

**ADDIS ABABA UNIVERSITY
FACULTY OF MEDICINE
SCHOOL OF GRADUATE PROGRAMME**

**ROLE OF DENDRITIC CELLS IN THE INITIATION OF IMMUNITY
TO *MYCOBACTERIUM TUBERCULOSIS* INFECTION**

**BY
ADANE MIHRET, DVM**

*A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE PROGRAMME OF ADDIS
ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE IN MEDICAL MICROBIOLOGY*

JULY 2005

I the undersigned declare that this is my original work and not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged.

Name: _____ Year: _____

Acknowledgements

I would like to thank Dr. Shreemanta K. Parida and Professor Asrat Hailu, my advisors, for their interest, carefully reading the manuscript, valuable input and suggestions for improving this thesis both in developing the proposal and the final write up.

I want to thank Dr. Abraham Aseffa and Dr. Howard Engers, for always being enthusiastic and encouraging. Moreover they have taught me to not give up, work hard, and think independently and for these and many more things I thank them.

I also want to thank Dr. Mariolina Salio and Dr. Nickolaus Romani for their guidance during my laboratory work. All their e-mails with protocols, suggested readings and general information regarding the FACScan analysis of dendritic cells and other related issues are much appreciated.

My sincere gratitude to Dr. Rowleigh Howe for guiding me through a forest of FACScan, for all helpful discussions and for needed criticism

I would like also to extend my heartfelt thanks to S/r Genet Amare for her unreserved support in collecting blood samples

I am very grateful to the Amhara Region Agriculture Bureau, the Addis Ababa University, Department of Medical Microbiology, Immunology and Parasitology and Armauer Hansen Research Institute (AHRI), who have helped me a lot in one way or the other to make my graduate study possible.

I am also greatly indebted to Dr. Yimtubeznash W/amanuel, Head of Department of Medical Microbiology, Immunology and Parasitology, for her advice, suggestions, support, encouragement, understanding and, in particular, patience.

I want to thank all AHRI staffs and lab mates, present and past, for all their scientific discussions, hard work, and for being so thoughtful and a lot of fun in and outside of the lab.

I would also like to thank all my friends who have been a source of inspiration and who have all made my time in graduate school very enjoyable.

I am also greatly indebted to my parents, Mihret Bekele and Senetsehay Anteneh. Thank you for all your efforts and support.

Finally, I wish to express my deepest gratitude to my dear wife S/r Etsegenet Eyayu and our beloved daughter Mahlet. I feel very fortunate to have such a wonderful and supportive family around me while I was in graduate school. Thank you for all your patience and for having endured all these years.

Table of Contents	Page
ACKNOWLEDGEMENTS -----	I
DEDICATION -----	VI
LIST OF FIGURES -----	VII
LIST OF TABLES -----	IX
LIST OF APPENDIXES -----	X
ABSTRACT -----	XIV
CHAPTER I -----	1
1 INTRODUCTION -----	2
1.1 The Pathogen -----	2
1.2 Epidemiology of <i>Mycobacterium tuberculosis</i> Infection -----	2
1.3 Immunology of Tuberculosis -----	5
1.3.1 Macrophages-----	5
1.3.2 Toll-Like Receptors (TLR)-----	7
1.3.3 Cytokines-----	8
1.3.3.1 Proinflammatory Cytokines-----	8
1.3.3.2 Anti inflammatory cytokines -----	10
1.3.4 Chemokines-----	11
1.3.5 T Cells -----	12
1.3.5.1 CD4 ⁺ T Cells -----	12
1.3.5.2 CD8 ⁺ T Cells -----	13
1.3.6 Dendritic Cells -----	14
1.3.6.1 Dendritic Cell Development and Trafficking Pathways -----	14
1.3.6.2 Antigen Capture, Processing and Presentation by Dendritic Cells -----	15
1.3.6.3 Dendritic Cell Role in T cell Memory, Effector Function and Tolerance-----	19
1.3.6.4 Dendritic Cells and Mycobacterium tuberculosis-----	22

1.4 Hypothesis -----	23
1.5 Objectives -----	23
1.5.1 General Objective-----	23
1.5.2 Specific Objectives -----	23
CHAPTER II -----	24
2. MATERIALS AND METHODS -----	25
2.1 Source of Dendritic cells -----	25
2.2 Sample Size -----	25
2.3 Study Design -----	25
2.4 Study Period -----	25
2.5 Materials -----	26
2.5.1 Media and Reagents -----	29
2.5.2 Mycobacteria -----	29
2.6 Laboratory Methods -----	30
2.6.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC) -----	30
2.6.2 Isolation of Monocytes-----	30
2.6.4 Generation of immature human DC <i>in vitro</i> -----	32
2.6.5 Infection of DC with <i>M. tuberculosis</i> -----	32
2.6.6 Stimulation of DC with LPS -----	33
2.6.7 7-Amino-Actinomycin D (7-AAD) Staining Assay -----	33
2.6.8 Flow Cytometric Analysis of Surface Markers-----	34
2.6.9 Mixed Leukocyte Reaction -----	34
2.7 Ethical Consideration -----	35
2.8 Data Analysis -----	35
CHAPTER III -----	36
3 RESULTS AND DISCUSSION -----	37

3.1 Results	37
3.1.1 Magnetic Cell Sorting of CD14 ⁺ Monocytes	37
3.1.2 Generation of Immature Dendritic cells	40
3.1.3 Viability of Dendritic Cells Following Infection with H37Rv	41
3.1.4 Maturation of Dendritic Cells	43
3.1.5 Mixed Leukocyte Reaction	47
3.2 Discussion	49
3.3 Conclusion and Recommendation	57
4 REFERENCES	58

Dedication

Dedicated to,

My parents

&

in loving memory of my Sister, Gedam.

List of figures	Page
Figure 1: Components of immune system involved in TB immunology.	5
Figure2: Cytokines and Cytokine Receptors Involved in type I Immunity in Tuberculosis. .	8
Figure 3: Schema for derivation of human dendritic cell (DC) subsets	15
Figure 4: Degradation and transport of antigens that bind major histocompatibility complex (MHC) class I molecules.	18
Figure 5: Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules	19
Figure 6: The Interaction between Dendritic Cells (DC) and T Cells	20
Figure 7: The Life Cycle of Dendritic Cells.....	22
Figure 8: Positive selection of cells using VarioMacs.....	31
Figure 9: Dot-Plot flow cytometric analysis of total peripheral blood mononuclear cells: (A) forward scatter and side scatter (B) anti CD14 FITC and anti CD3 PE.....	37
Figure 10: Dot-Plot flow cytometric analysis of positively selected CD14 ⁺ cell fraction: (A) forward scatter and side scatter (B) anti-CD14 FITC and anti- CD3 PE.	38
Figure 11: Dot-Plot flow cytometric analysis of CD14 depleted cell fraction: (A) forward scatter and side scatter (B) anti CD14 FITC and anti CD3 PE.....	38
Figure 12: Micrograph of DC by phase contrast microscopy (40x): (a) CD14 ⁺ cells at day 1, (b) Immature dendritic cells at day 7 (c) mature dendritic cells.....	40
Figure 13: Dot-Plot flow cytometric analysis of analysis of immature DC stained with anti CD14 FITC and anti CD54PE.	41

Figure 14: Viability of DC following *M. tuberculosis* infection from the FACScan analysis of DC: (A) Uninfected, (B) infected with MOI of 3, (C) infected with MOI of 5 and, (D) infected with MOI of 10.....42

Figure 15: *M. tuberculosis* H37Rv wild type strain infected DC stained with Ziehl-Neelsen. DCs were cultured with live H37Rv for 48 hrs.43

Figure 16: Histogram Flow cytometric analysis of cell surface phenotype of immature dendritic cells (CD40 and CD80 when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*.44

Figure 17: Histogram from flow cytometric analysis of cell surface phenotype of immature dendritic cells (HLA DR and CD54) when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*.45

Figure 18: Histogram from flow cytometric analysis of cell surface phenotype of immature dendritic cells (CD83 and CD86) when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*..46

Figure 19: Effect of *M. tuberculosis* infection on surface phenotype of dendritic cells.....47

Figure 20: Autologous T cell proliferation at 1:10 and 1:100 of DC: T cell ratio by CFSE dilution technique.48

Figure 21: Overlay histogram of unstimulated and H37Rv stimulated dendritic Cells.....54

List of tables	Page
Table 1: WHO Report on Ethiopia TB Profile	3
Table 2: Proportion and purity of CD14 ⁺ cells from total PBMC isolated in each experiment	39

List of Appendixes	Page
Appendix A Mean fluorescence intensity of different surface markers of dendritic cells.....	69
Appendix B Viability and proportion of DCs after infection with H37Rv.....	70
Appendix C Cell division index values.....	71

List of abbreviation

$\gamma\delta$	Gamma/delta
α/β	Alpha/Beta
β_2m	β_2 microglobulin
7AAD	7-amino-actinomycin D
AIDS	Acquired Immuno Deficiency Syndrome
AFB	Acid-Fast Bacilli
APC	Antigen Presenting Cells
BCG	Bacillus Calmette and Guérin
BSL	Bio Safety Level
CD	Cluster of Differentiation
CDI	Cell Division Index
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CMI	Cell-Mediated Immunity
CTL	Cytotoxic T lymphocyte
(DC-SIGN)	DC-specific C-type lectin intercellular adhesion molecule-3-grabbing nonintegrin
DMSO	dimethyl sulphoxide
DNA	Deoxyribo-Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
H ₂ O ₂	Hydrogen peroxide
iDC	Immature Dendritic Cell

IFN- γ	Interferon gamma
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-4	Interleukin-4
KO	Knock Out
LAM	Lipoarabinomannan
LJ	Löwenstein-Jensen
LPS	Lipopolysaccharide
M	Molar
mM	Milli Molar
M ϕ	Macrophage
MCP-1	Monocyte Chemoattractant Protein 1
MDR	Multi-Drug Resistant
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
NF- κ B	Nuclear Factor B
NO	Nitric Oxide
NOS ₂	Nitric Oxide Synthase
CHO	Chinese Hamster Ovary
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PE	Phycoerythrin
PFA	Paraformaldehyde
PGL-1	Phenolic Glycolipid I

PPD	Purified-Protein Derivative
PTB	Pulmonary Tuberculosis
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
rGM-CSF	Recombinant Granulocyte Macrophage Colony Stimulating Factor
rIL-4	Recombinant Interleukin-4
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
ROI/RNI	Reactive Oxygen Intermediate/Reactive Nitrogen Intermediate
SS ⁺	Smear positive pulmonary cases
SSC	Side Scatter
TAP	Transporter with antigen Processing
TB	Tuberculosis
TCR	T-Cell receptor
TGF- β	Tumor Growth Factor Beta
TLR	Toll-Like Receptor
TNF α	Tumor Necrosis Factor-alpha
WHO	World Health Organization

Abstract

M. tuberculosis is one of the most ubiquitous and extraordinary effective human pathogens, with one-third of the world's population being infected and is second to HIV in the number of deaths per year from a single infectious agent. These high numbers of deaths occur despite effective therapy available through the WHO DOTS (Directly Observed Therapy, short course) program, widespread vaccination with BCG, and the host's ability to mount a protective immune response.

Numerous studies in humans and animal models over the years have shown that cell-mediated immunity is necessary for protection against *M. tuberculosis* infection. In cell-mediated immunity, antigens should be presented by Antigen Presenting Cells (APCs) in the context of MHC I or MHC II molecules to T cells. Dendritic cells (DC) are unique among all APCs in the adult immune system in many critical ways and play an essential role in the initiation and maintenance of immune response to pathogens, moreover DC-based immunization protocols have been shown to mediate protection against a wide spectrum of infectious diseases caused by viral, bacterial, parasitic and fungal pathogens as well as cancer. However, whether the interaction between the human DC and *M. tuberculosis* represents a defence mechanism by the invaded host, or a smoke screen, masking the presence of an invader is still not clearly understood.

