thus MDMs from donors positive for *HLA DRB1*1501* secreted less TNF-α at 14h post infection.

Treatment with drug containing microparticles induced significantly higher TNF-α compared to treatment with drugs in solution (p<0.05 for *DRB1*1501*⁻ and <0.01 for *DRB1*1501*⁺). These results demonstrate that microparticles are more effective in eliciting macrophage TNF-α across *DRB1*1501* and non-*DRB1*1501* genotypes. A study by Yadav *et al* using phorbol-differentiated THP-1 monocytes and primary macrophages also reported the sustained release of TNF-α compared to drugs in solution (Yadav, Muttil et al. 2010).

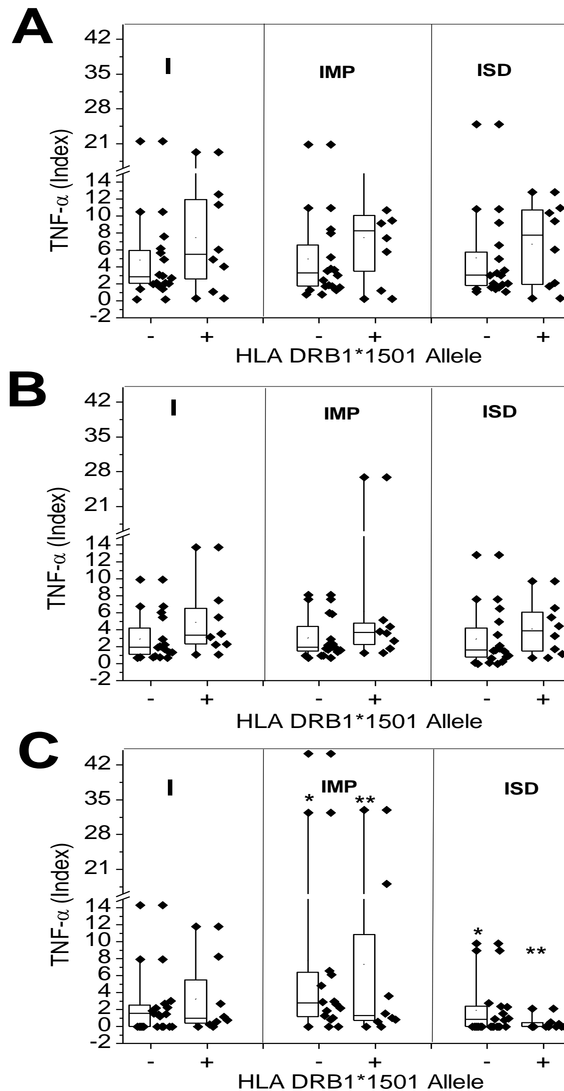


Figure 3.11: Median TNF-α secretion by MDMs from 24 healthy volunteers at 0 **(A)**, 6 **(B)** and 12 h **(C)** post treatment respectively. *HLA DRB1*1501* negative (-) IMP vs ISD; P<0.05 (*) and in positive (+) IMP vs ISD; p< 0.01 (**) using Mann-Whitney one tailed P test.

IL-6

The median index of IL-6 secretion by *DRB1*1501*$^{-}$ MDMs remained similar, i.e., around 3.2 at all time points in infected cells, while in *DRB1*1501*$^{+}$ MDMs, infection increased the median index from 5.8 to 18 at 6 h. At 12 hrs post treatment in *DRB1*1501*$^{+}$ cells, this value was around 10.6, significantly higher than the allele negative group ($p<0.05$) as shown in Figure 3.12. It has also been shown that *HLA DRB1*04*$^{+}$ TB patients secrete higher levels of IL-6 upon stimulation with Mtb, suggesting that other alleles in combination with *DR 15* regulate the IL-6 response to infection (Selvaraj, Nisha Rajeswari et al. 2007).

Treatment with micropaticles did not change IL-6 secretion by *DRB1*1501*$^{-}$ MDMs at any time point compared to untreated infection. There was a non-significant decreasing trend in the *DRB1*1501*$^{+}$ treatment groups and the magnitude were lower compared to untreated infection. Treatment with soluble drugs elicited more IL-6 compared to microparticles at 6 h. At 12 h, IL-6 declined in the *DRB1*1501*$^{+}$ group but microparticles maintained higher indices in both groups though differences from the ISD group were not statistically significant.

IL-10

IL-10, the Th2 cytokine was significantly elevated following infection in *HLA DRB1*1501*$^{+}$ at 0 h ($p<0.05$) and 6 hrs ($p<0.1$) as depicted in Figure 3.13. Selvaraj *et al* also reported higher spontaneous IL-10 in *DRB1*1501*$^{+}$ positive subjects. *DRB1*12*$^{+}$ normal human subjects showed increased cytokine response upon infection, suggesting multifactorial control in cytokine regulation (Selvaraj, Nisha Rajeswari et al. 2007). At 14 h post infection, the median IL-10 in both the positive and negative allele groups became almost equal if infection was left untreated.

Magnitude-wise, treatment with drugs in solution led to decrease in IL-10 at 6 h post treatment compared to infection and treatment with microparticles. At 12 h, the median index following treatment with microparticles was lower compared to treatment with drugs in solution. In the allele negative group the decrease was statistically significant ($p<0.1$).

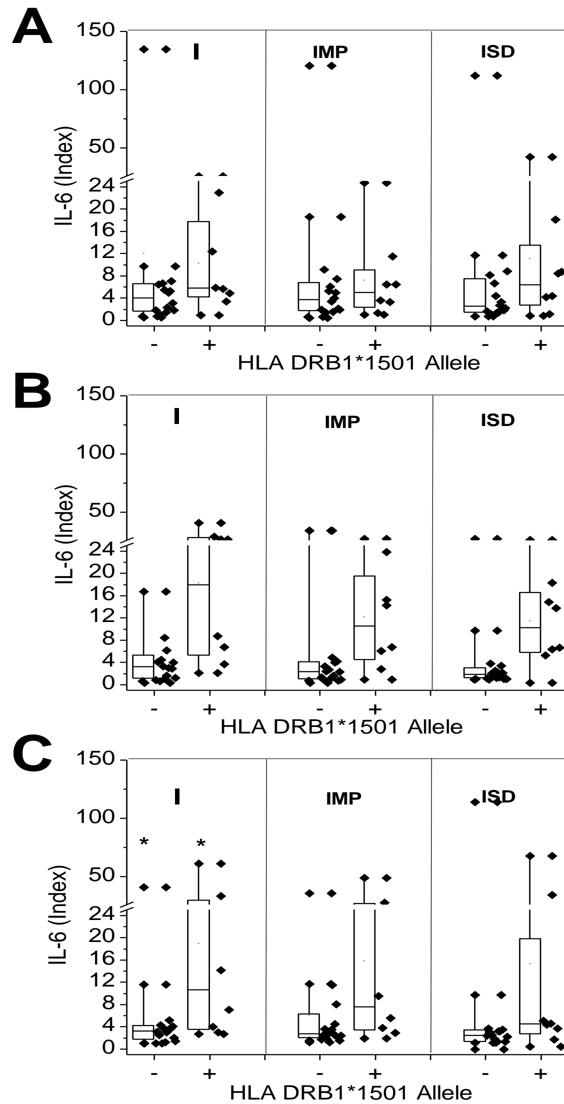


Figure 3.12: Median IL-6 secretion by MDMs from 24 healthy volunteers at 0 **(A)**, 6 **(B)** and 12 h **(C)** post treatment respectively. Panel **C**: *HLA DRB1*1501*$^{-}$ vs *DRB1*1501*$^{+}$ infection (**I**) group; P<0.05 (*) in Mann-Whitney one tailed P test

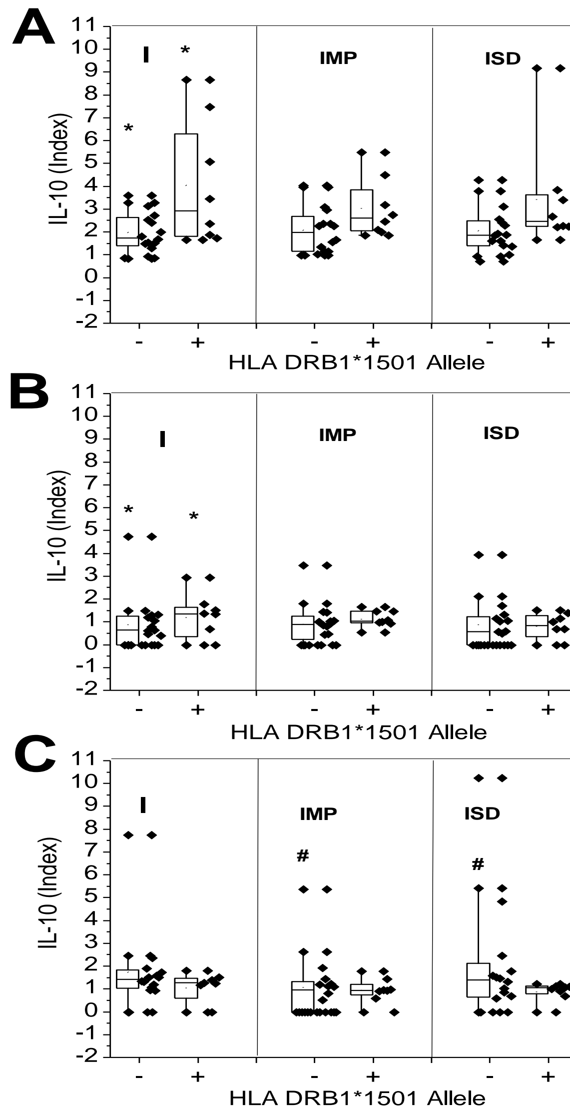


Figure 3.13: Median IL-6 secretion by MDMs from 24 healthy volunteers at 0 **(A)**, 6 **(B)** and 12 h **(C)** post treatment respectively. Panel **B**: **I** (-) vs **I** (+); p<0.1 (*), Panel **C**: HLA DRB1*1501^{-} **IMP** vs **ISD**; P<0.1 (#) in Mann-Whitney one tailed P test.

IL-4

Figure 3.14 depicts the effect of infection and different modes of treatment on IL-4. The cytokine was produced on almost equal median levels in both groups following infection up till 8 h post treatment. At 14 h post infection, levels were higher in the allele positive group (p =0.07). Increase in magnitude in allele positive donors was about 1.7 times compared to 6 h indices. In

the allele negative donor group, median levels were even lower than uninfected controls (ranged around 0.76 to 1) throughout the study. Treatment with either the drugs in solution or microparticles significantly ($p<0.05$) decreased the median value in the allele positive group compared to infection alone.

The delay in IL-4 responses compared to TNF-α secretion may have resulted from homeostatic or pathogen-induced mechanisms to dampen the effect of Th1 cytokines produced earlier in the couse of infection. The role of Th1/Th2 cytokine cross-regulation, including via IL-4 is currently under debate with regard to establishment of Mtb infection. Rook and collegues (Hernandez-Pando, Aguilar et al. 2004) have suggested that IL-4, in the presence of TNF-α, drives macrophages to necrosis rather than apoptosis. North and colegues have proposed that the role of IL-4 lies in antagonizing the effect of Th1 cytokines and thus resolving TB infection (Jung, LaCourse et al. 2002). IL-4, by decreasing TNF-α and NO production could also help Mtb bacilli to survive in the macrophage phagosome (Bogdan, Vodovotz et al. 1994; Nemoto, Otsuka et al. 1999) (Riendeau and Kornfeld 2003).

In the *HLA DRB1*1501* positive group, there was concomitant decrease in TNF-α and increase in IL-4 median levels, suggesting a pathway of downregulation of Th1 responses and increased susceptibility to TB. Treatment with drug containing microparticles decreased IL-4 levels across genotypes when compared to untreated infection and was equal or marginally better than treatment with soluble drugs.

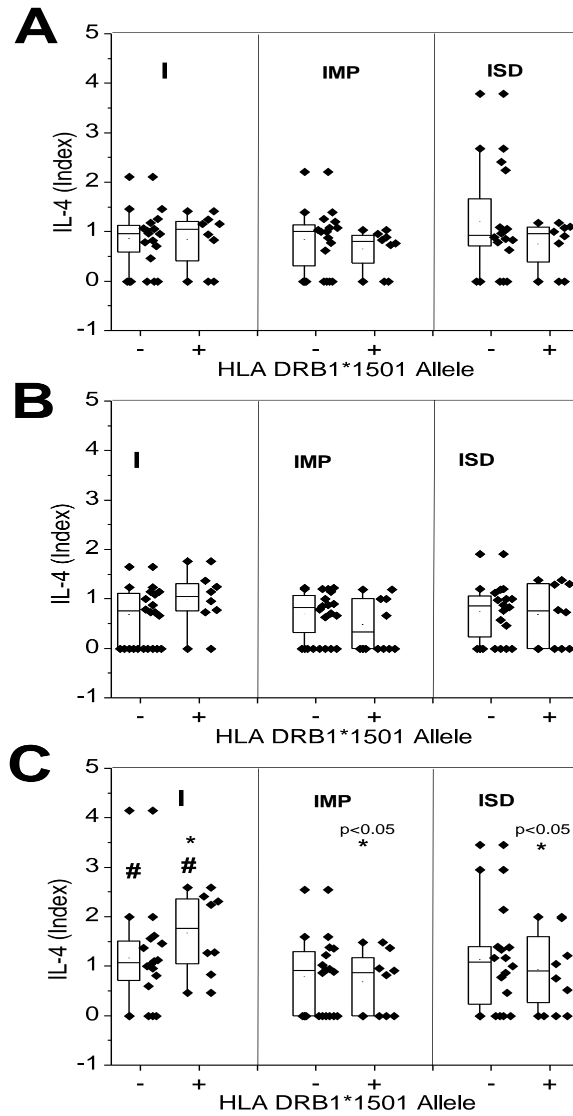


Figure 3.14: Median IL-4 cytokine indices (A, B and C) by peripheral blood mononuclear cell-derived macrophages from twenty four healthy volunteer with nomenclature same as figure 3.10. *HLA DRB1*1501* negative (-) vs positive (+) infection **(I)** group; P<0.1 (#), **+ (I)** vs **+ (IMP)** and **+ (I)** vs **+ (ISD)**; p<0.05 using Mann-Whitney one tailed P test

3.2.2 Cytokine secretion in context of S and R Scores

TNF-α

Median TNF-α indices in donors scored as S=0; i.e., non susceptible; ranged from 0.62 to about 4 following infection. For MDMs from donors with S=1, 2 and 3, it ranged from 1.5-2, 1.2-11.4, and 1.3-5.5 respectively from 2 to 14 h post infection (Figure 3.15). Although MDMs from more 'susceptible' donors initially responded to infection with increased TNFα secretion, a steep fall in TNF secretion ensued. A previous study has shown that infection of phorbol-differentiated THP-1 cell line ('susceptible'; S=3, R=0) initially resulted in high TNF-α secretion upon infection, but was followed by a steep fall later during the study (Yadav, Muttil et al. 2010). Donors in less susceptible group produced lower magnitude of response following infection than higher score group.

Treatment of infected cells with drug containing microparticles changed the median cytokine indices to 2-3.8, 1.8-2.6, 1.5-8.4, 1.5-9.9 while treatment with soluble drugs modulated the cytokine index from 0-3.4, 0.8- 2, 0.5-10.4 and 0- 6.3 respectively. From the above results it is clear that treatment of Mtb infected MDM's with drug containing microparticles induced and sustained higher TNF-α levels than treatment with drugs in solution. These results were similar to previous observations from THP-1 cells and primary macrophages of undefined genotype (Yadav, Muttil et al. 2010). Proinflammatory cytokines contribute both to innate and acquired immunity against TB and TNF-α play pivotal role in controlling the infection and granuloma formation. Thus, an advantage in chemotherapy of TB might be gained through the use of microparticles containing anti-TB drugs, since these evidently elicit TNF responses favouring innate bactericidal activity in addition to delivering the incorporated drugs to the intracellular compartment.

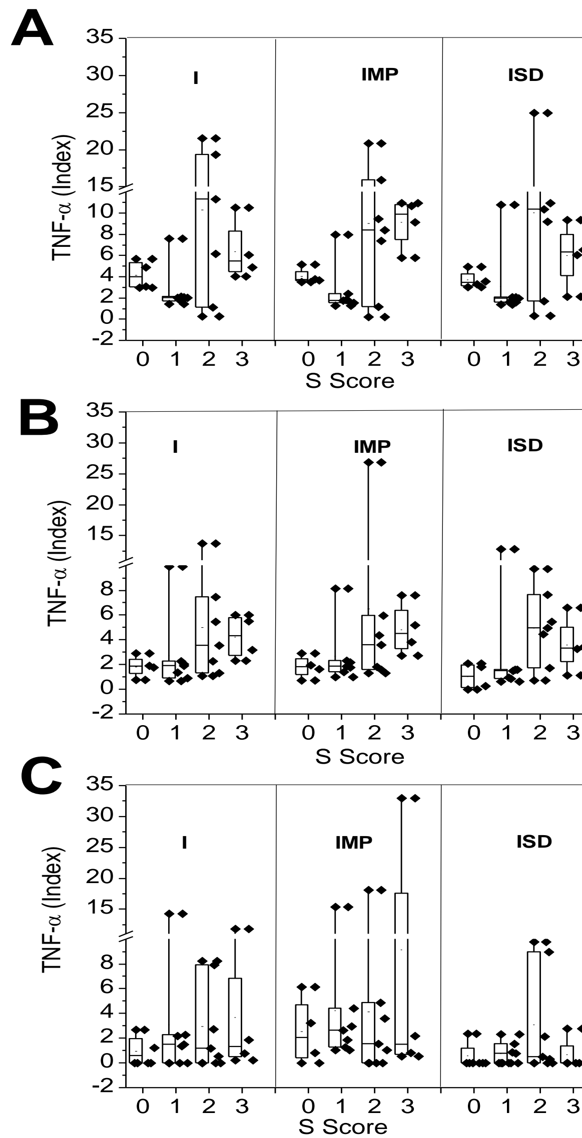


Figure 3.15: TNF-α secretion by MDMs of 24 healthy donors at 0 **(A)**, 6 **(B)** and 12 h **(C)** post treatment. Indices were calculated donor-wise, considering the concentration observed in the case of uninfected, untreated cells at each time point as 1. Group nomenclature: **I**, infected, untreated cells; **IMP**, infected cells treated with drug-containing microparticles; **ISD**, infected cells treated with drugs in solution.

IL-6

Figure 3.16 shows the secretion profile of IL-6 following infection and treatment at different time points.

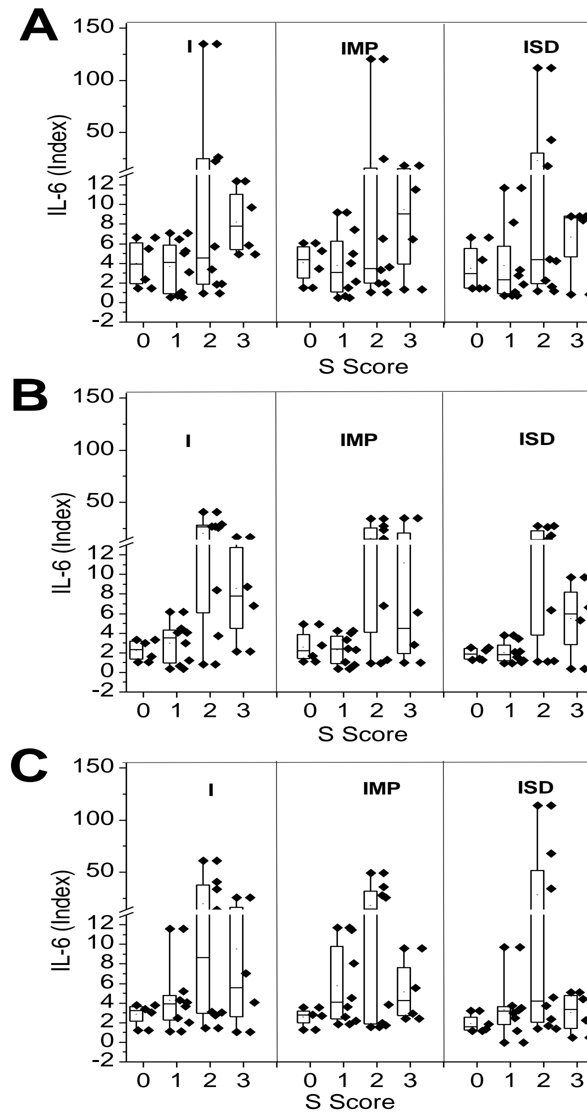


Figure 3.16: IL-6 secretion by MDMs of 24 healthy donors at 0 **(A)**, 6 **(B)** and 12 h **(C)** post treatment.

Constant levels of IL-6 were maintained till 12 h (median indices ranged from 3- 4) in infected cells from less susceptible donors while cells of more susceptible donors responded to infection with increased IL-6 secretion till 6 hrs. Indices increased from 4 to 26 for S=2 while S=3 showed constant indices (7.8). Secretion of this cytokine declined at 12 h (median indices 8.7 and 5.6 respectively for S=2 and S=3).

Treatment with drug-containing microparticles did not produce significant changes in IL-6 secretion when compared to untreated infection. Less IL-6 was secreted in the absence of treatment, but microparticles induced more IL-6 than treatment with soluble drug. One donor with S=2 consistently secreted higher IL-6 at all the points upon infection and following treatment.

IL-10

This Th2 cytokine was initially higher in infected cells from donors scoring S=2 and 3 but at 12 hrs there was no difference in cytokine indices across S scores (Figure 3.17). These results are consistent with the known lack of effect of the *DR 15* allele on IL-10 secretion. Treatment with drug containing microparticles decreased the median cytokine secretion indices at all the time points compared to infection and was almost equally effective when compared to drugs in solution in decreasing the cytokine.

IL-4

Median cytokine indices for IL-4 were almost constant if infection was left untreated in MDMs of donors scoring S=0, 1 and 2 at all the time points during the experiment. S=0 was an exception, wherein IL-4 increased at 12 hrs (Figure 3.18). For S=3, the median index followed an increasing trend at all the time points in the case of untreated infection. These results are in accordance with the known effect of the *DR 15* allele on IL-4 production. Treatment with drug containing mictoparticles decreased IL-4 secretion compared to untreated infection and was more effective than soluble drugs at 12 hrs post treatment across all groups.

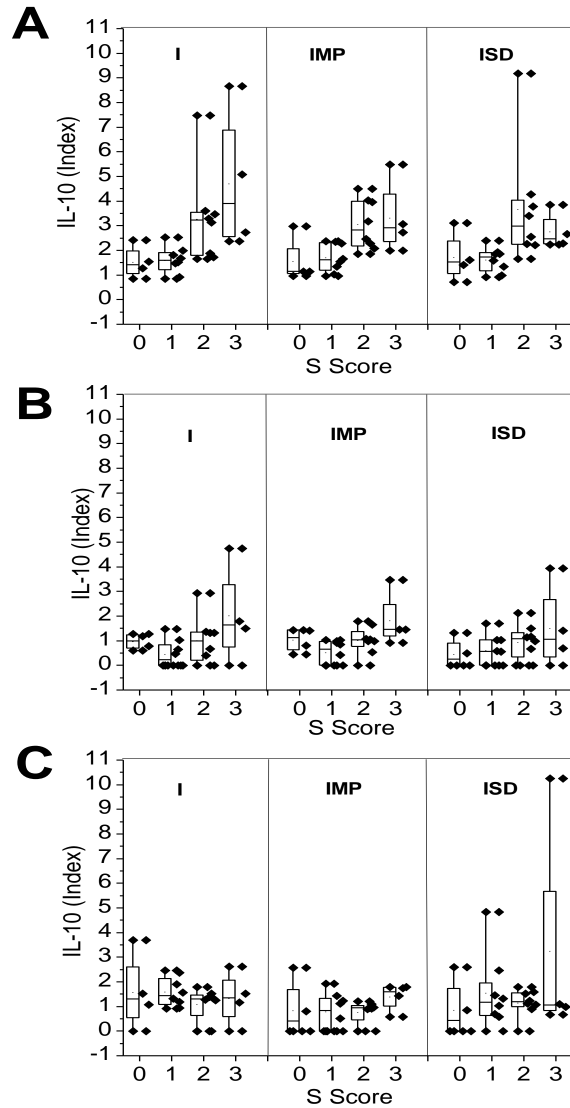


Figure 3.17:- Median IL-10 cytokine mean indices (A, B and C) by peripheral blood mononuclear cell-derived macrophages from twenty four healthy volunteers with nomenclature same as figure 3.15.

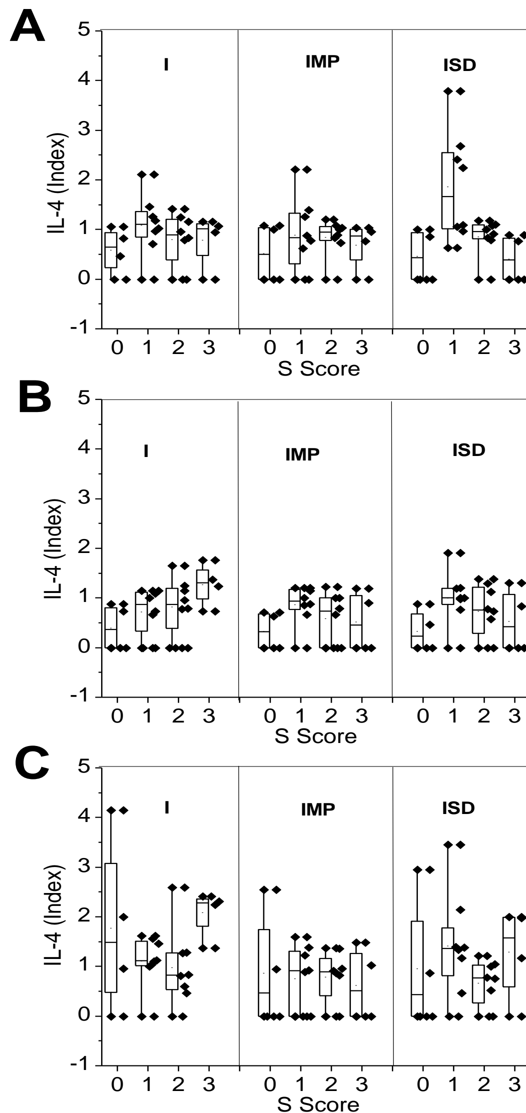
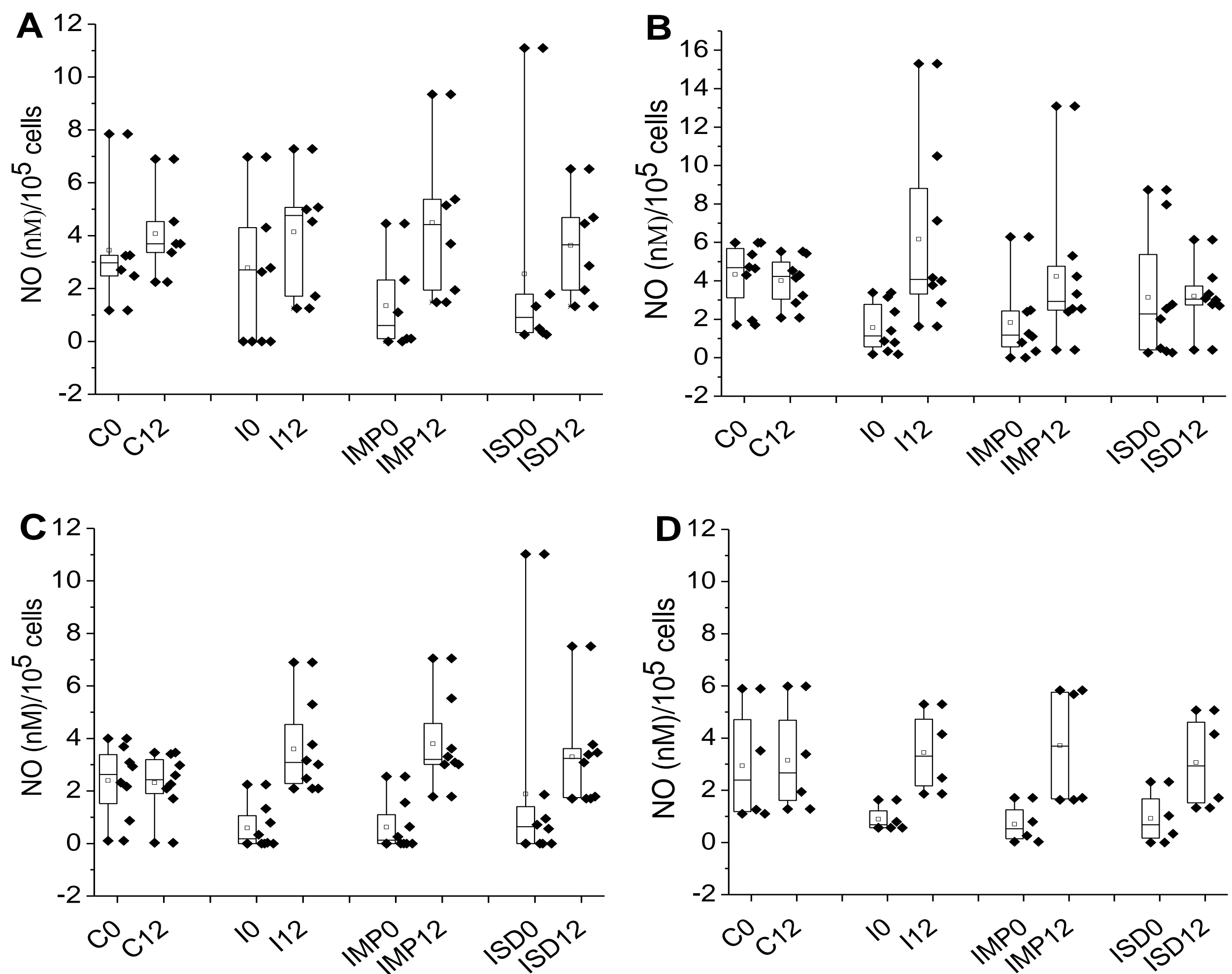


Figure 3.18: Median IL-4 cytokine indices (A, B and C) by peripheral blood mononuclear cell-derived macrophages from twenty four healthy volunteers with nomenclature same as figure 3.15.

3.3 Nitric oxide production by primary MDMs after infection and treatment

Accumulation of nitric oxide was estimated in culture supernatant of the primary macrophages after infection and treatment. The basal median level of NO in groups S=0 and S=1 ranged from 3-3.7 nM and 4.7-4.2 nM at 0 and 12 h post treatment respectively. For S=2 and 3, basal NO ranged from 2.4-2.7 nM at both time-points. Thus, MDMs of donors of lower or non susceptible group secreted higher basal level of NO. Upon infection, there was an initial dip at 0

h. The median level of NO in groups S=0, 1, 2 and 3 was 2.7/4.8, 1.1/4.1, 0.2/3.1 and 0.7/3.3 respectively at 0h/12 h. Thus, infection initially decreased NO production by primary macrophages across the groups and the dip was more prominent in more susceptible groups (S≥2). At 12 hrs post treatment, infected MDMs from all the volunteers showed increasing trend of NO production. Interestingly, cells from donors of lower 'susceptibility' produced more NO than more 'susceptible' ones. Treatment with drug containing microparticles sustained or increased the level of NO across the groups and induced either higher or equal NO compared to treatment with soluble drugs. The rate of increase was higher in microparticle-treated cells.