To analyze the interactions between *M. tuberculosis* and immune cells, human peripheral blood monocyte derived immature DC were infected with *M. tuberculosis* H37Rv *wild type* strain. DC were found to internalize the mycobacteria and show dose dependent infection and necrosis with different multiplicity of infection. In this study we investigated whether *M. tuberculosis* induced maturation of DC. Fluorescence activated cell analysis of mean fluorescence intensity of cell surface expression markers CD40, CD54, CD80, CD83, CD86 and HLA DR in infected DC revealed significant ($p < 0.05$) upregulation following infection with *M. tuberculosis* in comparison to immature DC with no stimulation. Lipopolysaccharide (LPS) from *Salmonella abortus equi*, a known DC maturation agent, was used as a positive control and showed a comparable upregulation of cell surface markers as observed with *M. tuberculosis* infected DC. We have also investigated the ability of the *M. tuberculosis* infected DC to induce T cell proliferation. Fluorescence activated cell analysis of mixed leukocyte reaction using 5 and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution technique revealed that the *M. tuberculosis* infected DC induced T cell proliferation. These data clearly demonstrate that *M. tuberculosis* induces activation and maturation of human monocyte derived immature DC *in vitro*.

CHAPTER I

1 INTRODUCTION

1.1 The Pathogen

Mycobacterium tuberculosis (*M. tuberculosis*) is an acid fast, facultative intracellular aerobic pathogen that has straight or curved rod morphology and exists either singly or in clusters. *M. tuberculosis* is a particularly slow growing bacterium and divides once every 18-24 hours and it takes between 18-21 days to view visible colonies on solid medium (Salyers and Whitt, 1994).

The cellular envelope of *M. tuberculosis* consists of a plasma membrane and a highly unusual cell wall. The plasma membrane consists of a classical bilayer structure. The elaborate distinctive features of the mycobacterial cell walls include the lipoarabinomannan (LAM), lipomannan, mycolyl-arabinogalactan, phosphatidyl-myoinositol mannoside, sulfatide, cord factor, and other acylated trehaloses, phenolic glycolipids, lipoligosaccharides, and other attenuated lipids. Many of these have been shown to be involved in the virulence and pathogenesis of this bacillus. LAM, a predominant component of the cell wall, is a virulence factor for *M. tuberculosis*, which activates macrophages and scavenges reactive oxygen intermediates (Brennan, 1995; Nigou *et al.*, 2003).

1.2 Epidemiology of *Mycobacterium tuberculosis* Infection

Mycobacterium tuberculosis was first identified as the causative agent of tuberculosis (TB) by Robert Koch in 1882. It is one of the most ubiquitous and extraordinary effective human pathogens, with one-third of the world's population being infected. Primary infection leads to active disease in only a minority (about 10%) of infected individuals, in most cases within the first two years. In the remaining 90% of cases, the immune system contains the infection and the individual remains non-infectious and symptom free. TB is the world's second commonest cause of death from all infectious diseases, after HIV/AIDS. In 1993 the WHO declared TB as a 'global emergency' (WHO, 1994).

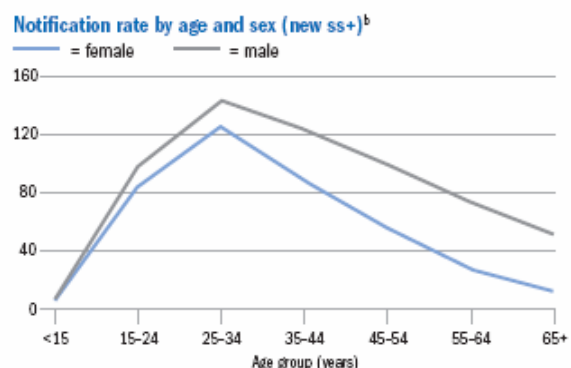
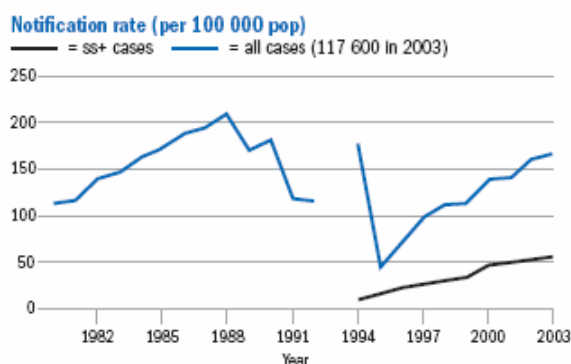
There were 8.8 million new cases of TB in 2003 (140/ 100,000 population), of which 3.9 million (62/100,000) were smear positive and 674,000 (11/100,000) were infected with HIV. There were 15.4 million prevalent cases (245/ 100,000), of which 6.9 million were smear-

positive (109/100,000). An estimated 1.7 million people (28/ 100,000) died from TB in 2003, including those co-infected with HIV (229,000) (WHO, 2005).

Ethiopia ranks 7th globally in the estimated number of TB cases among the 22 high burden countries. According to the estimates by WHO, the incidence of all cases of TB per 100,000 populations is 356 whereas the incidence of new smear positive pulmonary cases (SS⁺)/100,000 is 155. The mortality rate of all cases of TB per 100,000 is estimated to be 79 per year (WHO, 2005).

Table 1: WHO Report on Ethiopia TB Profile (Source: WHO, 2005)

LATEST ESTIMATES ^a		TRENDS	2000	2001	2002	2003
Population	70 678 002	DOTS coverage (%)	85	70	95	95
Global rank (by est. number of cases)	7	Notification rate (all cases/100 000 pop)	139	141	160	166
Incidence (all cases/100 000 pop/year)	356	Notification rate (new ss+/100 000 pop)	47	49	53	56
Incidence (new ss+/100 000 pop/year)	155	Detection of all cases (%)	45	43	47	47
Prevalence (all cases/100 000 pop)	533	Case detection rate (new ss+, %)	35	35	36	36
TB mortality (all cases/100 000 pop/year)	79	DOTS case detection rate (new ss+, %)	35	35	36	36
TB cases HIV+ (adults aged 15–49, %)	21	DOTS case detection rate (new ss+)/coverage (%)	41	50	38	38
New cases multidrug resistant (%)	2.3	DOTS treatment success (new ss+, %)	80	76	76	–



Through the WHO DOTS (Directly Observed Therapy, short course) program, effective therapy is now available in 182 countries and DOTS covers 69% of the world's population. However, the likelihood of DOTS therapy resulting in the eradication of TB is limited by the large reservoir of latently infected individuals as well as delays in diagnosis. Therefore, a vaccine that prevents infection and/or disease is necessary to control or eliminate TB worldwide (WHO, 2005).

Unfortunately, an effective vaccine against TB has not been developed. The only available TB vaccine, Bacillus Calmette-Guerin (BCG), has been in use since the 1920s and more than three billion people have received this vaccine. Although BCG can be effective in reducing the incidence of childhood TB, particularly meningitis, it is relatively ineffective in protecting against adult TB, and doesn't prevent infection with the organism. Thus BCG immunized persons still become infected with *M. tuberculosis*, can develop active TB, and can harbour the organism and reactivate later in life. The efficacy of BCG in human trials has varied from 0 to 80% (Fine, 1995).

It should be stressed that although the majority of the world's population is BCG vaccinated, the incidence of TB is still staggering. Clearly, a more effective vaccine against this disease is needed. A greater understanding of the immune response is necessary in order to develop a vaccine that will confer complete protection from infection and, hopefully, from reactivation of a pre-existing infection.

1.3 Immunology of Tuberculosis

The protective response to *M. tuberculosis* is complex and multifaceted involving many components of the immune system, both from innate and adaptive immune response (Fig.1).

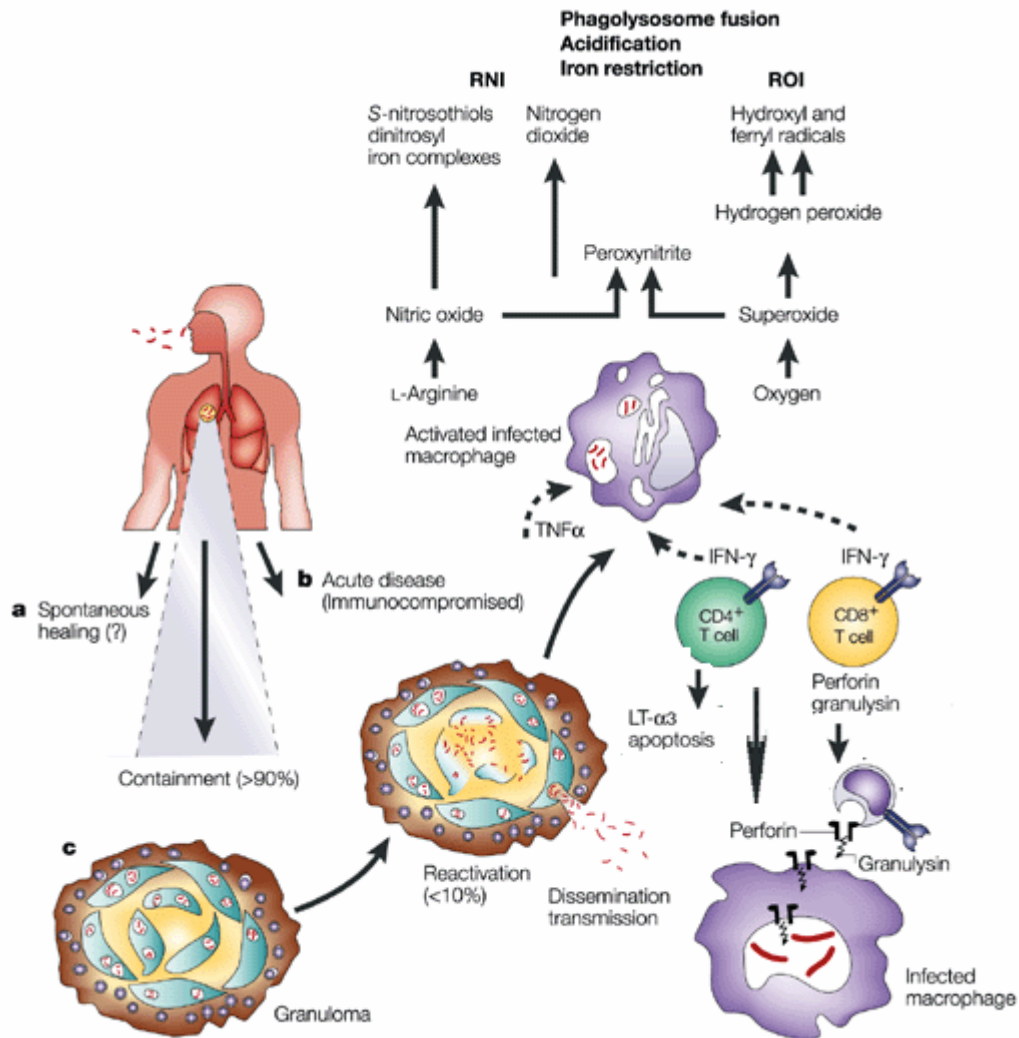


Figure 1: Components of immune system involved in TB immunology. (Source: Kaufman, 2001).