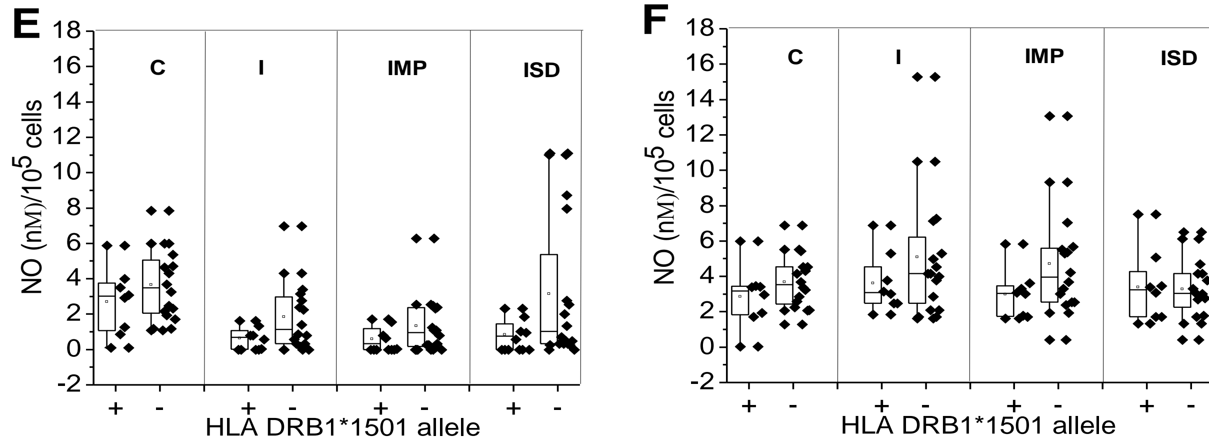


Figure 3.19: NO production by MDMs of healthy donors at 0 and 12 h post treatment. **(A-D):** S=0, S=1, S=2 and S=3. **(E)** NO production at 0 h by HLA *DRB1*1501*+ and *HLA DRB1*1501*⁻ donors **(F)** NO production at 12 h by HLA *DRB1*1501*+ and *HLA DRB1*1501⁻ donors*

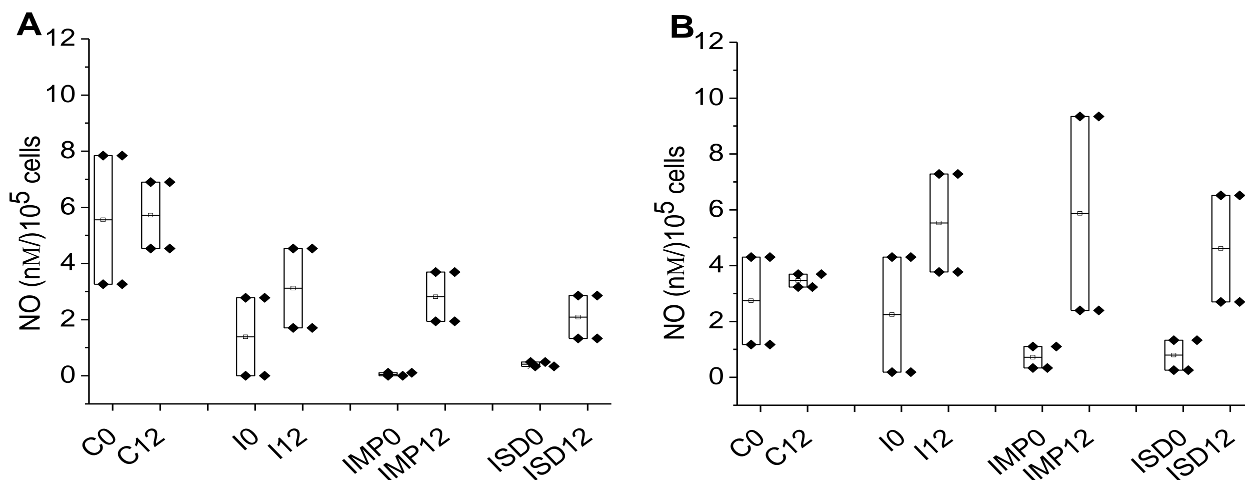
When volunteers were stratified according to presence or absence of *HLA DRB1*1501* allele, the basal level of NO was slightly higher in the allele negative group. There was no change in the median level of NO upon infection with Mtb in the allele positive donor group while the allele negative group responded to infection by increasing the median level of NO. Treatment of infected cells with drug containing microparticles maintained the median level of NO production while treatment with drug in solution leads to a decrease compared to infection and treatment with micropaticles.

It was observed that infected cells left untreated showed increased median NO at 12 hr post treatment or 14 hrs post infection. Thus primary cells across groups produced NO upon infection at 2 hrs post infection and cells from volunteers of lower 'susceptibility' apparently resisted infection more efficiently by maintaining higher level of NO at 14 hrs post infection. In the absence of *HLA DRB1*1501* allele, a marginal increase in basal NO level was observed in unifected cells, thus indicating that beside HLA, other factors also regulate the production of NO production. A study by Rockett *et al* has shown the inducible effect of vitamin D3 on NO production (Rockett, Brookes et al. 1998). Keeping in view that vitamin D3 action is affected by the presence of receptor variants (*VDR*) it is likely that *VDR* could be one such factor in the present study, since the S score took VDR variants into account. Thus, a combination of HLA

and *VDR* genotypes might have lead to more pronounced difference between basal NO levels of donors with different S scores.

Treatment with drug containing microparticles maintained or increased median NO across groups when compared to infection alone or treatment with soluble drugs. This observation is similar to those by Yadav *et al* and Sharma *et al* who observed increased NO production upon treatment with microparticles than soluble drugs (Sharma, Muttil et al. 2007; Yadav, Muttil et al. 2010).

When donors were further rearranged on the basis of the net susceptibility score, i.e S-R, not much difference was observed between NO release across scores ranging from -3 to +3 in uninfected, untreated cells at both 0h and 12h post treatment (Fig. 3.20). Two hrs after infection (0 h post treatment), MDMs scoring +1 produced smaller amounts of NO as compared to those scoring +3, though differences were not significant. At 12 h, for all scores, there was a rising trend in infection alone. As before, treatment with drug containing microparticles sustained or marginally increased NO production compared to treatment with drugs in solution. The possible reasons for lack of correlation between the arbitrary score employed here and NO production may include: (*a*) the lack of a sufficient number of donors in each group to produce statistically significant differences, or (*b*) the very weak association (OR around 0.25) of the protective allele AA (*ApaI*) and BB (*BsmI*) with disease resistance. Weak association was also observed in the case of the *HLA DR15* allele and NO production.



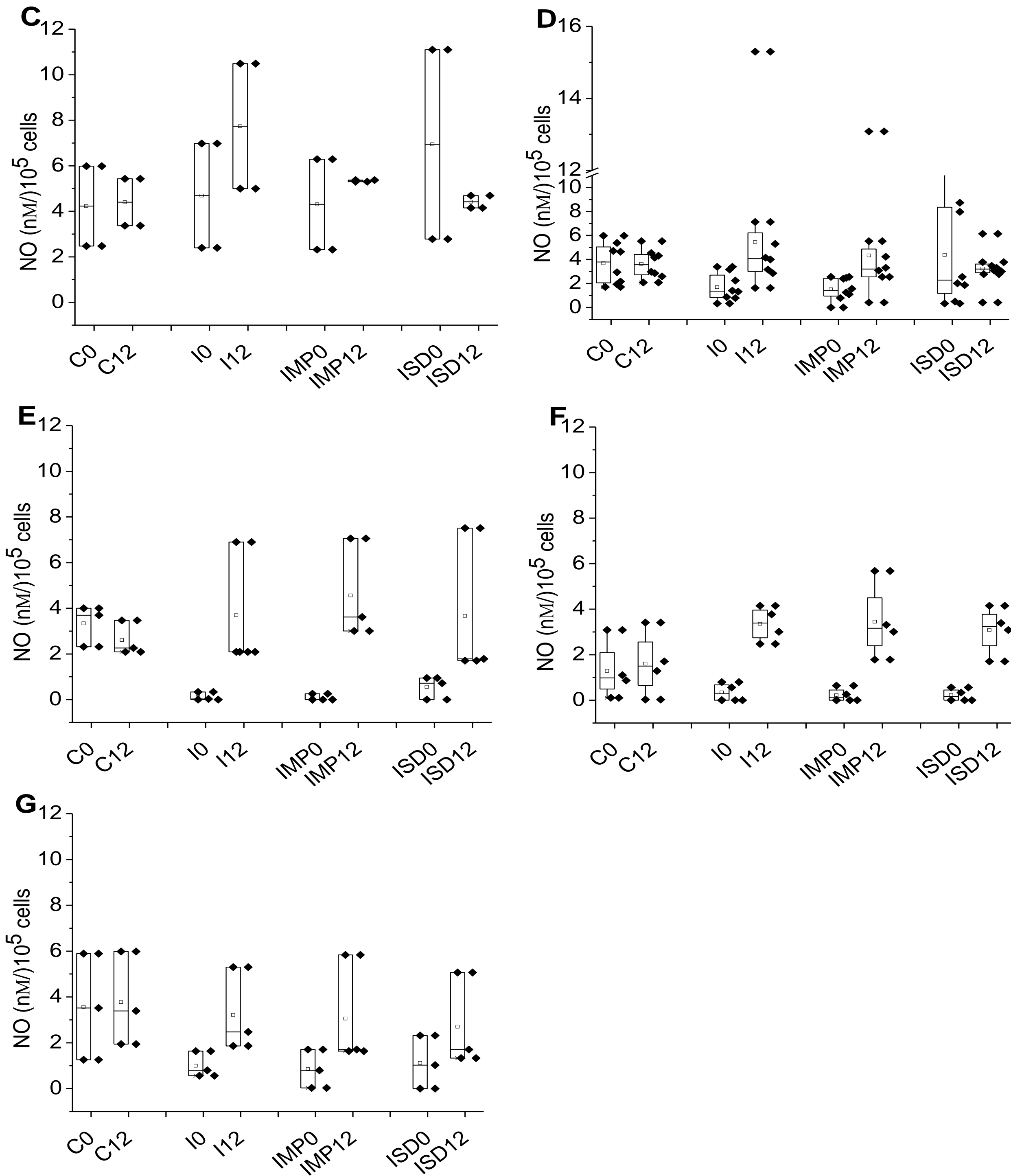


Figure 3.20: NO production by MDMs of healthy volunteers based on net 'S' score. **(A-G):** S=-3, S=-2, S=-1, S=0, S=1, S=2 and S=3

3.4 Survival of intracellular bacteria

In order to relate the production of proinflammatory cytokines and NO production to intracellular survival of mycobacteria, the viable bacterial count was determined by plating the cell lysate prepared 48 h after treatment (50 h post infection). CFU were estimated 3 weeks later. Both soluble drugs and drug-containing microparticles demonstrated bactericidal action across groups. In the absence of treatment, surviving bacteria recovered from the cell lysate increased proportionately with the S score. Thus, log 3.9, 4.1, 4.2 and 4.5 CFU were recovered from untreated infected cells having S=0, 1, 2 and 3 respectively. Treatment with drug-containing microparticles reduced the CFU count to log 1.9, 2.6, 2.4 and 2.5 while soluble drugs reduced CFU to log 2.7, 2.9, 2.8 and 2.7 respectively.

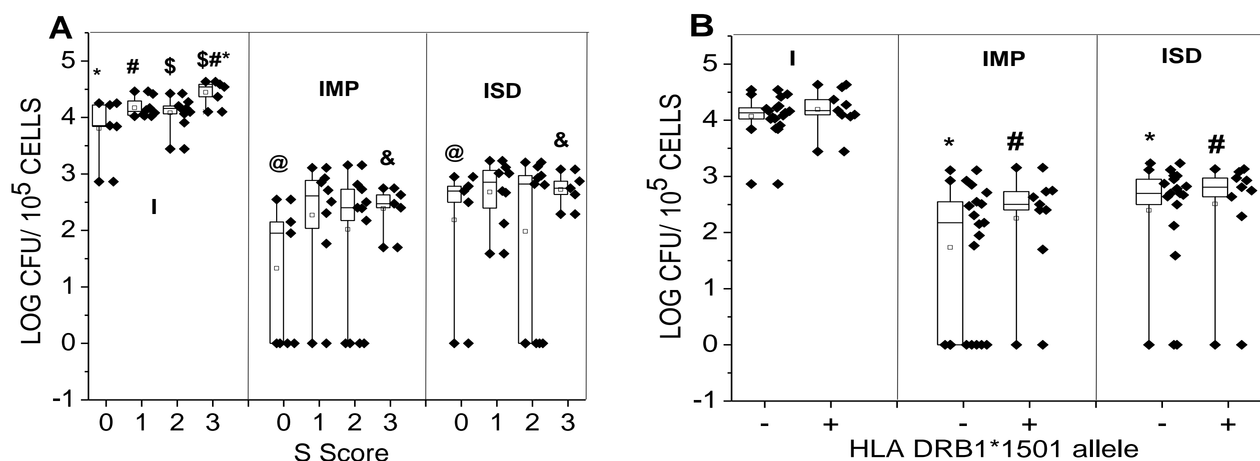


Figure 3.21: Box plot of Mean (*n*=2) $\log_{10}$ CFU at 48 h post treatment in cell lysates of MDMs derived from healthy donors. (**A**): CFU versus S scores. (**Left**) Untreated infected cells. (* S=0 vs S=3; p< 0.05, # S=1 vs S=3; p< 0.05, $ S=2 vs S=3; p< 0.05). (**Center**) IMP: treatment with microparticles. (**Right**): ISD: treatment with soluble drugs. IMP vs ISD (@ S=0; p< 0.1, & s=3; p< 0.1) in Mann-Whitney one tailed P test. (**B**) Box plot based on donors arranged according to presence (+) or absence (-) of *HLA DRB1*1501* allele. IMP vs ISD; * represents p<0.05 and # represents p<0.1 in Mann-Whitney one tailed P test)

When donors were rearranged according to the presence or absence of the *HLA DRB1*1501* allele, bacterial survival in the negative group was lower than in the allele positive group. But the magnitude of difference was less, indicating that VDR also plays a role in affecting intracellular viability of Mtb. Treatment with microparticles reduced the CFU count to zero in a few volunteers in all groups. It is clear from the above observations that genetic factors

do play a role in intracellular bacterial survival and that both *HLA* and *VDR* variants are contributing factors to intracellular bacterial survival. Decrease in intracellular viability by soluble drugs can be ascribed to drug intrinsic action while drug containing microparticles utilized innate immunity component of macrophages to exhibit bactericidal action.

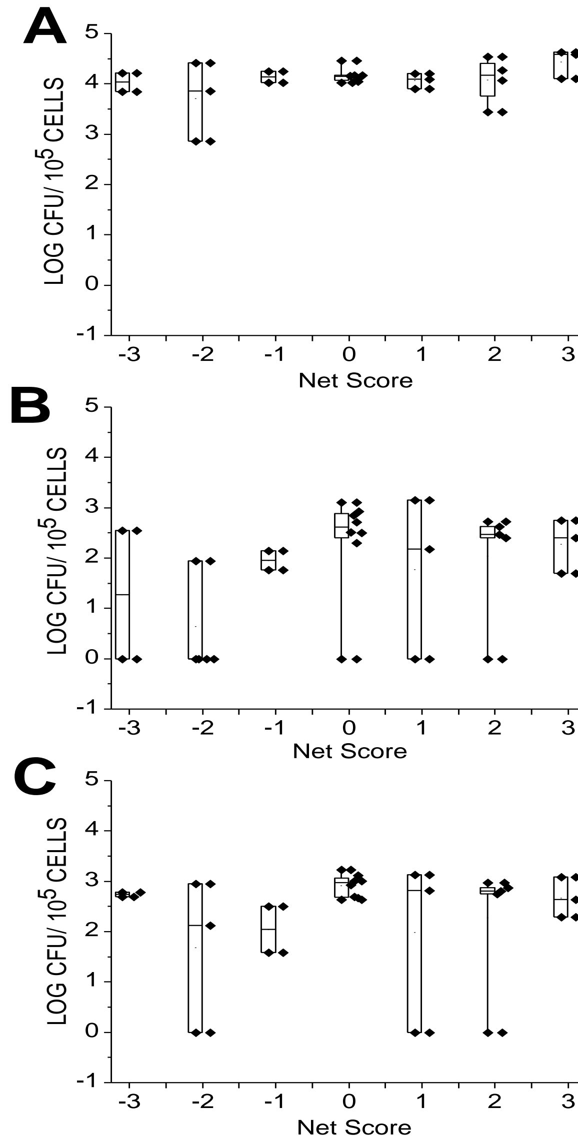


Figure 3.22: Log_{10} CFU at 48h post treatment vs net 'S' i.e. S-R Score. **(A):** CFU in the absence of treatment. **(B):** In infected cells treated with drug-containing microparticles. **(C):** in infected cells treated with drugs in solution (Infection alone: P≤0.1 for net 'S' -3 to +1 vs net 'S'+3, IMP vs ISD: P< 0.05 in 'S'0 and 'S' +2 in Mann-Whitney one tailed P test)

In order to determine whether net susceptibility 'S' score have any effect upon infection and response to treatment, donors were regrouped. Figure 3.22 illustrates box plots of log_{10} CFU count across different groups based on net 'S' score. In the case of untreated infection, infection (panel A), the CFU count ranged from log 3.9 to 4.1 for S-R= -3 to +1, while for S-R= +2 and +3, it was log 4.2 and log 4.5 respectively. Thus the net S score suggests a putuative contribution of the absence of the *HLA DRB1*1501* allele and concomitant presence of protective *AA* and *BB* alleles of the VDR subtype in reducing intracellular bacterial survival ($P \leq 0.1$ for S-R= -3 to +1 vs S-R=+3). Treatment with both drug containing microparticles (panel B) and drugs in solution (panel C) reduced bacterial count by about 2 log in all the donors irrespective of S-R score. However, microparticles elicited greater reduction in viable bacterial count as compared to equivalent amounts of drugs in solution, irrespective of the net 'susceptibility' of the donor ($p < 0.05$ in 'S'0 IMP vs ISD and 'S' +2 IMP vs ISD).

Bacterial survival was also assessed simaltenously using the Bactec assay. The Bactec system offers the advantage of assessing the bacterial viability at the earliest and also allows the growth of such bacteria as are unable to grow on solid medium. It works on the principle of measurement of radioactive $^{14}CO_2$ released due to catabolism of radioactive ^{14}C palmitate included as substrate in the broth. The BACTEC 460 system measures the amount of $^{14}CO_2$ accumulated in the head space above the liquid medium of culture vials in terms of growth index (GI). GI are the arbitrary units ranging from 0 to 999 and correlate microcuries of radioactivity detected.

In Figure 3.23, values on the X-axis represent the number of days reqired to attain GI of 999 and are thus inversely proportional to surviving Mtb in cell lysate. In some vials, no growth occured even after 60 days post inoculation. Therefore, to represent those donors and treatment methods an arbitrary number of 60 days was selected to facilitate depiction in the Figure. Median number of days required to attain GI of 999 in lysate from infected cells ranged from 14.5- 16 in donors having S=0,1 and 2 respectivley, while for S=3 it took 10.5 days to attain GI of 999. Treatment with drug containing microparticles signicantly reduced the bacterial viability and increased the numbers of days to attain GI of 999. MDMs of donors with S=2 and 3 responded equally well and the median number of days required for GI 999 increased to 24. Differences between different modalities of treatment were pronounced in more susceptible MDMs and the

number of days required to attain GI of 999 fell to 21 and 20.5 respectively for S=2 and 3. This observation also suggests that microparticles had higher bactericidal activity even in more 'susceptible' MDMs.

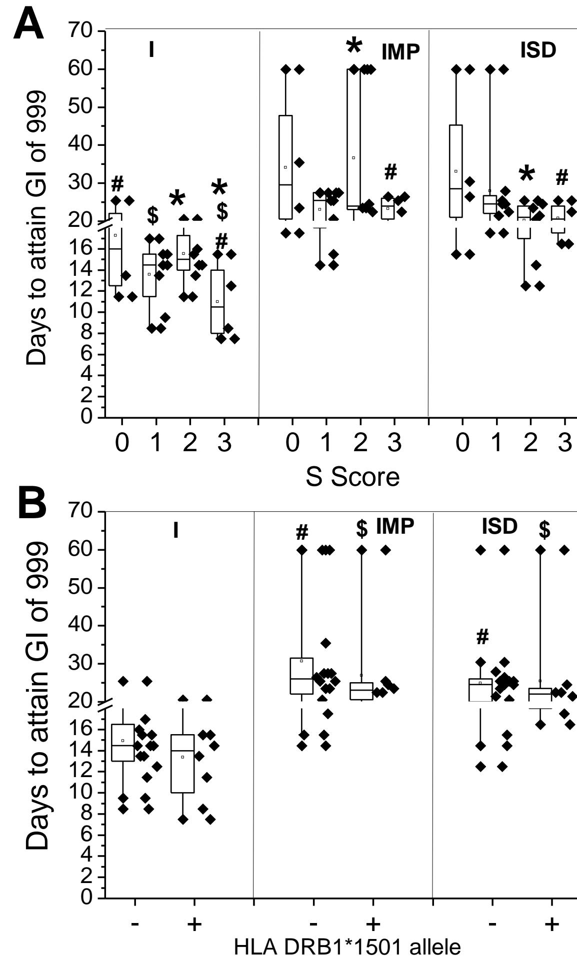


Figure 3.23: Survival of Mtb H37Rv in cell lysate expressed as days required to attain growth index (GI) of 999 in different groups. **(A)** Donors arranged on the basis of S score (* S=2 vs S=3: $p<0.05$; # S=0 vs S=3: $p<0.1$; $ S=1 vs S=3: $p< 0.1$ in Mann-Whitney one tailed P test), for IMP vs ISD: (* S=2: $p<0.05$; # S=3: $p< 0.17$ in Mann-Whitney one tailed P test). **(B)** Donors arranged according to presence (+) or absence (-) of *HLA DRB1*1501* allele (# represents $p<0.13$ and $ represents $p<0.17$ in Mann-Whitney one tailed P test). (**I**): infected, untreated MDMs, (**IMP**): infected cells treated with drug containing microparticles. (**ISD**): infected cells treated with drugs in solution.

When donors were rearranged according to presence or absence of the *HLA DRB1*1501* allele, the difference in the number of days required to attain GI of 999 was almost equal i.e. 14

and 14.5 days respectively, thus highlighting the role of VDR variants in TB susceptibility. After treatment with drugs in solution in the allele negative and positive MDMs it increased it to 24.5 and 22. Treatment with drug containing microparticles further increased GI 999 to 26 and 23 days respectively and differences were significant when compared to drugs in solution ($p<0.13$ and $p<0.17$ for *DRB1*1501* $^{-}$ and *DRB1*1501* $^{+}$ respectively).

This result demonstrates that genotype affects the intracellular bacterial survival and treatment with drug containing microparticles are equally effective across the group and difference with drugs in solution treatment were more pronounced in more susceptible group thus highlighting its efficacy through the process of "stimulat(ing) the phagocytes".

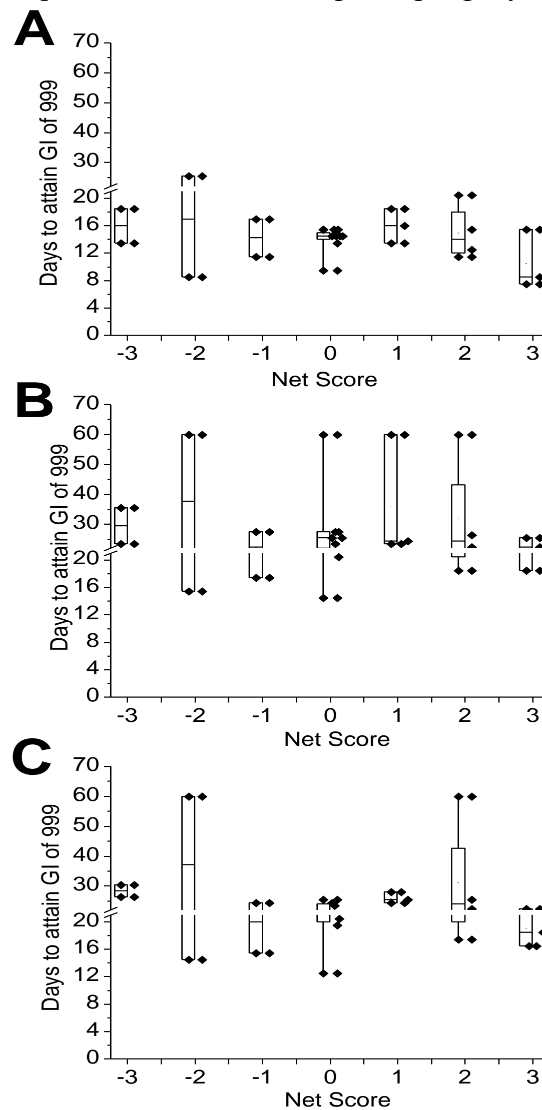


Figure3.24 (facing page): Box plot of growth indexes reflecting the survival of Mtb in MDMs of healthy donors after infection and / or treatment. Donors are arranged based on net 'S' score. **(A)** Infection. **(B)** Infected cells treated with drugs containing microparticles. **(C)** Treatment with drugs in solution

Median number of days required to attain GI of 999 in -3 to +2 'S' group ranged from 14 to 16 days while 'S'+3 group required 8.5 days to attain GI of 999. Treatment with drugs containing microparticles increased the median number of days (range 22.5 to 37.75) across the group and was effective than drugs in solution in reducing Mtb intracellular viability (median range 18.5 to 37.25) though differences were not significant.

3.5 Genome-wide transcription analysis of THP-1 cell line by Illumina

Genotyping study of THP-1cell line assigned it an arbitrary 'S' score of 3 which was highest among human donors studied. Therefore selecting THP-1 as representative of 'susceptible' individuals, gene expression profiling to assess the effect of infection and various treatment modalities at transcription level was investigated at 0 h and 24 h.

3.5.1 RNA isolation and quality assessment

Total RNA was isolated at 0 h and 24 h post infection from control and infected macrophages. RNA preparations showing OD260/OD 280 of 1.8 and above were further assessed for quality using an Agilent 2100 Bioanalyzer. Only RNA samples showing good yield and quality (RNA Integrity Number (RIN) >7.5) were used further for cRNA amplification and subsequent hybridization.

3.5.2 RNA amplification, cRNA fragmentation and chip hybridization with biotin labeled RNA

Biotinylated cRNA was prepared from 500 ng of total RNA using Illumina Totalprep RNA amplification kit. Following fragmentation, cRNA was hybridized to Illumia HumanHT-12v4 Beadchip containing probes for more than 47,000 transcripts, following manufacteruer's protocol and scanned using Illumina scanner (iScan). Array data export, processing and analysis were performed using Illumina Bead Studio 2.0 software.

3.5.3 Microarray data analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of internal quality control check and the raw scanned data. Raw data were extracted using the software provided by the manufacturer. The data was average normalized and and only those genes whose average signal intensity was greater than +30 and -30 cut off were analysed further. Fold change was calculated by dividing the average signal obtained in the case of infected and/or treated cells with that from the control group, i.e., cells that were neither infected nor treated. Only genes with two or above fold changes were considered stastically significant and analysed further.

Figure 3.25 summarizes the numbers of differentially regulated genes. Infection alone did not diffentially regulate any gene significantly compared to control while treatment with drugs in solution upregulated more genes compared to treatment with drug-containing microparticles. Microparticles downregulated more genes compared to drugs in solution. Blank microparticles caused more differential upregulation of genes at 0 h compared to 24 h post treatment. More genes were differentially regulated at 24 h in infected, untreated cells compared to treatment with different modalities.

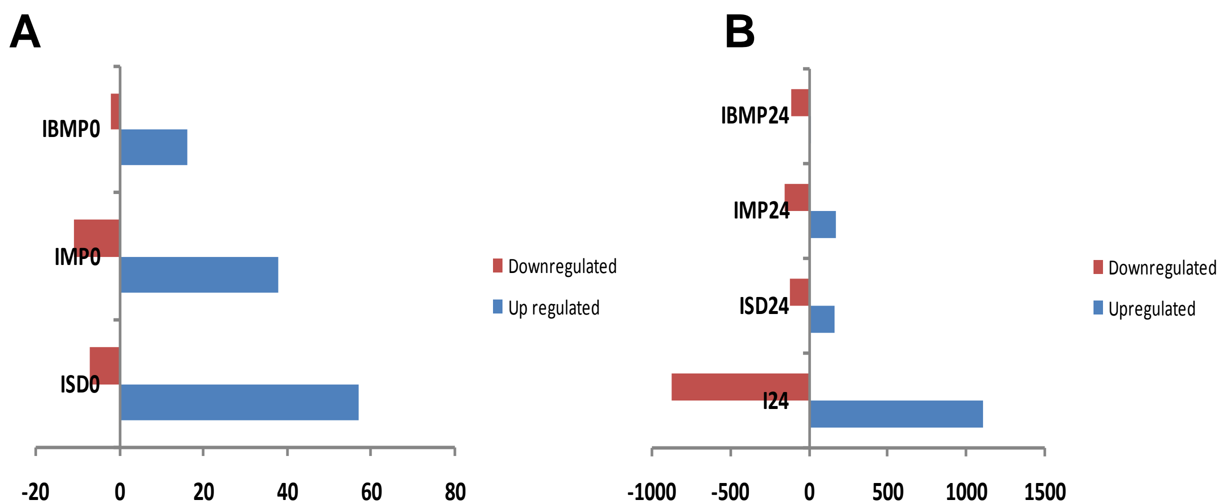


Figure 3.25: upregulated and downregulated genes at 0 h and 24 h

Functional profiling of biological process for differentially regulated genes in different group following infection and treatment with different drug modalities was performed using GENECODIS 2.0 software. Results for each group are represented from figure 3.26 to 3.33.

Biological processes are expressed as pie charts with numbers indicating the genes involved in that particular process. Functional profiling showed infection alone preferentially upregulated genes involved in metal ion binding, protein kinase activity and downregulated genes involved in RNA metabolic process and gene regulation. Treatment with both forms of drug modalities upregulated genes involved in immune response, signal transduction and inflammatory responses and downregulated expression of genes involved in rRNA, cell cycle and cell proliferation. Treatment of infected cells with blank microparticles upregulated genes of immune response, ERK1, ERK2 and JNK pathways while downregulating genes involved in cell membrane transport, carbohydrate metabolic process and DNA repair and cell proliferation. Figure 3.34 shows the heat map of genes upregulated or downregulated by at least two fold at 24 h following infection and treatment with different drug modalities.

It is evident from Figure 3.30 that at 24 h post treatment drug-containing microparticles were more effective in upregulating genes involved in inflammatory response, chemotaxis and cell signaling.

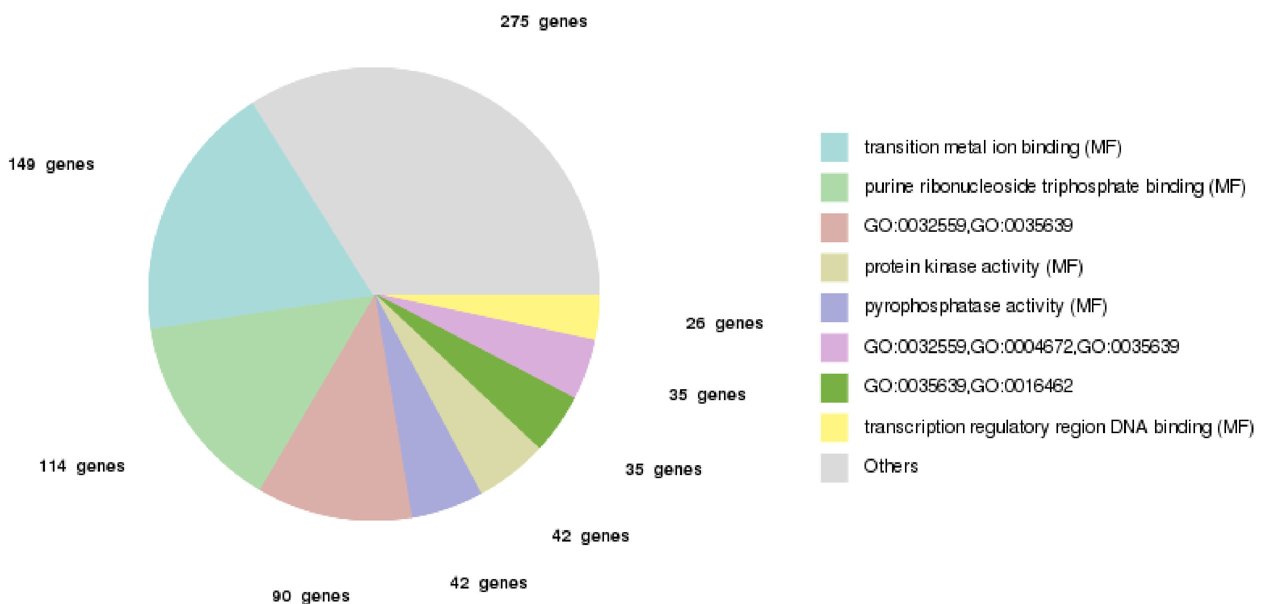


Figure3.26: Functional profiling of biological processes represented in a set of genes differentially upregulated following infection at 24 h.

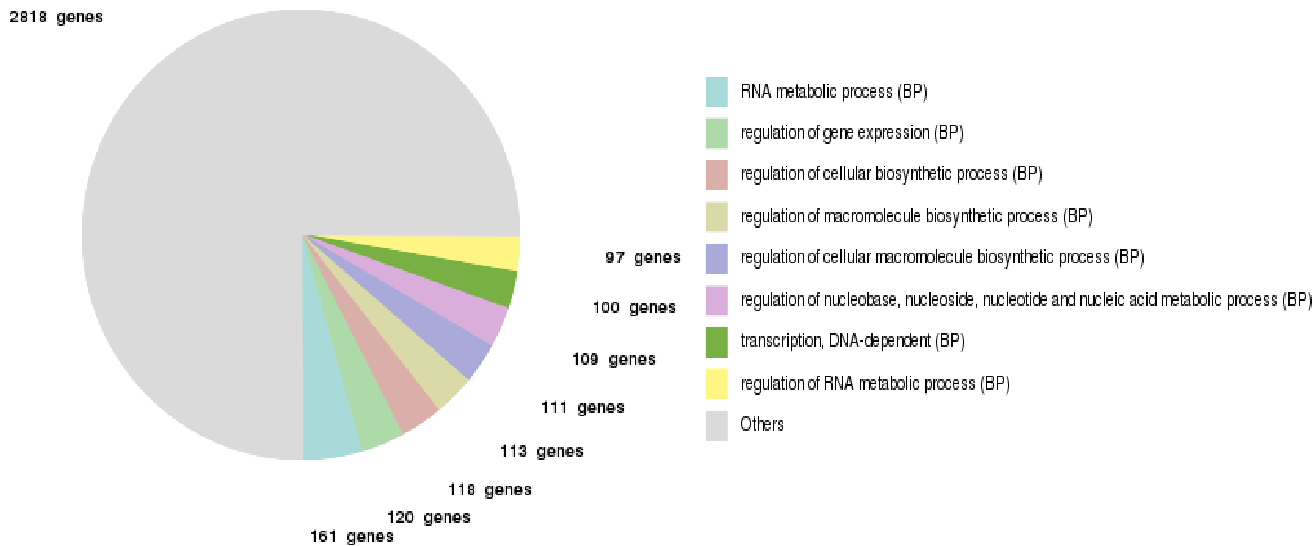


Figure3.27: Functional profiling of biological processes represented in a set of genes differentially downregulated following infection at 24 h.