1.3.1 Macrophages

The mononuclear phagocyte constitutes a potent antimicrobial component of cell-mediated immunity. The best characterized effector mechanisms include phagosome –lysosome fusion, the reactive oxygen intermediate (ROI) burst, and the production of reactive nitrogen intermediates (RNI) by an L-Arginine dependent pathway.

Phagolysosome Fusion

It is well established that phagosomes, the product of the endocytic pathway initiated by phagocytosis of large particles including microbes, can fuse with lysosomes. Phagocytosed microorganisms are subject to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes. It appears that the antimicrobial activity of the phagolysosome is mediated, at least in part, by the degradative function of lysosomal hydrolases and / or direct and indirect effect of acidification (Russell, 2001).

Studies have shown that *M. tuberculosis* can inhibit both phagosome acidification and phagosome-lysosome fusion. However, upon macrophage activation there is some phagosome-lysosome fusion resulting in bacterial death (Flynn and Chan, 2003).

Reactive Nitrogen Intermediates

Macrophages (MØ) have been shown to produce nitric oxide (NO) and other reactive nitrogen intermediates (RNI) via the NOS₂ enzyme using L-arginine as a substrate. NOS₂ is induced by IFN- γ and a second signal such as TNF α or bacterial products such as LPS or LAM (MacMicking, *et al.*, 1997).

Nitric oxide production within macrophages has major anti-microbial mechanisms. RNI can inflict damage to the bacterium by modifying DNA, proteins and lipids. In the murine system, toxic nitrogen compounds have been shown to play a role in protection in both an acute and a chronic *M. tuberculosis* infection. NOS₂ derived RNIs are the only clearly demonstrated mycobacterial effector function in macrophages (Shiloh, 2000).

Reactive Oxygen Intermediates

While the role of NOS₂ in host defence against *M. tuberculosis* is well established, the significance of toxic oxygen species in the control of TB remains controversial. Despite the demonstration that H₂O₂ generated by cytokine activated macrophages was mycobacteriocidal and ROI were shown to kill *M. microti*, the ability of ROI to kill *M. tuberculosis* remains to be confirmed. Indeed, mycobacteria are capable of evading the toxic effect of ROI by various means. A major mycobacterial cell wall component, LAM, was effective at scavenging ROI generated by the respiratory burst of infected macrophages. It was also shown that ROI could

inhibit protein kinase C which downregulates the respiratory burst. Tubercle bacilli also produce both superoxide dismutase and catalase that may interfere with toxic oxygen radical production. Other mycobacterial components such as sulfatides and phenolic glycolipid I (PGL-1) have also been suggested to interfere with ROI dependent antimicrobial mechanisms within macrophages (Flynn and Chan, 2001).

1.3.2 Toll-Like Receptors (TLR)

Toll-Like Receptors (TLRs) are believed to represent key receptors for the recognition of mycobacterial antigens and activation of macrophages and dendritic cells (DC) as well as other cells of innate immunity, thereby likely modulating the adaptive immune response. TLRs provide signal for NF- κ B-mediated cytokine cascades involved in anti-microbial mechanisms and likely play a role in regulating *M. tuberculosis* infections.

TLR2, TLR4 and, more recently TLR1/TLR6 that heterodimerise with TLR2, have been implicated in the recognition of mycobacterial antigens. Over expression of either TLR2 or TLR4 in Chinese Hamster Ovary (CHO) cells confers cellular activation by viable *M. tuberculosis*. Soluble heat-stable mycobacterial fraction, distinct from the mycobacterial cell wall LAM, signals through TLR2 whereas heat-labile cell associated ones signal through TLR4. However, most purified mycobacterial antigens tested so far signal through TLR2. *M. tuberculosis* induced TNF α production by macrophages is blocked by a TLR4 antagonist (Quesniaux, *et al.*, 2004) and other researchers also reported that *M. tuberculosis* induced nitric oxide production via signalling through TLR (Modlin, 2001).

1.3.3 Cytokines

1.3.3.1 Proinflammatory Cytokines

Different cytokines and cytokine receptors are involved in type I immunity (Th_1) in TB (Fig. 2).

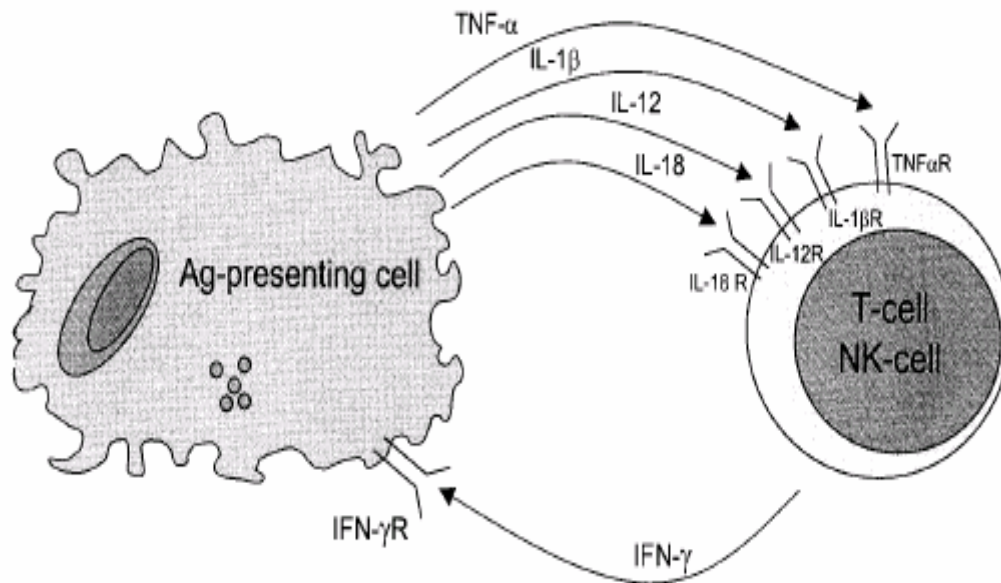


Figure2: Cytokines and Cytokine Receptors Involved in type I Immunity in Tuberculosis (Source: van Crevel *et al.*, 2002).

Interleukin 12 (IL-12)

To control *M. tuberculosis* infection, a Type 1 T cell response must be generated, as in the case of most intracellular infections. Exogenous administration of therapeutic IL-12 and the finding of susceptibility in IL-12p40 gene deficient mice to *M. tuberculosis* strongly support an important role for this cytokine in the protective immune response against *M. tuberculosis* (Cooper, *et al.*, 1997; Flynn, *et al.*, 1995). Vaccination in conjunction with IL-12 DNA has been reported to reduce the bacterial load in *M. tuberculosis* in chronically infected mice (Lowrie, 1999).

Interferon- γ (IFN- γ)

IFN- γ is a critical cytokine involved in the control of *M. tuberculosis* infections and produced by CD4⁺ and CD8⁺ T cells. NK cells are another source of IFN- γ , but they have not been shown to directly produce this cytokine in response to *M. tuberculosis* (Lalvani, *et al.*, 1998; Serbina and Flynn, 1999). Mice with a genetic deficiency for IFN- γ are very susceptible to infection with virulent *M. tuberculosis* with a mean survival time of 14 days (Flynn, *et al.*, 1993). Notably there was also less NOS₂ production in these mice, indicating that macrophage activation was defective, contributing to the susceptibility of IFN- γ gene knockout mice (KO). The importance of this cytokine has also been confirmed in humans who have a mutation in their IFN- γ receptor and this was associated with a heightened susceptibility to mycobacterial infections (Jouanguy *et al.*, 1999). IFN- γ has been detected in the granulomas of patients and their T cells have been shown to produce IFN- γ in response to challenge with *M. tuberculosis* (Fenhalls *et al.*, 2000).

Tumor Necrosis Factor α (TNF α)

TNF α is required for the control of an acute *M. tuberculosis* infection. TNF α is secreted by DC and macrophages in response to *M. tuberculosis* infection (Tascon *et al.*, 2000). Mice deficient in TNF α or the 55-kDa TNF receptor succumb more quickly to *M. tuberculosis* infection (Bean, *et al.*, 1999; Flynn, *et al.*, 1995). Again, these mice have a high bacterial burden compared to wild-type controls. TNF α acts as a necessary second signal in addition to IFN- γ to activate macrophages to produce RNI. NOS2 expression is delayed in the TNFRp55 gene KO mice but not in TNF α gene KO mice. Overall, the role of TNF α in *M. tuberculosis* infection is complex since it appears to contribute to both protection and pathology (Flynn and Ernst, 2000).

Interleukin 1 β (IL-1 β)

IL-1 β is mainly produced by monocytes, M ϕ , and DC. In TB patients, IL-1 β is expressed in excess and at the site of disease (Bergeron and Soler, 1997). Studies with mice suggest an important role of IL-1 β in TB: IL-1 β and IL-1 α double gene KO mice (Yamada, *et al.*, 2000) and IL-1R type I-deficient mice (which do not respond to IL-1) display an increased

mycobacterial outgrowth and also defective granuloma formation after infection with *M. tuberculosis* (Juffermans *et al.*, 1996).

1.3.3.2 Anti inflammatory cytokines

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine, which is produced by macrophages and T cells, leading to down regulation of IL-12 and consequently IFN- γ secretion by T cell and M ϕ activation. Inhibition of IL-10 can help reverse the suppression of T cell proliferation *in vitro* by M ϕ s isolated from human TB patients (Gong *et al.*, 1996). IL-10 has been shown to directly inhibit APC functions and T cell responses to mycobacterial infection (Rojas, *et al.*, 1999).

Tumor Growth Factor β (TGF- β)

TGF- β also seems to counteract protective immunity in TB. Mycobacterial products induce production of TGF- β by monocytes and dendritic cells. Interestingly, LAM from virulent mycobacteria selectively induces TGF- β production. Like IL-10, TGF- β is produced in excess during TB and is expressed at the site of disease (Toossi *et al.*, 1995). TGF- β suppresses cell-mediated immunity: in T cells, TGF- β inhibits proliferation and IFN- γ production; in macrophages it antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation. In addition, TGF- β may be involved in tissue damage and fibrosis during TB, as it promotes the production and deposition of macrophage collagenases and collagen matrix (Toossi *et al.*, 1998).

Interleukin 4 (IL-4)

The deleterious effects of IL-4 in intracellular infections (including TB) have been ascribed to this cytokine's suppression of IFN- γ production, and macrophage activation (Lucey *et al.*, 1996). In mice infected with *M. tuberculosis*, progressive disease (Hernandez Pando *et al.*, 1996) and reactivation of latent infection (Howard and Zwilling, 1999) are both associated with increased production of IL-4. Similarly, over-expression of IL-4 intensified tissue damage in experimental infection (Lukacs *et al.*, 1997). Conversely, inhibition of IL-4 production did not seem to promote cellular immunity: IL-4 KO mice displayed normal

instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of TB development (Erb *et al.*, 1998). In contrast, a recent study on IL-4 KO mice showed increased granuloma size and mycobacterial outgrowth after airborne infection. Compared with control mice, production of proinflammatory cytokines was increased in these animals and accompanied by excessive tissue damage. Researchers have detected increased production of IL-4 in human TB patients, especially those with cavitary disease. However, this is not a consistent finding and remains to be determined whether IL-4 causes or merely reflects disease activity in human TB. Thus, the role of IL-4 in TB susceptibility is not yet entirely resolved (van Crevel, *et al.*, 2002).