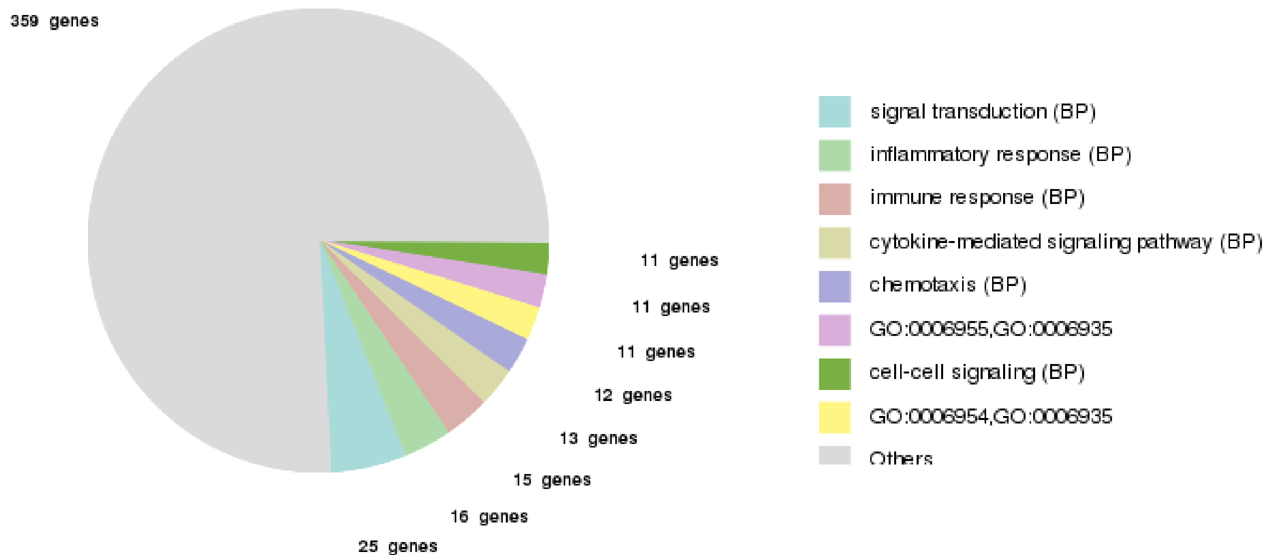


Figure3.28: Functional profiling of biological processes represented in a set of genes differentially upregulated following treatment of infected cells with drugs in solution at 24 h.

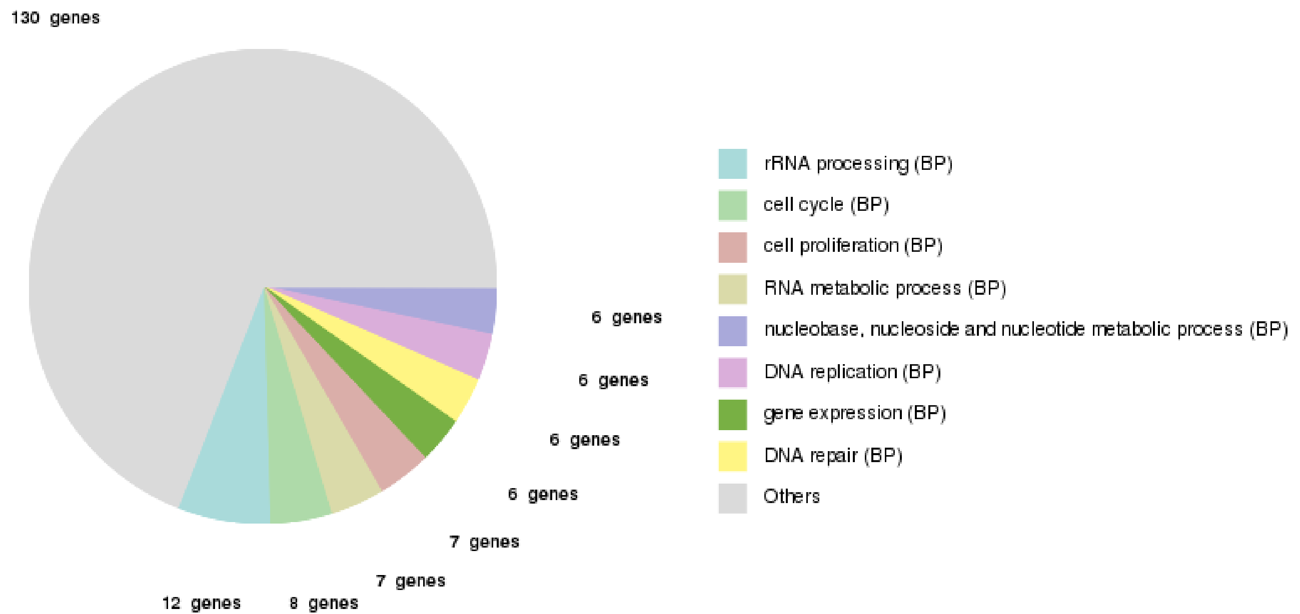


Figure3.29: Functional profiling of biological processes represented in a set of genes differentially downregulated following treatment of infected cells with drugs in solution at 24 h.

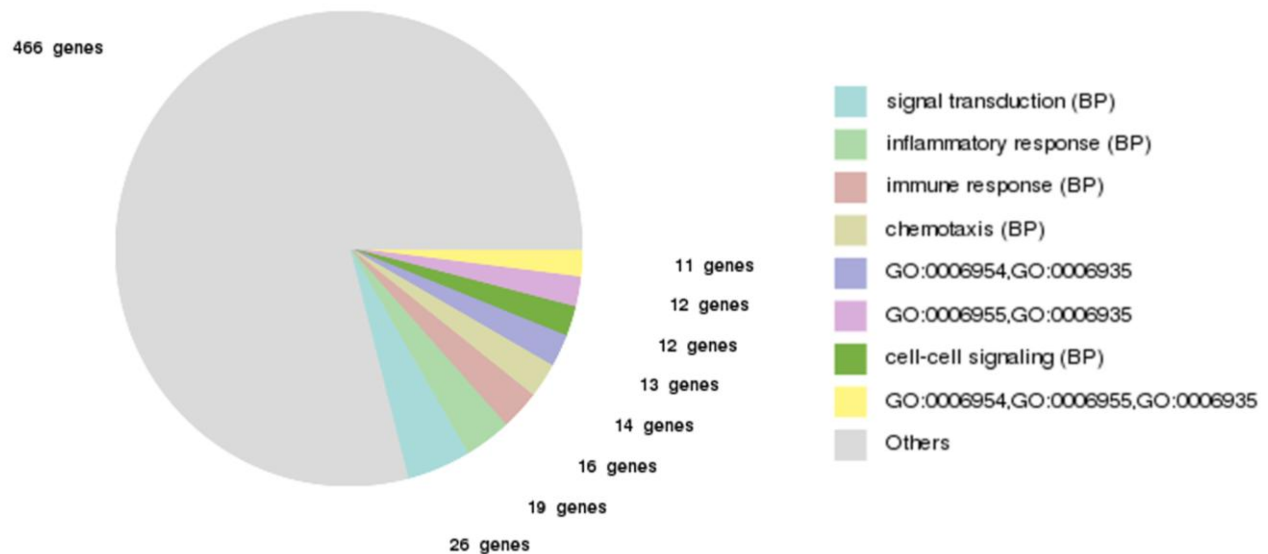


Figure3.30: Functional profiling of biological processes represented in a set of genes differentially upregulated following treatment of infected cells with drug- containing microparticles at 24 h.

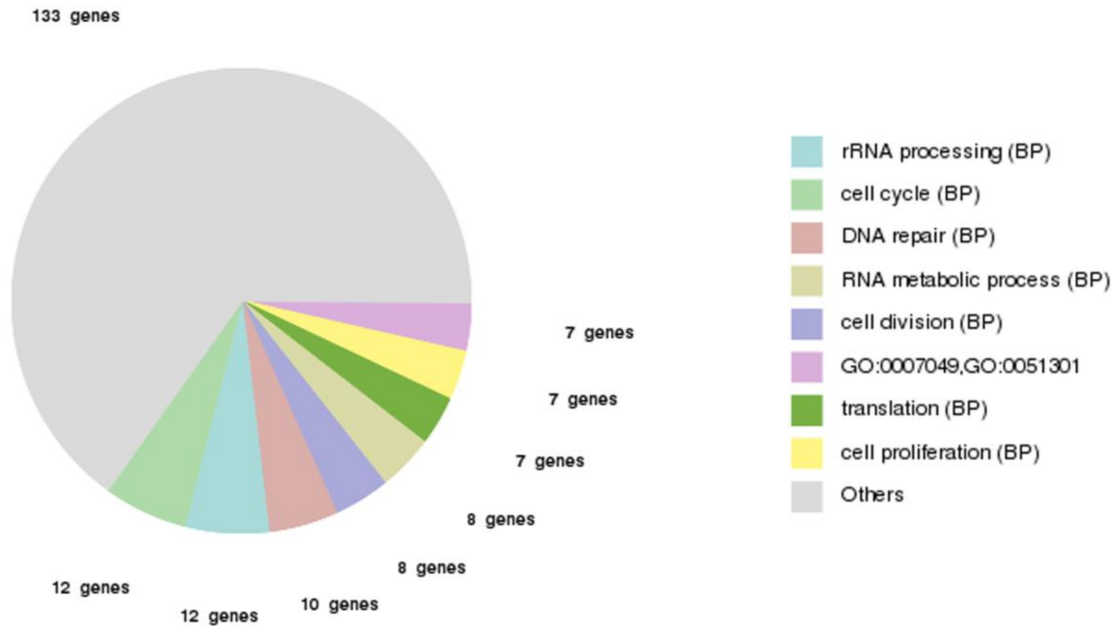


Figure3.31: Functional profiling of biological processes represented in a set of genes differentially downregulated following treatment of infected cells with drug-containing microparticles at 24 h.

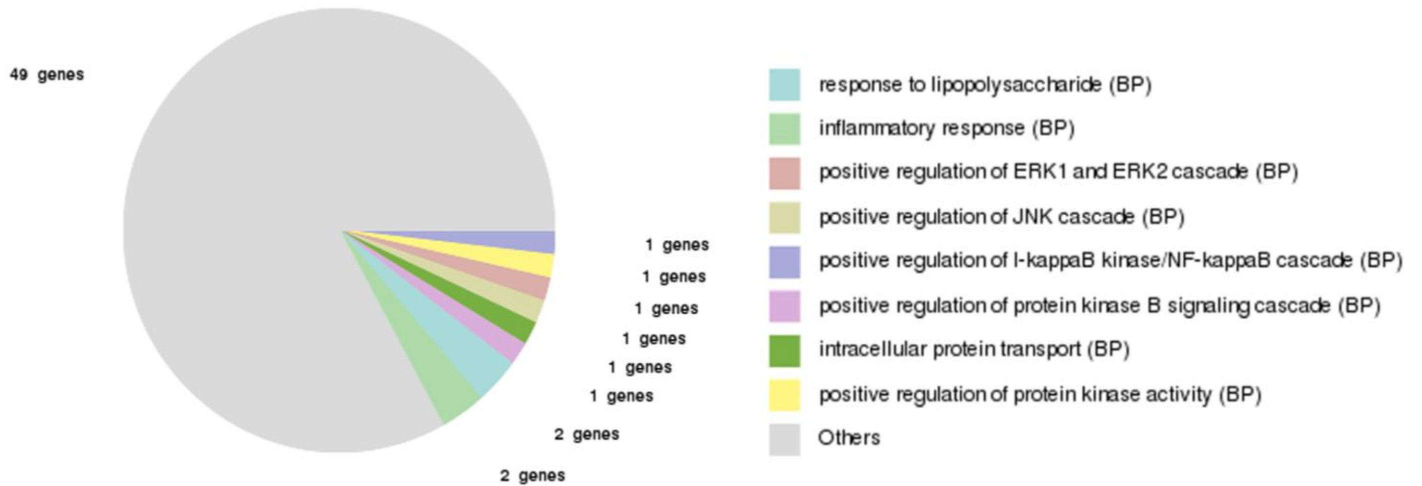


Figure3.32: Functional profiling of biological processes represented in a set of genes differentially upregulated following treatment of infected cells with blank microparticles at 24 h.

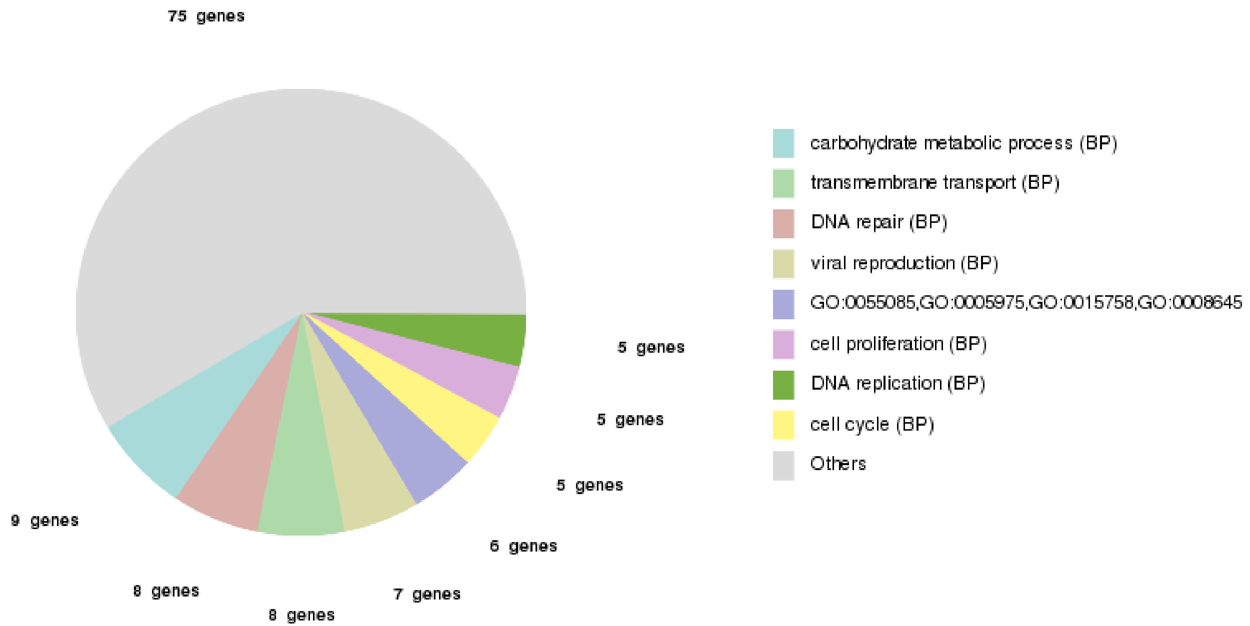


Figure3.33: Functional profiling of biological processes represented in a set of genes differentially downregulated following treatment of infected cells with blank microparticles at 24 h.