1.3.4 Chemokines

Chemokines (chemotactic cytokines) are largely responsible for recruitment of inflammatory cells to the site of infection. A number of chemokines have been investigated in TB. First, several reports have addressed the role of IL-8, which attracts neutrophils, T lymphocytes, and possibly monocytes. Upon phagocytosis of *M. tuberculosis* or stimulation with LAM, macrophages produce IL-8 (Zhang, *et al.*, 1995). This production is substantially blocked by neutralization of TNF α and IL-1 β , indicating that IL-8 production is largely under the control of these cytokines. Pulmonary epithelial cells also produce IL-8 in response to *M. tuberculosis* infection. In TB patients, IL-8 has been found in bronchoalveolar lavage fluid, lymph nodes, and plasma (Juffermans *et al.*, 1999).

A second major chemokine is monocyte chemoattractant protein 1 (MCP-1), which is produced by and acts on monocytes and macrophages. *M. tuberculosis* preferentially induces production of MCP-1 by monocytes. In murine models, deficiency of MCP-1 inhibited granuloma formation ((Lu *et al.*, 1998). Also, chemokine receptor 2-deficient mice, which fail to respond to MCP-1, display reduced granuloma formation and suppressed Th1-type cytokine production and die early after infection with *M. tuberculosis* (Peters, *et al.*, 2001). In alveolar lavage fluid, serum and pleural fluid from TB patients, concentrations of MCP-1 were found to be elevated (Juffermans, *et al.*, 1999).

A third chemokine is Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), which is produced by a wide variety of cells and which shows promiscuous binding to multiple chemokine receptors. In murine models, expression of RANTES was

associated with development of *M. bovis* induced pulmonary granulomas (Chensue, *et al.*, 1999). In human patients, RANTES has been detected in alveolar lavage fluid. Apart from IL-8, MCP-1 and RANTES, other chemokines may be involved in cell trafficking in TB (Ragno, *et al.*, 2001).

1.3.5 T Cells

M. tuberculosis is a classic example of a pathogen for which the protective response relies on cell-mediated immunity. This is primarily because the organism resides intracellularly within macrophages; thus T cell effector mechanisms, rather than antibody, are required to control or eliminate the bacteria. Studies in humans and animal models demonstrate that acquired immunity to *M. tuberculosis* requires contributions by multiple T cell subsets, which include a dominant role for CD4⁺ T cells and significant roles for CD8⁺ αβ and γδ T cells and CD1 restricted T cells. The reasons for involvement of these multiple T cell subsets are not known (Boom, *et al.*, 2003).

1.3.5.1 CD4⁺ T Cells

M. tuberculosis resides primarily in a vacuole within the macrophage and thus, MHC class II presentation of mycobacterial antigens to CD4⁺ T cells is an obvious outcome of infection. It is not surprising that these cells are among the most important in the protective response against *M. tuberculosis* (Flynn and Chan, 2001). Murine studies have shown by antibody depletion of CD4 T cells, adoptive transfer, or the use of gene-disrupted mice that the CD4⁺ T cells subset is required for control of infection. The mean survival time for CD4 gene KO mice infected with *M. tuberculosis* is 60 days and the mean survival time for MHC II gene KO mice is 40 days whereas *wild type* mice will not die from the infection. There is a heavy recruitment of CD4⁺ T cells to the lungs after intravenous infection with *M. tuberculosis* (Boom *et al.*, 2003).

In humans, the necessity for CD4⁺ T cells to help control infection is shown by the rapid acceleration of TB in HIV positive patients who have a loss of CD4⁺ T cells (Selwyn *et al.*, 1989).

Most studies have focused on IFN-γ production by CD4⁺ T cells in response to mycobacterial antigen, but other functions of CD4⁺ T cells are likely to be important in the protective

response and must be understood as correlates of immunity and as targets for vaccine design. Possible pathways which are still under active investigation include the perforin and granulysin, FAS-L or TNF α lytic and apoptosis pathways (Oddo, *et al.*, 1998) and the interaction of CD40-CD40L between CD4⁺ T cells and to enhance APC function (Andreasen, *et al.*, 2000).

1.3.5.2 CD8⁺ T Cells

The contribution of CD8⁺ T cells in infection has been debated for a long time. At the crux of this debate is whether *M. tuberculosis* is ever found outside the phagosome (*i.e.* cytoplasm) where it could be processed for MHC I presentation. Although it still has not been exhaustively determined as to how mycobacterial antigens are presented to the immune system, CD8⁺ T cells that are specific for mycobacterial antigens have been isolated from both infected humans and mice. CD8⁺ T cells have also been shown to accumulate in the lungs after *M. tuberculosis* infection in mice. These cells can produce IFN- γ and lyse infected macrophages (Serbina, *et al.*, 2000). Earlier studies with mice deficient in the genes for β 2-microglobulin, TAP, MHC I, and CD8⁺ T cells have shown that these mice exhibit heightened susceptibility to *M. tuberculosis* infection, and therefore CD8⁺ T cells must contribute to protection (Grotzke and Lewinsohn, 2005).

CD1 molecules are structurally similar to MHC class I molecules but have differences in their binding groove which allow for the binding and presentation of lipids and glycolipids instead of protein antigens (Porcelli and Modlin, 1999). Therefore, they are believed to serve as important presentation complexes for mycobacterial antigens and indeed, this has been shown for many mycobacterial lipids including LAM (Sieling, *et al.*, 1995). However the actual role of T cells in response to these complexes in infection has been unclear.

Indeed the roles of both CD4⁺ and CD8⁺ T cells may be multi-faceted and the two cell types likely collaborate in the generation of protective cellular immunity. Therefore understanding how to activate both T cell types using a vaccine is probably crucial to generating protection and long-term memory.

1.3.6 Dendritic Cells

Dendritic cells (DC) originally identified by Steinman and his colleagues (1972) represent the pacemakers of the immune response. They comprise a family of antigen presenting cells that act like ‘conductors’ of the immune response in their capacity to orchestrate signals derived from the different parts of the immune system. DC can take up an array of different antigens, including microorganisms, extracellular fluids and apoptotic bodies released by dying cells, which they can process and present in the form of peptides bound to both major histocompatibility complex (MHC) class I and class II molecules more effectively than any other antigen presenting cell. After microbial or inflammatory stimuli, DC undergo a process of maturation and migrate to secondary lymphoid organs where they can stimulate naïve T cells (Lipscomb and Masten, 2002).

The system of these cells is enormously complex. First, there is no single molecule that they uniquely express. Rather, the DC subsets as well as maturation stages are defined by a combination of markers. Second, DC comprises 2 distinct subsets, myeloid and plasmacytoid. Moreover, 3 stages of development have been delineated: 1) precursor DC patrolling through blood and lymphatics, 2) immature DC residing within virtually every tissue, and 3) mature DC residing temporarily within secondary lymphoid organs. *In situ*, as in the skin and lymphoid organs, DC can be identified as stellate-shaped cells with many dendrites that are long and thin, either fine or sheet like. The shape and motility of DC suit their functions, initially the efficient capture of antigen and subsequently the selection of rare antigen-specific lymphocytes (Hart, 1997).

1.3.6.1 Dendritic Cell Development and Trafficking Pathways

Dendritic cells are derived from haematopoietic stem cells in the bone marrow and form a network of heterogeneous cell populations. Human DC subsets are less well defined than murine ones. The best-characterized human DC populations are monocyte-derived DC (Myeloid DC) and lymphoid-derived DC (Plasmacytoid DC (pDC)) (Fig. 3). CD11c⁺ CD1a⁺ CD14⁻ monocyte-derived DC can be isolated from the blood, or they can be grown in culture in the presence of granulocyte/macrophage colony-stimulating factor and IL-4 from precursors isolated from the blood or the bone marrow. pDC, which are phenotypically characterized as CD11c⁻CD45RA⁺CD123⁺blood DC antigen 2 (BDCA2)⁺, are found in the blood and can be

generated *in vitro* from IL-3-supplemented cultures of CD11c⁻CD4⁺IL-3 receptor (IL-3R)⁺ precursors (Degli-Esposti and Smyth, 2005).

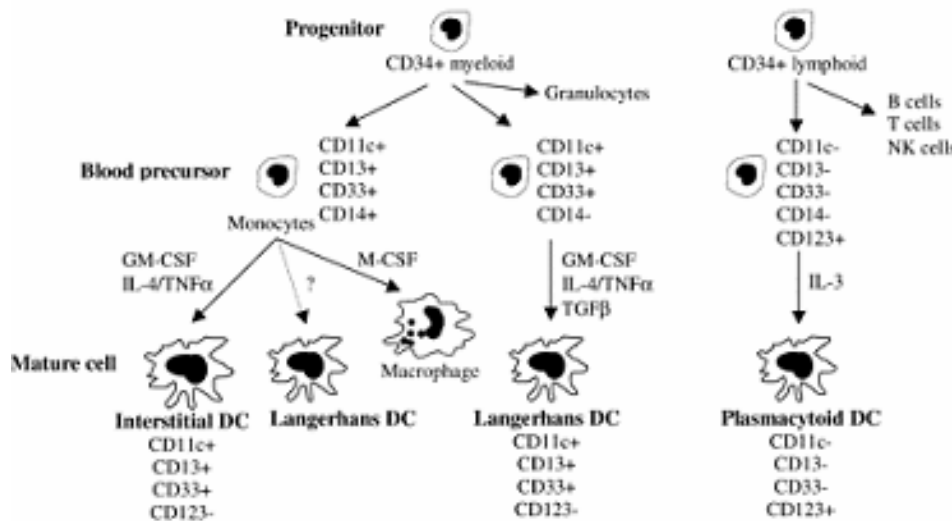


Figure 3: Schema for derivation of human dendritic cell (DC) subsets from CD34⁺ myeloid and lymphoid progenitors (adopted from Lipscomb and Masten, 2002).

Dendritic cells are migratory cells that traffic from one site to the next performing specific functions at each site. Bone marrow derived DC circulate as precursors in blood before entering tissue where they become resident immature DC that monitor their environment. The extravasation of DC from the blood to peripheral tissue and the movement from peripheral tissue into lymphoid tissue requires chemoattractants called chemokines. The anatomic location of inflammatory chemokines within peripheral tissue and lymphoid chemokines within lymphoid tissue regulates the migration of DC initially to sites of antigen and ultimately to lymphoid tissue to initiate an immune response (Lipscomb and Masten, 2002).

1.3.6.2 Antigen Capture, Processing and Presentation by Dendritic Cells

Dendritic cells are unique among all APCs in the adult immune system in many critical ways. Unlike M ϕ or B cells, DC' primary function appears to be antigen presentation. Both DC and M ϕ differentiate from circulating bone marrow derived precursors and complete their differentiation upon leaving the blood stream and taking residence in peripheral tissues. DC have a unique distribution within lymphoid organs, accumulating in regions where M ϕ and B cells are generally excluded. DC are enriched in the areas where naive T cells are activated, in

an optimal position to be interrogated by a continuously moving population of T cells seeking their cognate peptide- MHC complexes (Trombetta and Mellman, 2005).

Dendritic cells show a unique functional duality during their development, designed to ultimately provide secondary lymphoid tissues with useful information about the antigenic composition in the periphery. At the immature stages of development, DC resident in peripheral tissues are specialized in antigen capture, acting as sentinel cells (high intracellular MHC II, Endocytosis, phagocytosis, High CCR1, CCR5, and CCR6; low CCR7 and low CD40, CD54, CD80, CD83, CD86 and CD58). After antigen uptake, DC rapidly cross the endothelium of lymphatic vessels and migrate to the draining secondary lymphoid organ. During this migration, DC undergo a maturation process which is characterized by downregulation of the capacity to capture antigen and upregulation of antigen processing and presentation, expression of costimulatory molecules and of dendritic morphology (high surface MHC II, low endocytosis, low phagocytosis, low CCR1, CCR5, CCR6, high CCR7 and high CD40, CD54, CD80, CD83, CD86 and CD58) (Banchereau *et al.*, 2000).