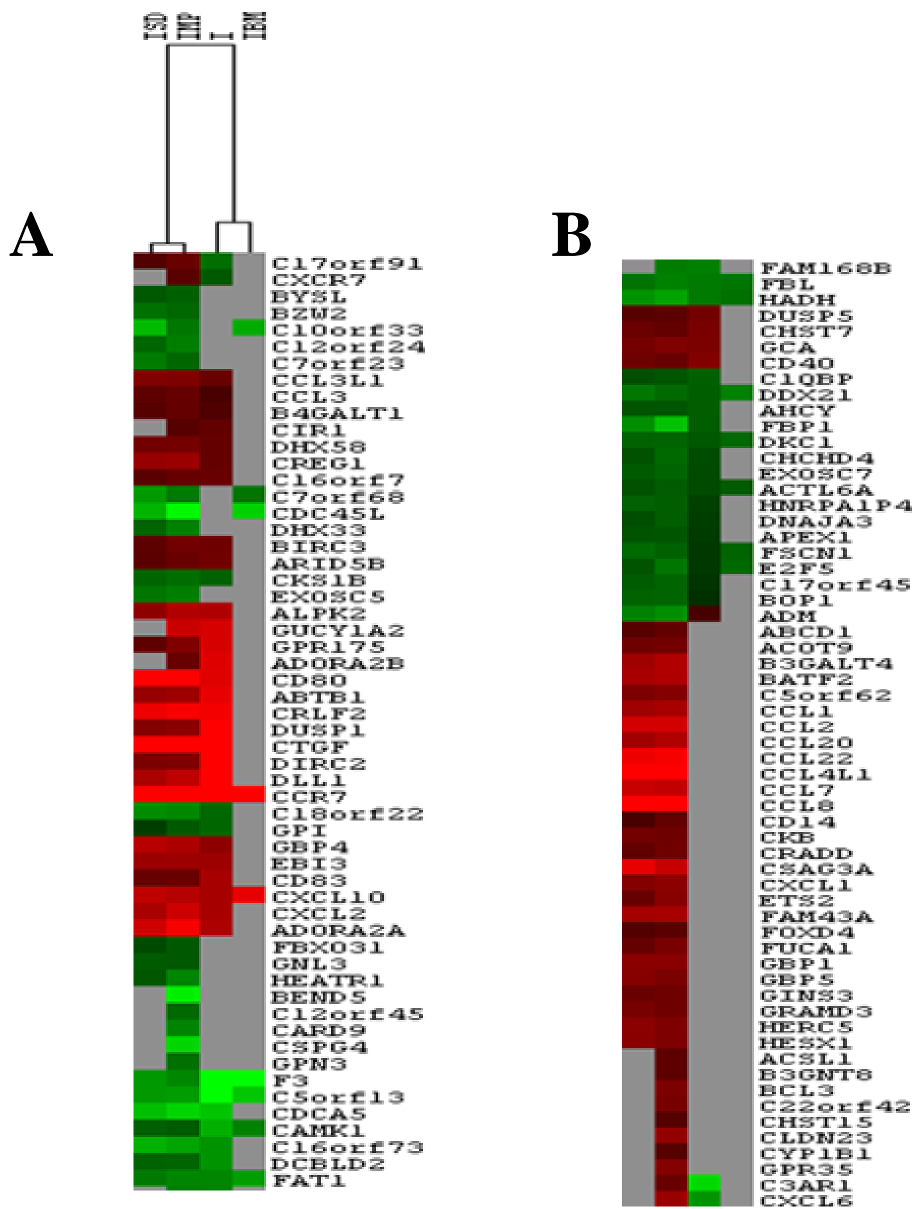


Figure 3.34: Heat map of genes regulated at least two fold following infection and treatment with different drug modalities at 24 h. Panel **A** and **B** are part of the cluster. Shades of green and red show downregulation and upregulation respectively.The experiment was performed in duplicate (n=2).

Table 3.9: Differential downregulated genes related to apoptosis following infection at 24h

YWHAB	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide
RXRA	retinoid X receptor, alpha
BCLAF1	BCL2-associated transcription factor 1
TRIAP1	TP53 regulated inhibitor of apoptosis 1
F3	coagulation factor III (thromboplastin, tissue factor)
TOP2A	topoisomerase (DNA) II alpha 170kDa
MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)
BCL2L2	BCL2-like 2
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)
GPI	glucose-6-phosphate isomerase
BIRC6	baculoviral IAP repeat containing 6
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining)
TSC22D1	TSC22 domain family, member 1
MLLT11	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11
ATF5	activating transcription factor 5
RPS6KB1	ribosomal protein S6 kinase, 70kDa, polypeptide 1
ACAA2	acetyl-CoA acyltransferase 2
CUL5	cullin 5
NRBP2	nuclear receptor binding protein 2
PRKDC	protein kinase, DNA-activated, catalytic polypeptide
SERBP1	SERPINE1 mRNA binding protein 1
PPIF	peptidylprolyl isomerase F
SERPINE1	serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1),

	member 1
PSME3	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)
CX3CR1	chemokine (C-X3-C motif) receptor 1
PHB	prohibitin
PRDX2	peroxiredoxin 2
RHOA	ras homolog gene family, member A
TMBIM6	transmembrane BAX inhibitor motif containing 6
PSMD8	proteasome (prosome, macropain) 26S subunit, non-ATPase, 8
SPRY2	sprouty homolog 2 (Drosophila)
CXCR7	chemokine (C-X-C motif) receptor 7
NUDT2	nudix (nucleoside diphosphate linked moiety X)-type motif 2
IGFBP3	insulin-like growth factor binding protein 3
BTG2	BTG family, member 2
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
SET	SET nuclear oncogene
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
ENDOG	endonuclease G
NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1
P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
MELK	maternal embryonic leucine zipper kinase

Table 3.10: Differential upregulated genes involved in anti- apoptosis following infection

PIM2	pim-2 oncogene
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
FOXO1	forkhead box O1
FOXC1	forkhead box C1

Table 3.11: Differential upregulated genes involved in immune response and apoptosis following treatment with drugs in solution

CCL3L1	chemokine (C-C motif) ligand 3-like 1
CCL7	chemokine (C-C motif) ligand 7
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CCL3	chemokine (C-C motif) ligand 3
CXCL10	chemokine (C-X-C motif) ligand 10
CXCL2	chemokine (C-X-C motif) ligand 2
CCL8	chemokine (C-C motif) ligand 8
CCL20	chemokine (C-C motif) ligand 20
CCL4L1	chemokine (C-C motif) ligand 4-like 1
CCL22	chemokine (C-C motif) ligand 22
DUSP1	dual specificity phosphatase 1
CASP9	caspase 9, apoptosis-related cysteine peptidase
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
CFLAR	CASP8 and FADD-like apoptosis regulator
CARD16	caspase recruitment domain family, member 16
BIRC3	baculoviral IAP repeat containing 3
IFIH1	interferon induced with helicase C domain 1

Table 3.12: Differentially upregulated genes involved in immune response and apoptosis following treatment with drug-containing microparticles

CXCL6	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
CCL3L1	chemokine (C-C motif) ligand 3-like 1
CCL7	chemokine (C-C motif) ligand 7
C3AR1	complement component 3a receptor 1
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
CCL3	chemokine (C-C motif) ligand 3
CXCL10	chemokine (C-X-C motif) ligand 10
CXCL2	chemokine (C-X-C motif) ligand 2
CCL8	chemokine (C-C motif) ligand 8
CCL20	chemokine (C-C motif) ligand 20
CCL4L1	chemokine (C-C motif) ligand 4-like 1
CCL22	chemokine (C-C motif) ligand 22
CCL2	chemokine (C-C motif) ligand 2
BCL3	B-cell CLL/lymphoma 3
DUSP1	dual specificity phosphatase 1
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
BIRC3	baculoviral IAP repeat containing 3
IFIH1	interferon induced with helicase C domain 1

Tables 3.9 to 3.12 show the differential regulation of genes involved in apoptosis and immune response following infection and treatment. Infection downregulated the genes involved in programmed cell death and upregulation of anti-apoptosis genes. Treatment with drug-containing microparticles and drugs in solution preferentially upregulated genes involved in apoptosis and immune responses.

3.6 Gene expression profile by Real time PCR (Q-RT-PCR)

Studies by Maertzdorf *et al* using whole blood microarray gene analysis identified the biomarkers predictive of susceptibility or resistance to TB (Maertzdorf, Repsilber et al. 2011). Fc gamma receptor 1B (FCGR1B) in combination with CD64, RAB33A, and LTF were reported to form a subset, on the basis of which high degree of accuracy was achieved in discrimating between active TB patients and latently infected donors.

It was investigated whether the expression of the above genes varies between different groups based on S score and allows identification of a separate cohort. Q-RT-PCR was used to determine the transcription level of each gene in the MDMs of different donors and data was normalized against GAPDH as it showed least variation in expression upon infection and treatment with various form of drugs in our previous studies (data not shown). Principal component analysis was performed using XLSTAT (limited version) to identify the similarities or differences between expressions within or between the groups. Figure 3.35(A) shows the PCA plot for FCGR1B, CD64 and RAB33A based on 'S' score. It is clear from the figure that donor across genotypes clustered together and did not segregate. Some volunteers had unique expression profiles for particular genes. Donor1 segregated in respect of FCGR1B, while donor 7 segregated from the cluster in respect of Rab 33A. Other than this, MDMs of all donors exhibited a similar expression pattern for these genes following infection with Mtb. When donors were rearranged on the basis of net susceptibility score i.e. S-R score (figure 3.35B), the donors did not clustered at a place and instead segregated with donors belonging to same net 'S' score group in general plotted near to each other with few exception same as for panel A. The possible reason may be the influence of protective alleles i.e. AA and BB and absence of HLA DRB1*1501 in transcription regulation affecting effective immune response.

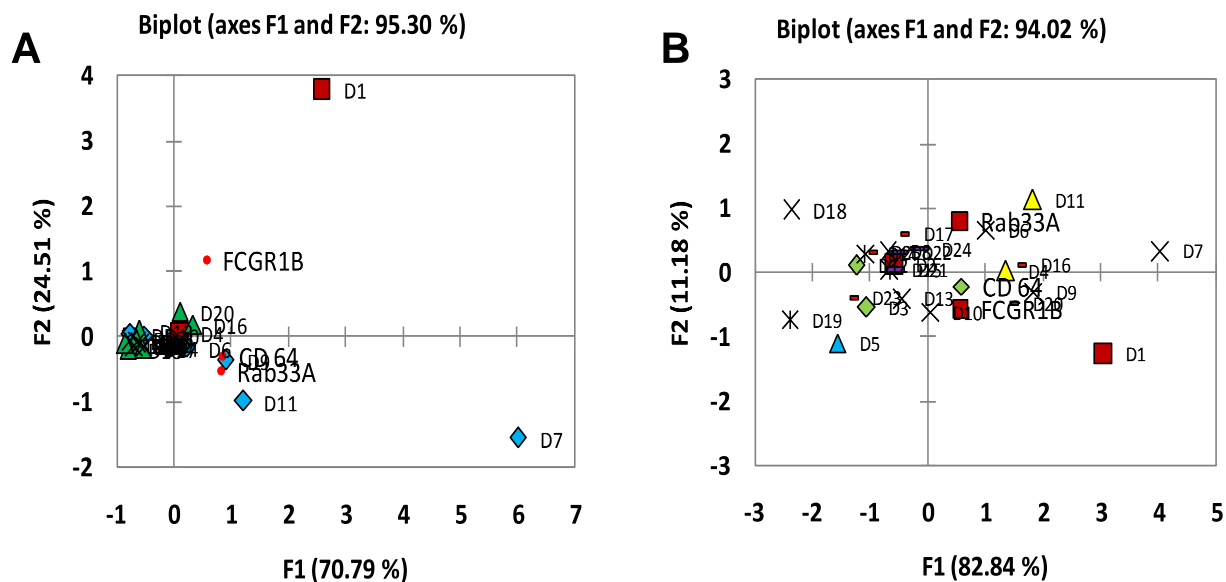


Figure 3.35: Two dimensional prinicipal component analyses computed using fold expression change compared to control for FCGR1B, CD 64 and Rab 33A in all donors. **(A)** Donors arranged on the basis of susceptibility score (S). **(B)** Donors arranged on the basis of net 'S' score.

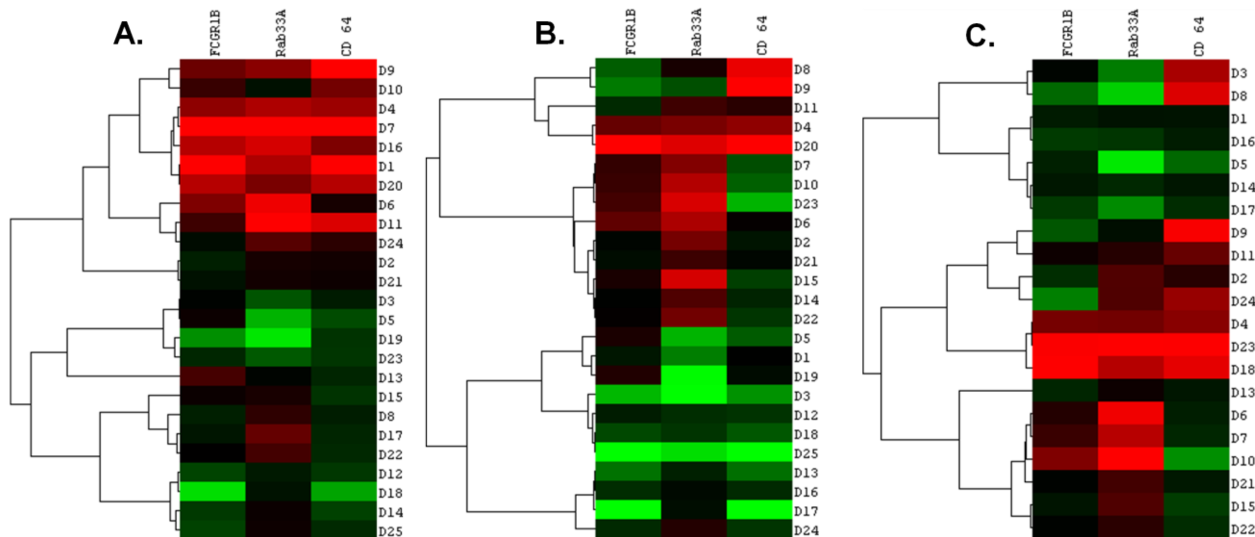


Figure 3.36: (A) Comparison of FCGR1B, Rab 33A and CD 64gene Mean Relative Expression (2-ddCt) normalized to their own controls in MDMs from healthy donors infected with Mtb H37Rv; (D1-D4: S=0; D5-D12: S=1; D13-D20: S=2 and D21-D25: S=3) **(B)** Mean relative expression after treatment of infected cells with drug containing microparticles. **(C)** Mean relative expression after treatment of infected cells with drugs in solution

Log transformed expression values were clustered using Cluster 3 software and the output was viewed using Tree View software. From Figure 3.36, it can be inferred that different groups did not cluster together and there was no group specific expression pattern. There was much inter-individual variation in expression profiles. It is also clear that there was no homogenous expression of different genes from the same donor, suggesting different pathways are utilized by individuals to counteract infection with Mtb.

It is also evident from heat map that transcription of FCGR1B, CD 64 and Rab 33 A upon infection with Mtb in most of the donors of group 3 and 4 were downregulated.

CD64 is involved in phagocytosis, respriratory burst, and antibody-mediated cytotoxicity in macrophages. New studies have also pointed towarded contribution of antibodies in disease control (Abebe and Bjune 2009). CD 64 expression is modulated by cytokines such as IFN-γ (Perussia, Dayton et al. 1983) and IL-10 (Capsoni, Minonzio et al. 1995). The transcription profile of CD64 following 2 hrs post infection or 0 hrs post treatment showed inter individual variation but followed a general consensus of downregulation in transcription level in more susceptible genotype with few exceptions. Thus donors from less susceptible genotype responded to infection initially by increasing CD 64 receptors and may be the contributory factor in antimycobacterial defense and reducing intracellular survival of mycobacteria. These results are similar to Kincaid *et al* who reported downregulation of CD 64 at both transcription and expression level in THP-1 cells infected with Mtb (Kincaid and Ernst 2003). Since THP-1 cells have S =3, reduced transcription of CD64 was observed even at 48 h after infection.

Rab 33A belongs to the family of Ras associated small GTPases, also known as S10. It is predominantly expressed in CD8+ T cells but is also found in monocytes and plays an important role in regulation of intracellular vesicle transport (Zerial and McBride 2001). Mtb has evolved the capacity to arrest phagosome maturation and thus persist intracellularly. Expression of Rab 33A was either downregulated or not changed in the MDMs of more 'susceptible' donors. Most of the donors from less 'susceptible' genotypes showed upregulation at 0 hrs post treatment. Jacobsen *et al* reported downregulation of Rab 33A in TB patients suggesting its involvement in disease (Jacobsen, Repsilber et al. 2005). MDMs of less 'susceptible' genotype in general increased Rab 33A expression following wxposure to Mtb, suggesting a putative role of these molecules in disease progession and intracellular survival of bacteria by increased phagocytosis.