DC have unique surveillance and migratory properties, enabling them to carry antigens captured in the periphery to secondary lymphoid organs, or to internalize them directly from the lymph (Itano, *et al.*, 2003). In peripheral tissues DC capture and process antigens via different pathways: (a) Macropinocytosis where fluid from the extracellular milieu is taken up into pinocytic vesicles and antigen is concentrated by expelling excess water (Sallusto, *et al.*, 1995); (b) Endocytosis via lectin receptors such as mannoseR, DEC-205, Langerin, or DC specific ICAM 3 grabbing non integrin (DC-SIGN) (Geijtenbeek, *et al.*, 2000; Sallusto *et al.*, 1995; Valladeau *et al.*, 2000) (c) Fc receptors (Fc γ RI, Fc γ RII, Fc γ RIII) and complement receptors (CR3) can mediate efficient internalization of immune complexes or bacteria (Rescigno, *et al.*, 2000) (d) Phagocytosis of apoptotic and necrotic cell fragments, viruses, bacteria including mycobacteria and /or intracellular parasites (Albert, *et al.*, 1998); (e) Toll like receptors (TLRs) in pathogen recognition including LPS, peptidoglycan, lipoteichoic acid, and lipoprotein (Visintin, *et al.*, 2001).

DC present virtually any form of protein antigen associated with MHC-I (Fig. 4) or MHC-II (Fig. 5) molecules. Indeed, the ability of DC to “cross-present” exogenous antigens on MHC-I molecules is arising as a distinguishing feature; M \emptyset are also capable of cross-presentation, but

with efficiencies that are orders of magnitude lower (Delamarre *et al.*, 2003). In addition to their distinct antigen handling and homing properties, DC exhibit a variety of other features that greatly enhance their capacity as APCs. Among these are exceptionally high levels of MHC-II and costimulatory molecules, certainly as compared with MØ in most tissues. DC also expresses CD86 greater than fivefold higher density than B cells. In addition, the extensive folds and dendritic extensions characteristic of DC enables them to form close contact with multiple T cells simultaneously. MHC-II molecules on the DC plasma membrane also appear to be laterally clustered, which may further facilitate the efficiency of T cell scanning by increasing the effective local density of MHC molecules at contact sites, even in the absence of antigen-specific recognition (Turley, *et al.*, 2000).

DC are also capable of presenting antigens on CD1 molecules. CD1 molecules present both endogenous and exogenous derived lipid and glycolipid antigens. Antigen processing and presentation by CD1 is different from that described above for MHC Class I and II. CD1 molecules are synthesized in the ER and are expressed on the plasma membrane following traffic to the surface via vesicular transport. CD1 molecules are subsequently incorporated into endosomes and become associated with lipid ligands and recycle to the plasma membrane (Shinkai and Locksley, 2000).

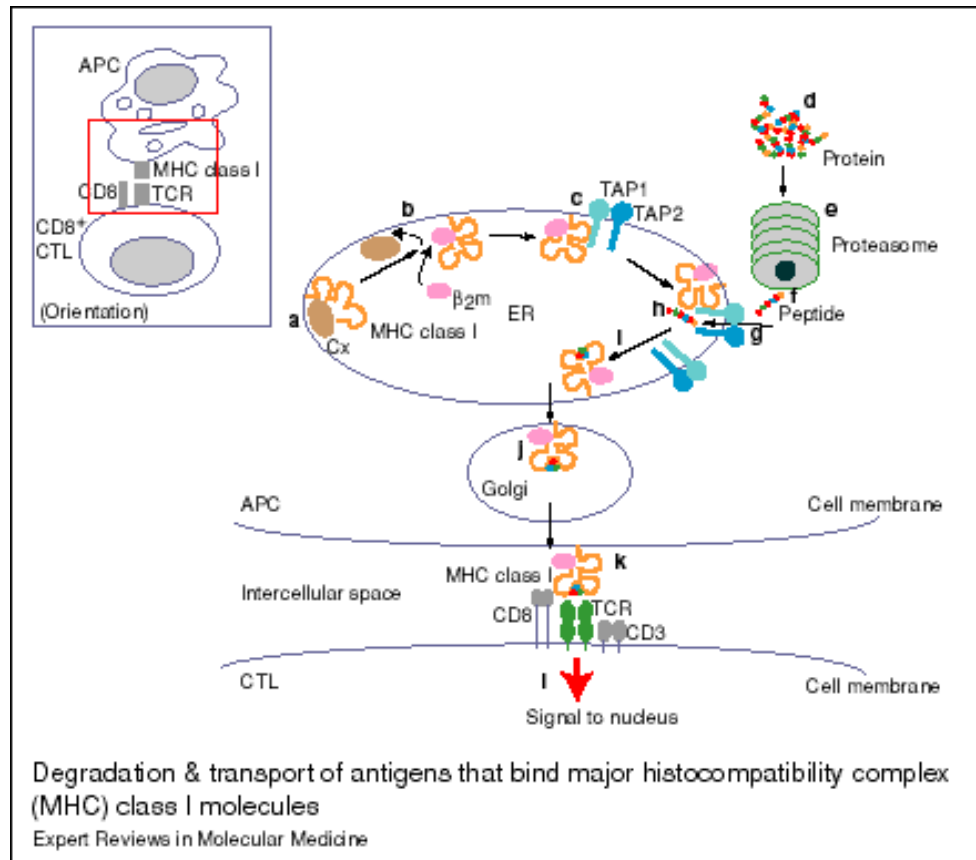


Figure 4: Degradation and transport of antigens that bind major histocompatibility complex (MHC) class I molecules (Source:<http://www-ermm.abcu.cam.ac.uk/figures.htm>). **(a)** In an antigen-presenting cell (APC), newly synthesised MHC class I molecules bind to calnexin (Cx), which retains them in a partially folded state in the endoplasmic reticulum (ER). **(b)** Binding of MHC class I molecules to β_2 microglobulin (β_2m) displaces Cx and allows binding of chaperonin proteins (calreticulin and tapasin; not shown). **(c)** The MHC class I- β_2m complex binds to the TAP complex (TAP1-TAP2), which awaits the delivery of peptides. **(d)** Peptides (e.g. from antigens) are formed from the degradation of cytosolic proteins ('self'-, pathogen- and tumour-derived proteins in the cytoplasm). **(e)** These are degraded by proteasomes into **(f)** short peptides. **(g)** Peptides are transported into the ER by the TAPs, where they meet the MHC class I- β_2m complex **(h)**. This peptide binding in the antigenic groove of the MHC stabilises the structure of the MHC class I molecule and **(i)** releases the TAP complex. **(j)** The fully folded MHC class I molecule with its peptide is transported to the cell surface via the Golgi apparatus. **(k)** Recognition of the MHC class I-peptide complex by the T-cell receptor (TCR) of an antigen-specific ($CD8^+$, $CD3^+$) cytotoxic T lymphocyte (CTL) takes place and **(l)** a signal transduction event activates effector functions in the MHC-class-I-restricted T cell.

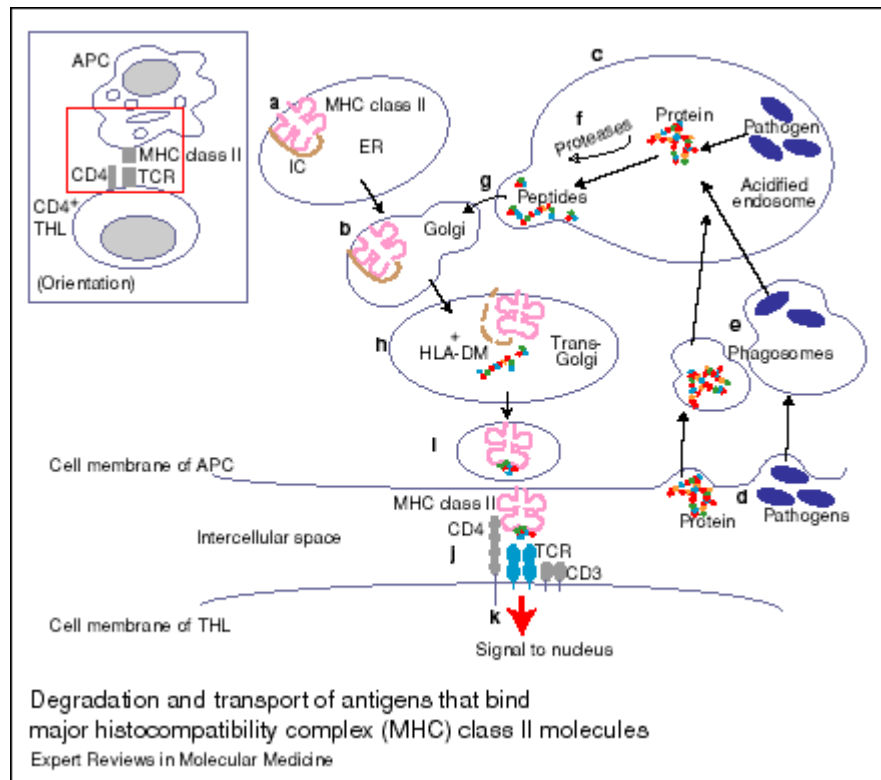


Figure 5: Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules (Source: <http://www-ermm.cbcu.cam.ac.uk/figures.htm>). **(a)** In an antigen-presenting cell (APC), newly synthesised MHC class II molecules bind the invariant chain (IC), which prevents binding of peptides that are present in the endoplasmic reticulum (ER). **(b)** The IC allows transport of MHC class II molecules from the ER into the Golgi apparatus to acidified endosomes. **(c)** Endosomes contain peptides that are derived from either resident pathogens (e.g. bacteria) or **(d)** engulfed extracellular proteins (or pathogens) **(e)** in the phagosomes. **(f)** Proteases within the endosome degrade proteins into peptides. **(g)** The endosome fuses with the Golgi to form the trans-Golgi. **(h)** Here, the IC is cleaved and released from the MHC class II molecule. This allows the binding of peptides within the endosome to the peptide-binding cleft of the MHC molecules. An MHC-class-II-like molecule (HLA-DM) binds to MHC class II molecules to facilitate the release of the IC (not shown). **(i)** The MHC class II–peptide complex is then transported to the cell surface of the APC for **(j)** recognition by the T-cell receptor (TCR) of (CD4⁺, CD3⁺) T-helper lymphocytes (THLs) and **(k)** intracellular signalling for activation.

1.3.6.3 Dendritic Cell Role in T cell Memory, Effector Function and Tolerance

DC regulate primary immune responses by directing antigen specific T cells to die or to become anergic, memory or effector T cells. CD4⁺ and CD8⁺ T cells respond to peptide

antigen displayed on MHC class II and MHC class I molecules respectively (referred to as signal 1). Accessory molecules on DC are required to ensure that T cells will divide and differentiate into effector cells (signal 2). In the absence of sufficient co-stimulation, T cells exhibit anergy or undergo apoptosis. Secretion or lack of secretion of factors by DC, particularly IL-12 (Fig. 4), are instrumental in the final differentiation of T cells into type 1 or type 2 effector T cells respectively (signal 3) (Banchareau *et al.*, 2000).

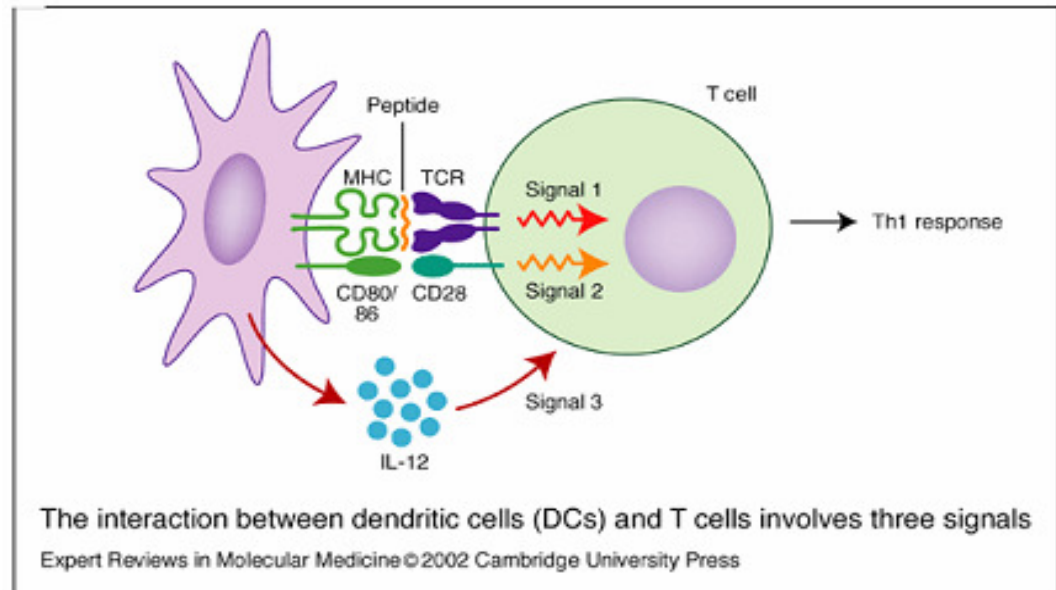


Figure 6: The Interaction between Dendritic Cells (DC) and T Cells

(Source: <http://www-ermm.cbcu.cam.ac.uk/figures.htm>).

The length of time that T cells and DC interact defines effector function, homing and survival of responder T cells with only the fittest T cells maintaining a DC-T cell interaction and surviving to become memory T cells (Lanzavecchia and Sallusto, 2000). Excessive stimulation causes responding naïve T cells to proliferate and develop effector function, but many of these responders soon die, some within the lymphoid organ and others after they migrate to tissue sites (Jacob and Baltimore, 1999). Some effector T cells survive and persist as effector memory cells as the primary cells wanes, although the survival signals these cells receive are not certain. Inadequate stimulation, either through a poor fit of the T cell receptor (TCR) for the DC's MHC/peptide complex or lack of costimulation as a result of a low level

of DC costimulatory molecules or both, would lead to T cell anergy or programmed cell death (Jameson and Bevan, 1995). A second type of memory T cell, a central memory T cell, develops if the strength of DC stimulation falls somewhere intermediate between that required for generating anergy and that required for polarizing effector T cells. Central memory T cells circulate between blood and lymphoid organs and respond to MHC/peptide complexes by further expansion and differentiation into effector cells, which subsequently migrate to relevant target sites. In contrast, effector memory T cells circulate between blood and peripheral tissues and, thus can quickly respond to antigen by displaying immediate effector function (Sallusto *et al.*, 1999).

DC also play a role in the development of tolerance both centrally and peripherally. Central tolerance mechanisms occur in the thymus for T cells. T cells that might inadvertently respond to DC carrying self-peptides are deleted during ontogeny in the thymus. T cells that fail to respond to stimuli in the thymus die from neglect, while T cells that recognize MHC/peptide with high avidity undergo apoptosis and are deleted (Brocker, 1999).

Peripheral tolerance mechanisms include T cell death, T cell anergy and active suppression by T regulatory cells. If T cells recognize DC with only low levels of MHC/peptide, have low affinity for their cognate ligand, or receive no costimulation from DC, they become anergic or undergo apoptosis (Steinbrink, *et al.*, 1997).

Apart from activating naïve T cells in secondary lymphoid organs, DC may directly or indirectly modulate B cell growth and differentiation (Fig.5). A critical indirect role for DC in B cell stimulation relates to their role in activating T cells through upregulation of CD40L and by secretion of B cell helper factors. However DC can also interact directly with CD40 activated naïve B cells to induce proliferation through an unidentified mechanism. Furthermore in an IL-12 dependent mechanism, DC affect the differentiation of B cells into IgM secreting plasma cells. Plasma cell differentiation is facilitated by DC secretion of the IL-6R α chain, gp80, which complexes to IL-6 and then binds to the IL-6R receptor on the responding B cell (Dubois, *et al.*, 1999). DC also stimulate CD40-ligand activated B cells to undergo isotype switching. DC can capture and present unprocessed antigen to B cells and induce an IgG switch both *in vitro* and *in vivo* (Wykes, *et al.*, 1998).

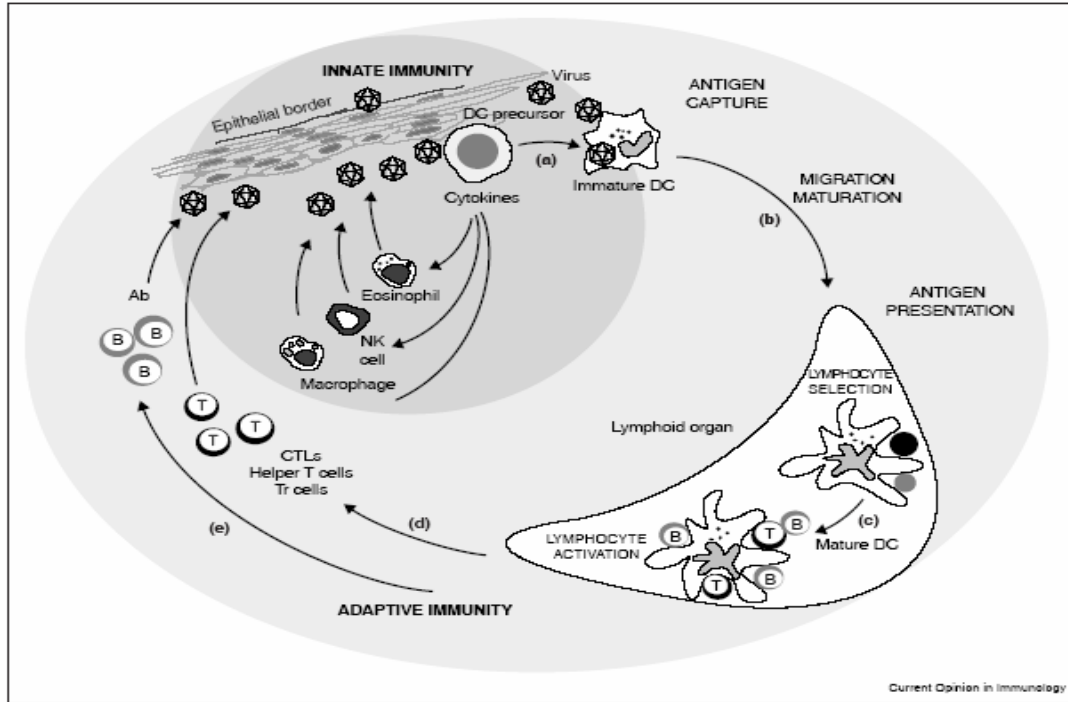


Figure 7: The Life Cycle of Dendritic Cells (Source: Banchereau *et al.*, 2000).

1.3.6.4 Dendritic Cells and *Mycobacterium tuberculosis*

Dendritic cells, the most potent antigen presenting cells, play a central role in induction and regulation of protective immunity against microorganisms. In the lungs, immature DC line alveolar spaces and readily take up foreign materials, presumably including inhaled *M. tuberculosis*. To present antigen efficiently, these immature DC must differentiate into activated, mature DC. During this process, DC phagocytic capacity is downregulated and antigen-presenting capacity is upregulated. Mature DC express high levels of the antigen presenting molecules MHC I, MHC II, and co-stimulatory molecules such as CD40, CD80, CD86 on their surfaces. These cells can migrate to regional lymph nodes by means of upregulated surface expression of the chemokine receptor CCR7. Within lymph nodes DC interact with the abundant T cells that traffic through this site, to activate both naïve and memory T cell immunity.

The fate of interaction between the human dendritic cell and *M. tuberculosis* is not clearly known. Whether this encounter represents a defence mechanism by the invaded host, or a smoke screen, masking the presence of an invader is still unknown. Henderson *et al* found that

DC phagocytose *M. tuberculosis* efficiently, suggesting a role for this important cell in early response to TB infection. Infection with this pathogen resulted in upregulation of MHC I and MHC II, CD40, CD54, CD58, and CD80 (Henderson, *et al.*, 1997), a phenotype consistent with the activation of the DC, suggesting that infected DC produce cytokines that lead to maturation, and possibly to migration and antigen processing and presentation. In contrast to these findings some workers (Hanekom, *et al.*, 2003) reported that DC infected with *M. tuberculosis* show some upregulation of DC surface expression of maturation markers such as CD25, CD83, CD 40, CD80, CD86 and the antigen presenting molecules MHC I and MHC II. This was minimal compared with that induced by the maturation cocktail of TNF α , IL-1 β , and PGE $_2$. *M. tuberculosis* infected DC are compromised in their ability to activate naive T cells and were poor inducers of autologous T cell proliferation and cytokine production.

1.4 Hypothesis

The immune response generated to *M. tuberculosis* infection is unable to eliminate a persistent infection and this is due to the different evasion mechanism(s) of the bacteria. In this study, we hypothesise that *M. tuberculosis* inhibits maturation of dendritic cells and thereby inhibits development of strong T helper 1-subset responses. This could be described as a novel evasion mechanism of *M. tuberculosis*.

1.5 Objectives

1.5.1 General Objective

To determine the role of dendritic cells in the initiation of immunity to *M. tuberculosis* infection.

1.5.2 Specific Objectives

1. To analyse whether human monocyte-derived dendritic cells mature after infection with *M. tuberculosis*.
2. To determine whether human monocyte-derived dendritic cells process and present *M. tuberculosis* antigens and thereby augment T cell proliferation.

CHAPTER II

2. MATERIALS AND METHODS

2.1 Source of Dendritic cells

DC were obtained from peripheral venous blood of apparently adult healthy volunteers from AHRI staffs/ medical students. From each volunteer, 40~50ml of peripheral venous blood was taken by venipuncture using heparinized BD vacutainer tube. Blood was collected from human volunteers after obtaining informed consent from each of them and each study subject was given 30 birr as compensation of their time and travel.

2.2 Sample Size

A convenient sampling technique was employed and a total of 15~20 set of experiments with DC were planned to be undertaken at the beginning of the project. Seventeen healthy individuals were bled as source of DC for the experiments.

2.3 Study Design

The study was an experimental *in vitro* study on DC generated from positively selected CD14⁺ cells isolated from peripheral blood mononuclear cells (PBMC) derived from the peripheral venous blood following density gradient method. The isolated immature DC were infected with wild type H37Rv *M. tuberculosis* under BSL 3 conditions and analysed for maturation by looking at the surface markers with flow cytometric analysis. The infected DC were assessed for their ability to promote T cell proliferation in mixed leukocyte cultures (MLC) with autologous CD14⁻ cells by using 5 and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution technique. The result was compared with the known DC activator lipopolysaccharide (LPS), and immature DC alone without any stimulus.

2.4 Study Period

The study was conducted at Armauer Hansen Research Institute (AHRI) from January 2004 to April 2005.