In the study by Maertzdorf *et al*, FCGR1B was the most strongly differentially expressed gene in TB patients versus controls (Maertzdorf, Repsilber et al. 2011). Expression of the FCGR1B is regulated by IFN-γ and performs similar function as CD64, i.e., binding of antibodies by their constant domain. Expression of this receptor in the present study followed a similar trend as that of CD 64 and was upregulated in donors of less susceptible genotype with few exceptions. These receptors trigger activating and inhibitory signaling pathways resulting in well balanced immune response.

Treatment with drug-containing microparticles in general reduced the transcription of FCGR1B, Rab 33A and CD 64 respectively when compared to untreated infection. The possible reason may be that treatment with drug containing microparticles or soluble drug initially induced very little TNF-α and little or no IFN-γ, but TNF-α was upregulated at 12 h post treatment. The transcription analysis was done at 0 h post treatment. The possible reason of downregulation of above genes may be due to the role of IFN-γ in upregulating the expression of above genes while mycobactericidal action of microparticles is ascribed to the induction of TNF-α and NO and not to IFN-γ and antibody mediated toxicity, therefore the expression level of above genes downregulaed when compared to infection alone (Yadav, Muttil et al. 2010). Other possible reason may be that the increased expression of above genes in TB infection may reflect the immune activation response due to bacteria while treatment with microparticles or soluble drug because of intrinsic antimycobacterial action reduce the need for sustained immune activation.

In sum, it can be hypothesized that infection with Mtb induced increased expression of FCGR1B, Rab 33A and CD 64 in less susceptible genotype to control infection and treatment with drug containing microparticles utilizes strategy to combat Mtb infection not relying on IFN-γ and antibodies and rather exert mycobactericidal effect through the TNF-α pathway.

Chapter 4

Summary & Conclusions

Summary & Conclusions

- Genotype profiling of donors with respect to *HLA DR2*, *HLA DRB1*1501*, *VDR* (*TaqI*, *BsmI*, *ApaI* and *FokI*), and cytokine gene polymorphisms (IFN-γ *+874A/T*, TNF-α *-308 G/A*, IL-10 *-1082 A/G* and IL-4 *-590C/T*) was performed using PCR-SSP, PCR-RFLP and ARMS-PCR respectively. Gene and allele frequencies were in Hardy-Weinberg equilibrium for all the genes except *FokI* and the IL-10 *-1082A/G* substitution, and were similar to results obtained in a previous study on a North Indian population. Thus, the sample of donors used in this study was representative of the North Indian population.
- Donors could be ranked with respect to assigned arbitrary 'susceptibility' (S), 'resistance' (R) and 'net susceptibility' (S-R) scores based on presence or absence of HLA and VDR factors associated with TB susceptibility.
- Genome wide transcription profiling was performed on THP-1-derived macrophages; after infection of these cells with Mtb H37Rv and no further treatment; and after treatment with either isoniazid (INH) + rifabutin (RFB) in solution or equivalent amounts incorporated in biodegradable microparticles. Using the Illumina microarray platform, no genes were found to be differentially regulated by ≥2 fold at 0 h post infection, but at 24 h 1,112 genes were upregulated and 881 genes were downregulated in response to infection. Most of the upregulated genes belonged to metal ions binding factors and anti-apoptosis pathways while downregulated genes were involved in apoptosis, RNA metabolic process and gene regulation, as indicated by GENECODIS 2.0 software.
- Treatment with drugs in solution differentially upregulated more genes (57) compared to treatment with drug-containing microparticles (38) at 0 h, but at 24 h microparticle treatment differentially regulated a larger number of genes (167 upregulated and 152 downregulated by microparticles while 159 genes upregulated and 125 genes downregulated by drugs in solution). Analysis mapped most of the upregulated genes with immune response, apoptosis, cytokine mediated immune response and inflammatory pathway. Downregulated genes were involved in rRNA processing and cell cycles.

- Q-RT-PCR was performed for RNA samples isolated from human donors for *FCGR1B*, *Rab33A* and *CD 64* gene products. Principal component analysis of gene expression did not segregate donors based on their S score, but segregation was observed when donors were regrouped on the basis of net S-R score. Thus the presence of protective alleles, i.e. *AA* and *BB* of *VDR* and the absence of the susceptibility allele *HLA DRB1*1501* affected expression levels of the studied genes related with immune response.
- Transcription of *FCGR1B*, *Rab33A* and *CD 64* gene following infection was higher in MDMs of donors with a lower S score with few exceptions thus implicating their role in disease progression and intracellular survival of Mtb. Treatment with drug-containing microparticles and drugs in solution in general reduced expression of the above genes across 'susceptibility' scores. The possible reasons for this observation may either lie in effective utilization of the TNF-α and NO dependent pathways by infected MDM for bactericidal actions, or in the fact that drug-induced killing of the intracellular Mtb resulted in homeostatic downregulation of these genes. The elucidation of these mechanisms was beyond the scope of the present study.
- Downstream immune activation was assessed in terms of cytokine and NO release following infection and treatment with different drug modalities. Infection of *HLA DRB1*1501*-positive MDMs initially induced TNF-α at 2 h post infection, but secretion decreased at 12 h post treatment. In the allele negative group, TNF-α was sustained throughout the time course of study. Treatment with drug-containing microparticles increased TNF-α level significantly than treatment with drugs in solution in both the allele positive and negative groups and was thus effective across 'susceptibility' genotypes. When donors were stratified on the basis of the S score, it was observed that MDMs of $S \geq 2$ responded to infection initially by producing higher indices of TNF-α but this was followed by a steep fall. MDMs from donors of $S \leq 1$ initially produced lower indices of TNF-α but at 12 h post treatment, levels were almost equal across S scores. Treatment with microparticles thus induced and sustained higher levels of TNF-α compared to drugs in solution.

- *HLA DRB1*1501* positive MDMs responded to infection by increasing median IL-6 indices at all time points compared to the allele negative group. Microparticles reduced IL-6 secretion compared to untreated infection, but drugs in solution reduced IL-6 more strongly at 12 h. When donors were stratified on the basis of the S score, IL-6 was produced at almost constant level throughout the course of experiment by MDMs of S≤1. MDMs of donors with higher S scores initially produced higher levels of IL-6, which decreased later though values were still higher than those with S≤1. Similar or higher levels were produced following treatment with drug-containing microparticles compared to infection alone. Microparticles induced higher levels than drugs in solution.
- IL-10 median indices were significantly higher in *HLA DR 15* positive MDMs at 0 and 6 h. Treatment with microparticles significantly reduced IL-10 at 12 h compared to drugs in solution. Similar trend was observed when donors were regrouped on the basis of S score and donors of S≥2 secreted higher IL-10 till 6 h post treatment.
- IL-4 was significantly higher in *HLA DR 15* positive MDMs at 12 h following infection and was reduced significantly by treatment with microparticles compared to drugs in solution. A similar trend was observed when donors were regrouped on the basis of S score. Donors with S score 3 showed an increasing trend during the course of study while there was no significant differences between donors with S≤2.
- Thus, MDMs from *HLA DRB1*1501* positive donors increased Th2 cytokine secretion upon infection and displayed an alternatively-activated or M2 phenotype. Treatment with microparticles rescued MDMs from alternative activation and led to a classically activated (M1) phenotype by increasing Th1 cytokines.
- MDMs of donors of lower 'S' score (S≤1) secreted higher basal level of NO. Infection initially decreased NO production by primary macrophages across S scores and the dip was more prominent in more susceptible individuals (S≥2). There was increase in NO production 12 h post treatment, and cells of lower 'S' score (S≤1) produced higher levels of NO. Treatment with microparticles either sustained or increased NO production across S scores. The increase was higher or equal to treatment with drugs in solution. When donors were stratified on the basis of presence or absence of *HLA DRB1*1501* allele,

basal NO secretion was higher in the allele negative group and infection rapidly induced NO. There was no change in NO production by allele positive MDMs in response to infection.

- Bacterial survival was estimated by counting CFU in cell lysates and by a Bactec assay. Donors with S≥2 permitted intracellular survival of significantly higher CFU than less susceptible ones. Treatment with microparticles decreased the count by about log 2-2.5 in all individuals and was significantly better than drugs in solution even for more susceptible genotypes. The Bactec assay showed the same trend.

It was concluded, despite wide inter individual variation, that in general:

- Donors of more 'susceptible' genotypes initially responded to infection through production of more Th1 cytokines, but could not sustain this response. Less 'susceptible' genotypes sustained Th1 cytokine production by MDMs throughout the course of study.
- Higher levels of Th2 cytokines were produced by donors assigned higher S scores. Treatment with drug- containing microparticles decreased Th2 cytokines and increased Th1 cytokines.
- Microparticles performed better than drugs in solution in inducing a proinflammatory cytokine response in more susceptible genotypes and reduced Th2 cytokine significantly.
- NO production in response to infection was observed to depend on S score. Microparticles elicited higher NO in all genotypes compared to drugs in solution.
- Survival of Mtb in MDMs correlated with host genotype. CFU recovered from cell lysates were directly proportional to the S score assigned. Microparticles exhibited better efficacy than drugs in solution in reducing bacterial load.
- No clear correlation of host gene expression profiles on infection with and the genotype of the host could be established. Expression profiling indicated that microparticles enhanced proinflammatory bactericidal responses, and reduced interference in host metabolism by the pathogen more appreciably than drugs in solution.

Appendix

1. ***Consent form***
2. ***Case record form***
3. ***Summary Sheet***

Consent Form and Volunteer Information Sheet

Macrophage gene expression

Sample ID No.: Date:

I, ____Age_______

Son/Daughter/Wife of ____

Resident of____

____

hereby give my consent freely to participate in the observational study aimed at understanding how immune cells taken from the blood of different people would respond if they were infected with TB-causing bacteria and treated with small particles containing anti-TB drugs and a biodegradable, bio-compatible plastic material. I am willing to donate initially 1 ml blood to assess my suitability for participation and if eligible will donate 20 ml of my blood for this study. It has been explained to me that all information provided to the investigators, my blood sample and analysis results shall be stored anonymously. I am willing to donate 20 ml more blood on two additional occasions over the next one year. My participation in this study is entirely voluntary and I am free to withdraw as and when I feel inclined.

Signature of Volunteer Date:

Certified that the above consent form has been signed in my presence. The purpose for which the blood sample shall be used has been explained to the satisfaction of the above volunteer. He/she is free to withdraw from participation at any time. He/she has given voluntary consent to store the sample anonymously and the result of the analysis on his/her blood sample confidentially.

Signature of Investigator Date:

CASE RECORD FORM

CONFIDENTIAL: DO NOT PUT DOWN VOLUNTEER'S NAME OR OTHER IDENTIFICATION DETAILS ON THIS SHEET

Sample ID Date

Age of donor Sex

Body mass index (BMI)

1. Have you visited doctor in last one year?
   a. if yes, number of visit
   b. reason of illness
   c. have you undergone X-ray, ultrasound sonography?
2. Have you had fever in last one year?
   a. Yes b. No
   If yes,how many days you took to recover?
   a. <7 daysb. 8-14 days c. >14 days
3. Do you have histroy of chronic illness in the past?
   a. Yes b. No
   if yes, what disease and how long did you take to recover?
4. Are you diabetic or have any familial history of diabetes?
   a. Yes b. No
   Familial history:-----------------------
5. BCG vaccination status
   a. yes b. No c. don't know
6. Volunteer occupation
   a. employed b. self employed c. unemployed
   if self employed , nature of job:
7. Personal abuse
   a. smoking i) Yes ii) No iii) occasionally
   b. alcohol i) Yes ii) No iii) occasionally

This information pertains to the volunteer bearing identical ID No. on the consent form. The volunteer meets inclusion criteria and does not meet any exclusion criterion as specified in the study protocol

Signature of Investigator

Donor I. D.	Cytokine Genotype				S Score	R Score	CFU count (CFU X10^3 /10^5 cells)		
	IFN-γ	*TNF-α*	*IL-10*	*IL-4*			*I*	*IMP*	*ISD*
D1	AA	AG	AG	CC	0	3	7.0	0.0	0.6
D2	AT	GG	AG	CC	0	3	16.7	0.4	0.5
D3	AA	GG	AA	CC	0	2	0.7	0.0	0.0
D4	AA	AG	AA	CC	0	1	17.9	0.1	0.3
D5	AA	GG	AA	TT	0	2			
D6	AA	GG	AA	CC	0	2	7.1	0.1	0.9
D7	AA	GG	AA	CC	0	2			
D8	AA	GG	GG	CC	0	2			
D9	AT	GG	AG	CC	0	1			
D10	AA	GG	AA	CC	0	1			
D11	AT	AG	AG	CT	1	1	29.0	1.3	1.3
D12	AA	GG	AA	CC	1	1	12.1	0.2	1.0
D13	AA	AG	AG	CC	1	1	14.7	0.5	0.5
D14	AT	GG	AG	CC	1	1	11.2	0.7	1.0
D15	AT	GG	AA	CC	1	1	10.5	0.8	1.7
D16	AT	GG	AA	CT	1	1	13.6	0.3	0.5
D17	AA	GG	GG	CC	1	2	10.7	0.1	0.0
D18	TT	GG	AA	CC	1	3	26.2	0.0	0.1
D19	AA	GG	AG	CC	1	1			
D20	AT	GG	AG	CC	1	2			
D21	AA	GG	GG	CC	1	1			
D22	AT	GG	AA	CC	1	1			
D23	AA	GG	AG	CT	1	1			
D24	AT	GG	AG	CC	1	1			

D25	TT	GG	GG	CT	1	2			
D26	AT	GG	AA	CC	1	1			
D27	AA	GG	AA	CC	1	1			
D28	AT	GG	AA	CC	1	1			
D29	AT	GG	AA	CC	2	1	8.1	0.0	0.7
D30	AA	GG	AA	CC	2	2	14.5	0.0	0.4
D31	TT	GG	AG	CC	2	1	12.5	1.4	1.4
D32	TT	GG	GG	CC	2	1	16.0	0.2	0.0
D33	AA	AG	GG	CC	2	0	11.6	0.5	0.9
D34	AA	GG	AA	CC	2	0	2.8	0.0	0.0
D35	TT	AG	AA	CC	2	2	14.8	0.3	0.9
D36	TT	GG	AA	CC	2	1	26.6	0.2	1.6
D37	AA	GG	AG	CC	2	0	18.8	0.3	0.6
D38	AT	GG	AA	CC	2	2			
D39	AA	AA	AA	CC	2	1			
D40	AA	AG	GG	CT	2	2			
D41	AA	GG	AA	CC	2	2			
D42	AA	GG	AA	CC	2	1			
D43	AA	AG	AA	CC	2	1			
D44	AA	AG	AA	CT	3	0	43.1	0.1	0.2
D45	AT	GG	AA	TT	3	0	38.5	0.6	1.2
D46	TT	GG	AG	CC	3	1	34.7	0.3	0.7
D47	AT	AG	GG	CC	3	0	12.6	0.3	0.4
D48	AT	GG	AG	CT	3	1	23.3	0.4	0.6
D49	AT	GG	AA	CC	3	1			
D50	TT	GG	AA	CC	3	0			
D51	AT	GG	AG	CT	4	0			
D52	AT	GG	AA	CC	4	0			