2.5 Materials

Chemicals and Reagents

Chemical/Reagent	Company	Cat. No.
RPMI-1640	Sigma, Germany	R-6504
LPS from <i>S. abortus equi</i>	Sigma, Germany	L-1887
β -2 Mercaptoethanol	Sigma, Germany	M-6250
Paraformaldehyde	Sigma, Germany	P-6148
Trypan blue	Sigma, Germany	T-8154
Dimethyl sulfoxide	Sigma, Germany	D-2650
Sodium bicarbonate	Sigma, Germany	S-5761
Ficoll-Paque	Amersham Bioscience, Sweden	17-1440-03
7AAD	BD Pharmingen, USA	559925
24 well Cell culture plate	BD Pharmingen , USA	353047
6 well Cell culture plate	BD Pharmingen , USA	353046
rHuman IL4	Cytolab/Peprtech , USA	200-04
rHuman GM-CSF	Cytolab/Peprtech , USA	300-03
CD14 microbeads	Miltenyi Biotech, Germany	130-050-201
LS separation columns	Miltenyi Biotech, Germany	130-041-306
Vybrant TM CFDA SE cell tracer	Molecular Probes, USA	V-12883

Monoclonal Antibodies

All listed below were obtained from BD Pharmingen, USA.

Chemical/Reagent	Recommended conc for staining	Clone	Antibody Composition	Cat. N0
Anti-CD3PE	20 μ l / 10 ⁶ cells	UCHT1	Mouse IgG1, κ	555333
Anti-CD40 FITC	20 μ l / 10 ⁶ cells	5C3	Mouse IgG1, κ	555588
Anti-CD54 PE	20 μ l / 10 ⁶ cells	HA58	Mouse IgG1, κ	555511
Anti-CD80 PE	20 μ l / 10 ⁶ cells	L307.4	Mouse IgG1, κ	557227
Anti-CD83 FITC	20 μ l / 10 ⁶ cells	HB15e	Mouse IgG1, κ	556910
Anti-CD86 PE	20 μ l / 10 ⁶ cells	FUN-1	Mouse IgG1, κ	555658
Anti-HLA-DR FITC	20 μ l / 10 ⁶ cells	G46-6	Mouse IgG2a, κ	555811
Anti-CD14 FITC	20 μ l / 10 ⁶ cells	M5E2	Mouse IgG2a, κ	555397
Mouse isotype control IgG1 κ -PE	0.5 μ g / 10 ⁶ cells	MOPC-21	Mouse IgG1, κ	559320
Mouse isotype control IgG1 κ -FITC	0.5 μ g / 10 ⁶ cells	MOPC-21	Mouse IgG1, κ	554679
Mouse isotype control IgG2a κ -FITC	0.5 μ g / 10 ⁶ cells	G155-178	Mouse IgG 2a, κ	554647

Buffers and Media

No.	Type of Media or Buffer	Composition
1	R0	<ul style="list-style-type: none">• RPMI 1640 with L-glutamine• 1% Sodium bicarbonate• 1% Penicillin – Streptomycin
2	R10	<ul style="list-style-type: none">• R0• 10% FBS
3	FACS Buffer	<ul style="list-style-type: none">• PBS• 1% FBS• 0.1% Sodium azide
4	MACS Buffer	<ul style="list-style-type: none">• PBS• 0.5% Bovine Serum Albumin (BSA)• 2 mM EDTA
5	PBS	<ul style="list-style-type: none">• Na_2HPO_4 (9.2 g/10L)• NaCl (81g/10L)• KH_2PO_4 (2.1g/10L)• pH adjusted to 7.2

2.5.1 Media and Reagents

The medium used throughout the study was R0 and R10. Human rGM-CSF and rIL-4 were purchased from Cytolab. LPS from *Salmonella abortus equi* was purchased from Sigma and CFSE was purchased from Molecular Probes.

The following fluorochrome conjugated monoclonal antibodies were purchased from BD Pharmingen: CD40 FITC, CD54 PE, CD80, CD83 FITC, CD86 PE, HLA DR FITC, CD14 FITC, CD3 PE, FITC conjugated mouse IgG1, κ Isotype control, PE conjugated Mouse IgG₁, κ Isotype control, FITC conjugated Mouse IgG_{2a}, κ and 7AAD. CD14 microbead and columns were purchased from Miltenyi Biotech, Germany.

2.5.2 Mycobacteria

M. tuberculosis H37Rv wild type strain was used (Source: ATCC#25618). The mycobacteria were cultured in LJ supplemented with 10% Oleic acid-Albumin-Dextrose Catalase (OADC) (Difco, BD Biosciences Mountain view, CA) and glycerol. Cultures were grown for up to 12 days and were then used for cell infection. To determine the number of bacteria, a loop of colony was taken and enumerated by plating on 7H10 media after eliminating clumps by vigorously vortexing with 5-7 sterile 2mm glass beads. The suspension was then passed through a 26 gauge needle up to 25 to 30 times and settled for 15 minutes, so that the homogenized suspension could be transferred to another tube with the beads and debris left in the tube. The bacterial suspension was adjusted to 0.3 OD value at 600 nm using Novaspec II photometer, Pharmacia Biotech Ltd, UK with R10 without antibiotics to be consistent in each experiment.

Tubes were set up with serial dilution series running from 10^{-1} to 10^{-7} . After mixing gently the bacterial suspension, 100 μ l of bacterial suspension was added into a 10^{-1} dilution tube containing 900 μ l of R10 without antibiotic. Transfer of 100 μ l of bacterial suspension repeated consecutively until the final dilution series include dilutions of 10^{-1} to 10^{-7} . In each experiment fresh 12-days-old culture cells were used to infect DC and the same protocol was used to determine the number and viability of *M. tuberculosis* used.

2.6 Laboratory Methods

2.6.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Peripheral blood (40~50ml) was collected in multiple 10ml heparinised BD vacutainer tubes. PBMC were isolated from the whole blood according to the standard procedures. In brief, whole blood was resuspended in two volumes of R0 and mixed well by pipetting. Two volumes of the diluted blood were gently overlaid on top of 1 volume of Ficoll-Paque™ Plus density gradient (Amersham Pharmacia Biotech, Sweden) and centrifuged at a speed of 1800 rpm (Allegra™ 6R Centrifuge, Beckman Coulter) for 30 min at room temperature with no brake. After centrifugation, the white ring of PBMC at the interface of the plasma and the Ficoll-Paque™ Plus was harvested aseptically and washed three times with R0 medium at 1500 rpm for 10 min at RT. The cells were finally resuspended in R10 and counted using haemocytometer under light microscope (40 x magnifications) using 0.2% trypan blue dye in PBS. Four corners of the WBC counting chambers -16 squares (Improved Neubauer cell counting chamber) were counted for refractile cells and the mean count of four chambers taken to calculate final cell number per ml by multiplying with dilution factor (10) and volume of the counting chamber (10^4) under the cover slip.

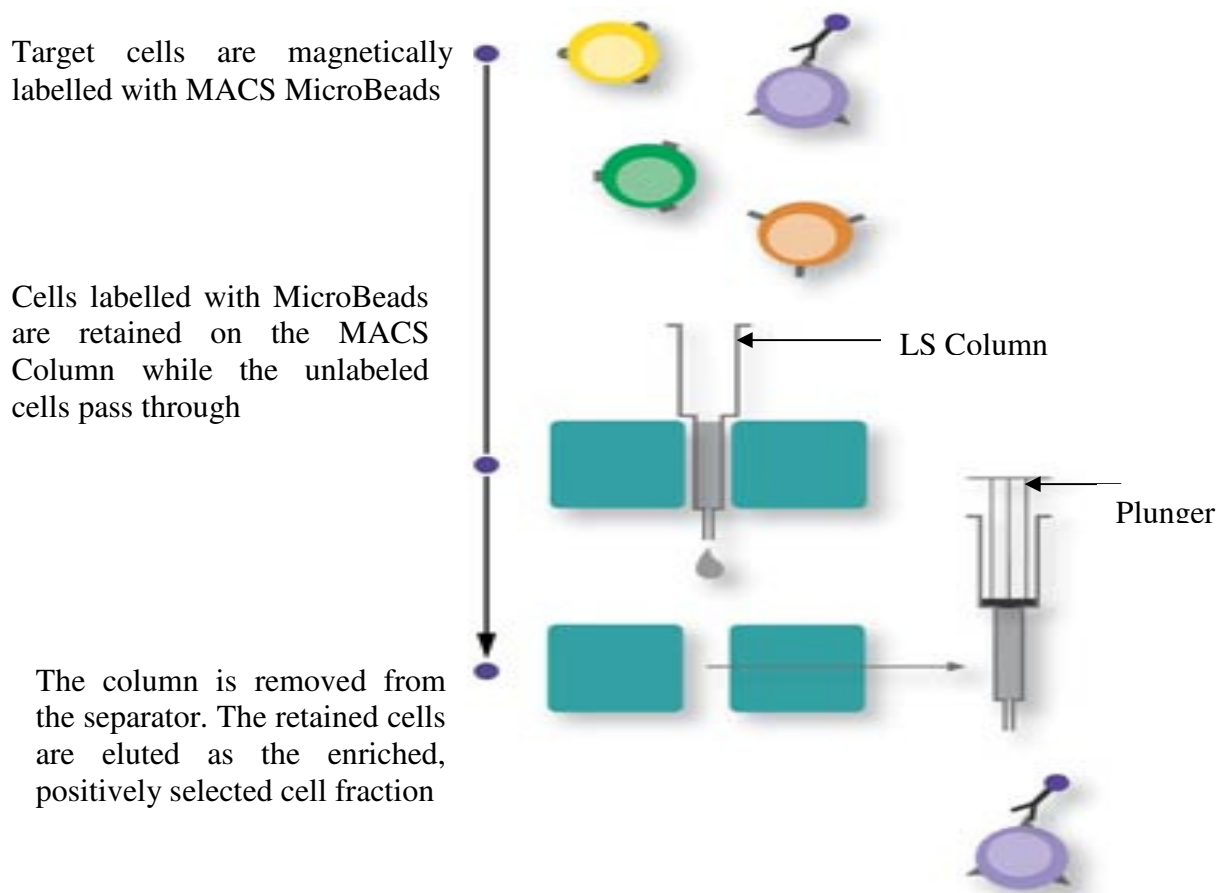
2.6.2 Isolation of Monocytes

Monocytes were purified by positive selection of CD14⁺ cells using a magnetic cell separator (VarioMacs, Miltenyi Biotech, Germany) according to the manufacturer's instruction (Fig. 7). In brief, PBMCs were resuspended in 80µl MACS buffer to which 20 µl of MACS anti- CD14 antibodies coated microbeads were added and incubated for 15 minutes at 4 °C. Cells were then washed by adding 2ml of MACS buffer at 1500 rpm (Allegra™ 6R Centrifuge, Beckman Coulter) for 10minutes at RT. After removing the supernatant completely, the cell pellet was resuspended in 500µl of MACS buffer and the cell suspension was applied onto the LS⁺ column at once. The column was rinsed 3x with 3 ml of MACS buffer each time to wash out all CD14⁻ cells. The column was then removed from separator and placed on a suitable collection tube to obtain CD14⁺ cells. Positive cells were flushed out by applying 5 ml of MACS buffer onto the column using the plunger supplied with the column. Purity was

checked by flow cytometry with the anti CD14 antibody. CD14⁻ cells were cryopreserved and used as a source of autologous T cells for mixed lymphocyte reaction.

After the cells were counted and adjusted to 5×10^6 cells/ml, cells were frozen in freezing media containing 10% dimethyl sulphoxide (DMSO) in FBS and stored at -80°C in the freezing Frost box with Isopropanol for graded freezing for 2 days before transferring to the liquid nitrogen until subsequent assays were done.