Donor I. D.	TNF-α cytokine level (pg/ml)											
	0h				***6h***				***12h***			
	C	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**
D1	6.0	17.8	22.8	18.3	2.2	4.3	6.5	4.0	2.1	0.0	1.8	0.0
D2	5.1	29.0	26.4	25.3	4.6	8.1	9.1	1.2	0.0	1.2	0.0	0.0
D4	10.1	31.1	35.8	33.4	9.3	7.1	6.7	0.0	0.0	3.2	0.0	0.0
D5	20.3	100.0	75.0	72.9	14.5	42.3	24.0	30.5	2.4	6.4	5.6	14.5
D11	45.3	91.6	71.9	73.6	9.2	21.3	12.6	9.0	1.8	4.8	4.0	4.2
D12	11.3	23.8	20.9	18.2	4.4	8.6	8.2	6.7	2.1	3.9	2.9	1.7
D13	67.6	12.1	54.4	76.4	2.2	15.2	13.2	14.7	0.0	6.6	3.0	0.0
D14	2.4	5.1	5.7	3.3	2.1	4.9	4.7	3.4	0.0	3.0	2.3	0.0
D15	7.6	15.6	13.4	15.9	3.0	4.2	2.7	1.9	0.0	0.0	0.0	4.4
D16	4.3	7.4	5.6	8.4	5.8	4.0	5.7	4.9	1.3	0.0	1.7	0.0
D17	3.1	4.4	5.4	6.5	1.4	2.9	2.6	2.3	2.0	3.0	2.1	1.7
D18	13.3	101.9	106.7	144.5	4.4	44.0	36.1	57.0	1.7	23.8	25.6	2.6
D29	4.0	25.1	34.0	37.2	2.7	3.6	4.3	20.5	1.3	0.0	0.0	12.0
D30	43.6	119.2	131.5	133.1	25.2	25.1	19.2	2.9	0.0	2.9	1.6	0.0
D31	4.0	50.4	38.0	44.0	3.2	24.3	11.7	17.8	10.6	16.2	6.0	3.6
D32	5.1	109.7	106.2	127.1	5.4	29.3	32.1	26.5	2.6	20.7	12.7	25.5
D33	1.6	18.0	11.7	16.4	5.6	12.9	10.2	9.9	3.3	3.9	3.4	1.7
D34	224.4	68.3	54.9	77.8	5.8	25.3	20.7	25.9	1.8	6.5	4.9	3.9
D35	8.2	9.2	9.9	14.2	2.0	2.3	2.7	1.5	0.0	0.0	0.0	0.0
D37	4.8	93.7	77.1	62.1	3.5	92.9	47.5	33.7	1.6	28.7	13.0	0.0
D44	7.0	74.3	28.1	15.0	3.7	14.0	8.5	4.3	0.0	33.0	11.8	0.0
D45	15.5	76.4	90.3	145.9	6.5	20.6	17.6	42.7	5.6	3.1	1.4	0.0
D46	2.9	30.5	31.7	19.0	3.1	18.5	23.3	10.4	6.0	11.1	13.2	16.6
D47	3.2	19.2	29.0	19.2	2.7	14.7	13.9	8.8	2.5	2.1	1.9	0.0

Donor I. D.	IL-6 cytokine level (pg/ml)											
	0h				*6h*				*12h*			
	C	I	IMP	ISD	C	I	IMP	ISD	C	I	IMP	ISD
D1	12.7	18.7	43.9	18.3	2.7	4.5	13.2	6.1	2.4	7.5	8.7	3.2
D2	10.6	70.9	56.3	70.9	10.3	34.9	28.7	26.5	2.7	10.2	7.2	5.0
D4	11.4	62.6	69.3	50.0	11.2	12.2	13.0	14.6	3.4	4.2	4.5	4.0
D5	13.4	32.0	20.6	19.9	4.2	12.7	7.1	5.9	3.1	10.5	8.7	10.1
D11	2.1	6.6	4.6	3.9	0.0	4.1	2.4	1.3	0.0	2.5	2.2	0.0
D12	10.3	54.7	52.0	34.4	4.7	21.1	19.9	10.2	2.2	25.5	25.2	21.3
D13	18.8	13.9	12.3	14.0	2.8	3.5	2.9	2.6	1.4	2.8	3.6	5.2
D14	21.0	22.1	31.6	22.3	4.0	12.0	9.8	8.5	3.1	11.4	24.9	7.8
D15	9.2	59.7	84.9	75.8	4.1	16.5	13.7	15.5	4.3	17.6	50.3	13.0
D16	6.2	43.9	46.2	72.3	11.5	7.7	9.2	12.3	2.3	9.8	8.3	8.0
D17	27.8	15.3	14.4	20.8	11.7	4.8	4.6	18.3	3.8	7.0	4.2	4.4
D18	9.7	49.0	38.9	27.1	3.9	24.4	15.9	13.5	3.1	16.1	13.9	10.1
D29	2.0	3.7	3.9	3.2	2.0	16.5	2.6	2.4	1.6	2.4	2.8	3.8
D30	26.9	51.3	53.6	60.8	44.8	38.3	43.5	50.0	4.0	12.5	6.3	5.6
D31	9.1	239.4	224.7	165.1	4.6	133.8	69.6	83.4	42.4	118.7	83.4	73.0
D32	6.6	886.5	793.5	737.3	3.7	94.2	126.6	101.5	2.5	103.4	90.3	286.8
D33	2.7	61.4	9.0	114.4	2.0	53.1	27.8	29.0	2.1	30.0	59.1	9.8
D34	823.3	779.1	903.0	968.0	8.6	233.0	204.4	228.8	3.3	201.6	162.1	224.7
D35	15.7	53.6	56.8	67.0	3.7	13.7	24.9	23.3	1.5	4.6	5.8	5.6
D37	18.6	106.3	121.0	82.6	4.7	194.4	130.2	65.1	3.2	108.3	82.6	110.8
D44	38.1	189.2	51.5	32.3	21.1	45.7	21.6	8.3	22.6	586.5	216.7	11.2
D45	4.2	52.0	27.2	37.0	2.3	19.9	6.5	15.1	2.4	9.6	7.0	12.0
D46	6.9	67.0	128.4	60.8	3.1	51.5	107.3	30.0	30.6	33.7	74.4	70.3
D47	7.7	45.3	89.0	65.1	4.1	28.0	25.2	21.8	3.2	22.3	17.7	14.0

Donor I. D.	IL-10 cytokine level (pg/ml)											
	0h				*6h*				*12h*			
	C	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**
D1	3.3	2.8	3.2	2.4	1.7	2.2	2.4	0.0	2.1	2.3	1.7	1.8
D2	2.6	4.0	2.9	4.1	2.4	1.5	1.1	0.0	0.0	0.0	0.0	0.0
D4	8.8	21.4	26.3	27.5	2.9	2.3	2.4	1.5	0.0	1.5	0.0	0.0
D5	3.3	4.2	3.8	4.6	2.1	2.6	3.1	2.8	2.1	7.8	5.4	5.4
D11	0.0	2.0	2.3	2.4	1.2	0.0	0.0	0.0	0.0	1.6	1.9	0.0
D12	3.3	5.1	7.9	6.5	3.2	0.0	3.0	1.8	1.9	2.3	2.7	1.3
D13	2.7	2.4	2.8	4.4	1.9	1.2	0.0	0.0	0.0	1.3	0.0	2.5
D14	1.7	2.5	2.6	1.7	2.2	2.3	2.2	2.3	0.0	1.9	0.0	1.5
D15	2.3	4.1	3.8	3.1	1.1	1.6	0.0	0.0	0.0	2.5	0.0	4.9
D16	4.2	7.1	5.7	7.9	3.0	0.0	1.3	1.8	0.0	2.4	1.2	1.3
D17	4.1	3.9	4.0	3.9	2.8	1.3	2.4	4.8	2.5	2.3	2.8	1.5
D18	1.5	3.7	3.5	2.7	2.1	0.0	2.2	2.2	2.1	2.0	1.1	2.2
D29	0.0	3.2	2.3	2.6	1.2	0.0	0.0	0.0	0.0	1.5	1.1	1.8
D30	14.2	51.2	57.1	60.6	3.3	4.3	5.9	7.0	0.0	0.0	0.0	1.5
D31	3.2	11.1	14.4	10.9	2.9	4.0	3.1	2.9	2.6	3.3	2.6	2.4
D32	6.1	19.9	24.0	23.0	3.3	1.3	3.5	3.8	2.2	2.9	2.6	3.5
D33	2.6	19.4	4.9	23.9	2.6	7.5	4.2	3.9	3.0	3.9	2.7	3.3
D34	6.2	10.3	19.7	14.0	2.9	2.0	2.9	2.0	2.1	3.8	2.0	2.4
D35	2.5	4.2	5.2	5.5	1.5	0.0	1.5	0.0	0.0	0.0	0.0	0.0
D37	2.1	4.0	5.2	3.5	2.0	2.7	1.1	2.4	1.7	2.4	1.6	2.1
D44	2.9	24.9	7.9	7.7	1.6	2.8	1.5	1.1	2.5	2.9	1.5	1.7
D45	0.0	2.4	2.0	3.9	0.0	0.0	1.5	0.0	0.0	0.0	1.8	1.1
D46	3.3	9.0	10.1	7.5	0.0	4.8	3.5	4.0	2.0	3.5	5.2	20.4
D47	5.7	28.8	31.1	12.7	1.9	2.9	2.8	2.7	2.3	3.5	3.3	2.3

Donor I. D.	IL-4 cytokine level (pg/ml)											
	0h				*6h*				*12h*			
	C	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**
D1	4.2	3.5	4.5	3.6	4.1	3.6	3.0	3.6	4.5	4.3	4.2	3.9
D2	3.1	1.5	0.0	0.0	3.5	0.0	0.0	1.6	0.0	2.0	0.0	0.0
D4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
D5	3.1	3.3	3.1	3.1	3.8	2.8	2.4	0.0	2.8	11.5	7.1	8.2
D11	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.0
D12	6.0	5.8	5.3	6.5	4.8	0.0	4.1	4.7	4.5	4.6	4.0	6.0
D13	0.0	1.5	0.0	2.7	2.0	2.0	2.4	2.0	0.0	0.0	0.0	3.5
D14	4.1	3.0	2.6	4.4	3.5	4.0	3.5	4.1	3.2	3.6	4.5	4.5
D15	3.8	3.9	3.0	2.4	3.3	3.8	4.0	4.0	3.5	3.5	0.0	7.4
D16	1.8	2.2	2.2	4.0	3.6	2.4	2.4	2.8	2.1	3.3	2.6	3.0
D17	4.8	5.6	6.7	4.6	3.0	3.2	3.4	5.6	3.0	4.8	4.7	3.5
D18	0.0	2.1	2.2	3.8	2.0	1.5	1.8	2.0	2.6	2.9	2.4	1.2
D29	3.3	2.6	3.3	2.6	3.3	2.6	2.6	1.9	2.4	1.5	3.3	2.4
D30	0.0	0.0	1.2	0.0	1.5	2.4	0.0	0.0	0.0	0.0	0.0	0.0
D31	4.8	6.0	5.0	5.6	4.6	5.3	4.6	3.6	3.1	4.0	4.3	3.8
D32	5.7	5.5	6.2	4.8	4.5	0.0	5.6	5.1	5.3	4.3	4.6	4.1
D33	4.6	5.4	3.4	5.1	4.1	4.0	0.0	0.0	4.0	3.3	3.6	4.1
D34	4.0	3.3	3.3	3.6	5.4	4.2	3.6	4.0	4.1	5.3	4.0	3.1
D35	1.8	0.0	0.0	1.8	1.5	0.0	1.5	1.9	0.0	2.6	0.0	0.0
D37	1.6	2.3	1.5	1.8	2.0	2.5	0.0	2.8	3.8	1.8	3.1	2.0
D44	3.1	3.0	2.4	0.0	1.5	2.0	0.0	0.0	0.0	2.0	2.4	0.0
D45	1.8	0.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0	0.0	2.3	0.0
D46	4.5	4.8	4.6	4.0	5.1	6.3	4.6	4.3	3.8	3.9	5.2	4.5
D47	4.3	5.0	4.1	3.3	3.8	2.8	4.5	5.0	2.2	3.3	5.0	4.4

Donor I. D.	NO (nM)/ 10^5 cells							
	0h				*12h*			
	C	**I**	**IMP**	**ISD**	**C**	**I**	**IMP**	**ISD**
D1	7.8	2.8	0.1	0.3	6.9	4.5	3.7	2.9
D2	3.3	0.0	0.0	0.5	4.5	1.7	1.9	1.3
D3	3.2	0.0	0.1	0.3	3.7	1.3	1.5	1.9
D4	2.5	7.0	2.3	11.1	3.4	5.0	5.4	4.7
D5	1.2	4.3	1.1	1.3	3.7	7.3	9.3	6.5
D10	2.7	2.6	4.5	1.8	2.2	5.1	5.1	4.5
D11	1.7	3.4	2.5	8.7	2.1	2.9	3.3	2.8
D12	4.6	1.4	1.3	0.3	4.3	15.3	13.1	6.1
D13	5.4	3.2	2.4	2.5	4.2	7.1	2.5	3.3
D14	6.0	0.3	0.0	2.0	5.5	4.0	2.5	3.0
D15	4.7	0.8	1.1	8.0	4.5	4.2	4.2	3.1
D16	1.9	0.9	0.8	0.5	2.9	1.6	0.4	0.4
D17	6.0	2.4	6.3	2.8	5.4	10.5	5.3	4.2
D18	4.3	0.2	0.3	0.3	3.2	3.8	2.4	2.7
D29	3.7	0.3	0.3	0.7	2.1	2.1	3.0	1.8
D30	2.2	2.2	2.5	11.0	2.6	5.3	5.5	3.8
D31	4.0	0.0	0.0	0.9	3.5	6.9	3.6	7.5
D32	2.3	0.0	0.0	0.0	2.3	2.1	7.1	1.7
D33	0.9	0.8	0.6	0.6	1.7	2.5	1.8	1.7
D34	3.1	0.0	0.0	0.0	3.4	3.8	3.3	3.4
D35	2.9	1.3	1.6	1.9	3.0	3.2	3.1	3.5
D37	0.1	0.0	0.0	0.0	0.0	3.0	3.0	3.1
D44	3.5	1.6	1.7	2.3	3.4	5.3	5.8	5.1
D45	5.9	0.8	0.8	1.0	6.0	2.5	1.7	1.7
D46	1.1	0.6	0.3	0.3	1.3	4.2	5.7	4.2
D47	1.3	0.6	0.0	0.0	1.9	1.9	1.6	1.3

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List of Publication

A. Paper published in reputed journals

International

1. Rahul K Verma, **Amit K Singh**, Mradul Mohan, Atul K Agrawal, Jatinder Kaur, Anuradha Gupta, Heikham Kajal, Kaushlendra Kumar, Awadh B Yadav, Pavan Muttil, Rolee Sharma, Anil K. Dwivedi, Pushpa Gupta, Umesh D Gupta, Uthirappan Mani, Bhushan P Chaudhari, Ramesh C Murthy, Sharad Sharma, Smrati Bhadauria, Sarika Singh, and Amit Misra. *Preclinical Efficacy and Safety of Inhalable Microparticles Containing Isoniazid and Rifabutin for Treating Tuberculosis* (communicated)

2. Yadav. A.B, Muttil. P, **Singh. A.K**, Verma. R.K, mohan. M, Agrawal. A.K., Verma. A.S, Sinha. S.K, and Amit Misra, *Microparticles induce variable levels of activation in macrophages infected with Mycobacterium tuberculosis*. Tuberculosis: 2010: **90**: 188-196

National

3. A. B.Yadav, R.Sharma, P. Muttil, **A. K. Singh**, R. K. Verma, M. Mohan, S.K.Patel & Amit Misra,' *Inhalable microparticles containing isoniazid and rifabutin target macrophages and 'stimulate the phagocyte' to achieve high efficacy* . Indian Journal of Experimental Biology: June 2009: **47**: 469-74

B. Review published in reputed journal

1. Rahul K Verma; **Amit K Singh**; Mradul Mohan; Atul K Agrawal; Amit Misra, *Inhaled therapies for tuberculosis and the relevance of activation of lung macrophages by particulate drug delivery system.* Therapeutic Delivery :(2011) **2(6)**, 753–768

2. Awadh B Yadav; **Amit K Singh**; Rahul K Verma; Mradul Mohan; Atul K Agrawal; Amit Misra , *The Devil's Advocacy: When and Why Inhaled Therapies for Tuberculosis May Not Work* . Tuberculosis: 2011: **91(1)**: 65-6

C. Abstracts Published in Conference proceedings

1. *Variations in macrophage responses to infection with Mycobacterium tuberculosis and treatment with microparticles affect bacterial survival.* **Amit K Singh,** Rajiv Garg and Amit Misra Tuberculosis: Immunology, Cell Biology and Novel Vaccination Strategies (J3) | **(**Keystone Symposia**),** January15-20, 2011
2. *Association of HLA and VDR variants with cytokine profile and bacterial viability in M. tuberculosis-infected human MDMs following treatment with anti tuberculosis drugs and drug-loaded microparticles,* **Amit K Singh**, Rajiv Garg and Amit Misra , International Symposium on TB Diagnostics: Innovating to Make an Impact December 16 - 17, 2010
3. *Validation of microarray transcription analysis of responses to treatment with inhalable microparticles containing anti-TB agents* , **Amit Kumar Singh**, Awadh B. yadav, Amit Misra; Inhaled tuberculosis Therapies and host pathoigen interaction, 2009
4. *Serendipitous activation of mouse and human macrophages infected with mycobacterium t tuberculosis on treatment with inhalable microparticles,* Rolee Sharma, pavan Muttil, **Amit Kumar Singh**, Awadh B. yadav,, Rahul Verma, Mradul mohan, Amitt Misra; Inhaled tuberculosis Therapies and host pathoigen interaction, 2009
5. *The Devil's advocacy: When and why inhaled therapies may not work*, Amit Misra , Awadh B. yadav, **Amit Kumar Singh**, ,, Rahul Verma, Mradul mohan; Inhaled tuberculosis Therapies and host pathoigen interaction, 2009