Figure 8: Positive selection of cells using VarioMacs



2.6.3 Processing of frozen cell samples

Frozen cells were rapidly thawed in 37°C water bath and the cells were washed three times in complete tissue culture medium (R10), centrifuged at a speed of 1500 rpm, for 10 min at room temperature (Allegra™ 6R Centrifuge, Beckman Coulter). After the first wash, cell clumps were disrupted by incubating the cell suspension with RNase-free DNase I, 10-units/μl (Roche Diagnostics GmbH, Germany) at a ratio of 1:1000 for an hour, in a humidified incubator containing 5% CO₂, at a temperature of 37°C. After washing, the cells were suspended in R10 and counted using haemocytometer under light microscope (40x magnification).

2.6.4 Generation of immature human DC *in vitro*

Immature DC were generated as described previously (Romani *et al*, 1996) with minor modifications. In brief, CD14⁺ cells were incubated in six-well tissue culture plates (at a concentration of approximately 2 x 10⁶/well in a volume of 3ml) for 7 days at 37 °C in an atmosphere of 5% CO₂ in R10 medium supplemented with 50μM 2-mercaptoethanol, 50 ng/ml human recombinant GM-CSF and 50 ng/ml human recombinant IL-4. After 7 days of incubation, the non-adherent cells were harvested and placed in 24-well plates containing R10 without antibiotics.

Initially twenty experiments were conducted to optimise all the conditions before adhering to the final protocol for the study.

2.6.5 Infection of DC with *M. tuberculosis*

Immature DC were plated in 24-well plates, at 2 x 10⁵ cells in 2 ml of R10 per well, without antibiotics, and then were infected with H37Rv at a multiplicity of infection (MOI) of 5 in BSL-3. After 6 hour of incubation at 37° C in CO₂ incubator, infected cells were washed three times in the 24-well plate with R10 without antibiotics at low speed (600rpm) to remove extracellular bacteria by manual pipetting and were incubated in fresh medium for a further 2 days. Infected DC were incubated in 0.02% EDTA for 15 minute at RT before harvesting to obtain the total cellular content of each well. This was followed by a single wash with R10 without antibiotics. The percentages of infected and necrotic cells were estimated in each experiment by staining aliquots of cells by Ziehl-Neelsen method for acid-fast bacteria and

7-amino-actinomycin D (7-AAD) respectively. Cells were fixed with 2% Paraformaldehyde (PFA) for overnight at 4° C before taken out of BSL-3 lab for flow cytometric analysis.

Moreover, infected DCs were evaluated after 48 hours for both cell surface phenotype and extent of autologous T cell stimulation using flow cytometry as described below at 2.5.8 and 2.5.9.

Twelve experiments were done as optimisation steps before adhering to the final protocol for the experimental study.

2.6.6 Stimulation of DC with LPS

Immature DC were plated in 24-well plates, at 2×10^5 cells in 2 ml of R10 per well, without antibiotics, and then were stimulated with 1 µg/ml LPS for 48 hours. Stimulated DC were incubated in 0.02% EDTA for 15 minute at RT before harvesting to obtain the total cellular content of wells. This was followed by a single wash. Stimulated DC were evaluated after 48 hours for both cell surface phenotype and extent of autologous T cell stimulation in tandem with the protocol of infecting DC with *M. tuberculosis* using flow cytometry as described below in 2.5.8 and 2.5.9.

2.6.7 7-Amino-Actinomycin D (7-AAD) Staining Assay

7-AAD is a fluorescent, DNA-binding agent that intercalates between cytosine and guanine bases. Disrupted and permeabilized cellular membrane allows 7-AAD to enter the cell and bind to the DNA. 7-AAD staining intensity reflects the loss of membrane integrity.

The degree of staining intensity can be measured by flow cytometry and discriminates between live and necrotic cell populations. In brief, the cells from one well of the 24-well plate which was plated with 2×10^5 DC followed by infection with *M. tuberculosis* and co-culture for 48 hrs were harvested into small 5ml Falcon tubes (BD, Phrmingen). Cells were washed once at 1500 rpm at 4 °C for 10 minutes (Allegra™ 6R Centrifuge, Beckman Coulter) with FACS buffer and cells were then incubated with 5µl of 7AAD for 30 minutes at 4° C. Stained cells were fixed with 2% PFA at 4° C overnight before taken out of the BSL3 lab for flow cytometric analysis. Apoptotic cells were identified and quantified by flow cytometry (Schmid *et al*, 1994). Briefly, the cells were first gated based upon size by forward scatter and granularity by side scatter plotting. These cells were then analyzed for their ability to take up

the 7-AAD. The 7-AAD dull are the live cells and 7-AAD bright are necrotic populations. The values were reported as percentages of the total events counted (n=10000 events).

2.6.8 Flow Cytometric Analysis of Surface Markers

All groups of DC (Immature DC alone, Immature DC infected with H37Rv *M. tuberculosis* strain, and stimulated with LPS for 48 hours) surface marker expression was assessed by flow cytometry (FACScan, BD). Direct double immunofluorescence labelling technique (CD40 FITC / CD80 PE, HLA DR FITC / CD54 PE, CD83 FITC / CD86 PE) was used. In brief, 2×10^5 cells were aliquoted into tubes and were washed once at 1500 rpm at 4 °C for 10 minute in FACS medium and cells were incubated with 15µl fluorochrome conjugated monoclonal antibodies of each surface antigen for 30 minutes at 4 °C. The cells were washed an additional three times with FACS medium. Dead cells were eliminated from the analysis by staining with 7AAD. Flow cytometric acquisition was performed on DC following overnight fixation with 2% PFA in P3 lab at 4 °C. The values were reported as Mean Fluorescence Intensity (MFI) of the total events counted (n=10000). Small 5ml Falcon tubes were used for staining and flow cytometric analysis.

2.6.9 Mixed Leukocyte Reaction

The ability of *M. tuberculosis* infected DC to stimulate T cells was assessed using a mixed DC-autologous-T-cell reaction. Infected dendritic cells were used to stimulate autologous CD 14⁻ PBMC (purified by magnetic beads following CD14⁺ positive selection). A total of 2×10^5 CFSE stained CD 14⁻ PBMC (responder cells) were added to each well in 100µl R10 on U bottom, 96 well plate (BD, Phrmingen). Dendritic cells (stimulator cells) were harvested following stimulation with medium alone/ LPS/ *M. tuberculosis*, washed twice, resuspended in complete medium and then added to the responder cells at a ratio of 1:10 and 1:100. After 5 days, cells were harvested, and CD3 positive cells were analysed for their CFSE staining intensity.

The T cell proliferation was quantified based on dilution of CFSE with which the cells are prelabelled as described previously (Mannering SI, 2003). In brief, 10×10^6 autologous CD 14⁻ T cells/ml in R10 were stained with 5µM CFSE (5 and 6 carboxyfluorescein diacetate succinimidyl ester) in DMSO for 8 minutes at 37⁰ C with occasional stirring. Stained cells

were cultured for 5 days in the presence of infected DC. CFSE labelled cultured cells were incubated (30 min, 4 °C) with 20µl PE-labelled anti CD3 monoclonal antibodies and washed in FACS buffer and analyzed by flow cytometry. Similarly negative controls (immature dendritic cells cultured with media only) and positive control (dendritic cells cultured with LPS) were assessed for their ability to stimulate autologous T cells.

The number of cells that had proliferated was determined by gating on the lineage positive CFSE^{dim} subset. The cell division index (CDI) was calculated based on a fixed number (usually 5000) of CFSE^{bright} CD3⁺ cells, with the following formula:

$$\text{CDI} = \frac{\text{Number of CD3}^+ \text{ CFSE}^{\text{dim}} \text{ cells with antigen}}{\text{Number of CD3}^+ \text{ CFSE}^{\text{dim}} \text{ cells without antigen}}$$

A CDI of greater than 2 was considered to represent a positive response. A CDI of 2.0 means that there are twice as many divided cells with antigen than without (Mannering *et al.*, 2003).

2.7 Ethical Consideration

The project has obtained ethical clearance from the Medical Faculty Research and Publication committee, AHRI/ALERT Ethical Review Committee and the National Ethical Review Committee before commencement of the study.

2.8 Data Analysis

The mean fluorescence intensity data were analysed using a non-parametric Wilcoxon signed rank test, and results were considered significant if $p \leq 0.05$ and for the proliferation test CDI values greater than 2 was taken as a positive result (Mannering *et al.*, 2003).

CHAPTER III

3 RESULTS AND DISCUSSION

3.1 Results

3.1.1 Magnetic Cell Sorting of CD14⁺ Monocytes

PBMC were used as a starting material for generating immature DC from positively selected CD14⁺ monocytes (Fig. 9). CD14⁺ cells were isolated from PBMC by magnetic cell sorting using microbead conjugated to anti-CD14 antibodies and magnetic cell separation columns. After MACS, the purity of the CD14⁺ cells was found to be 94 to 98% (Mean of 96%) based on the flow cytometric analysis (Fig. 10). Typical yields of CD14⁺ cells ranged from 9 to 18% of the original PBMC numbers (Table 2). Flow cytometric analysis of CD14⁺ depleted cells show that there were 3.5% of contaminant CD14⁺ cells (Fig. 11) among the CD14⁻ cells.

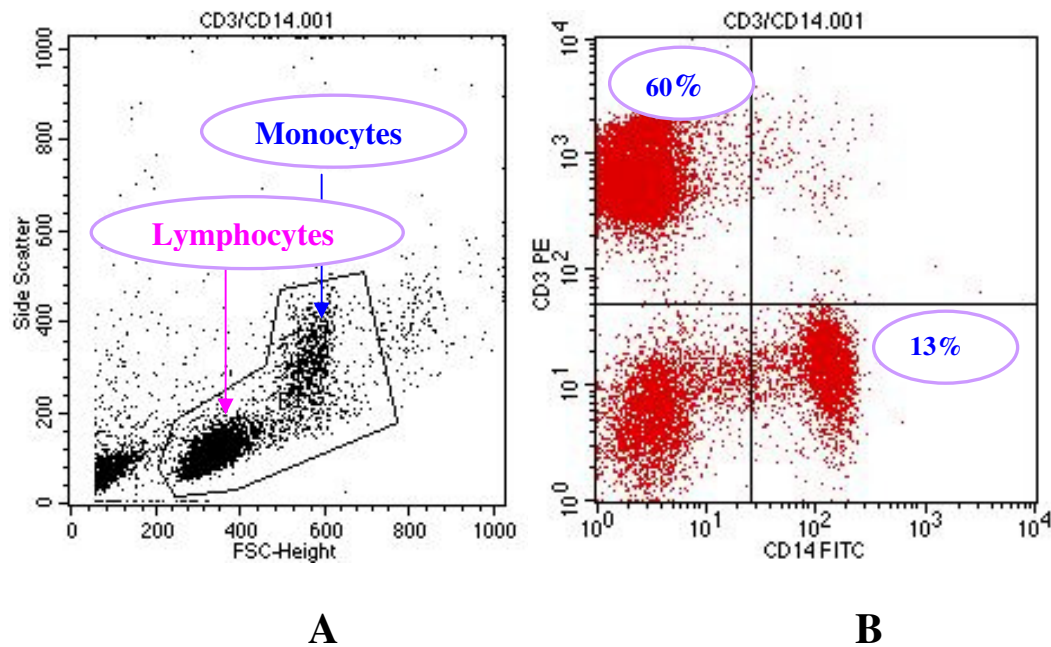


Figure 9: Dot-Plot flow cytometric analysis of total peripheral blood mononuclear cells: (A) forward scatter and side scatter (B) anti CD14 FITC and anti CD3 PE. The upper left corner represent CD3 cells and the lower right corner represents CD14 cells. This is a representative plot set from 17 independent experiments.

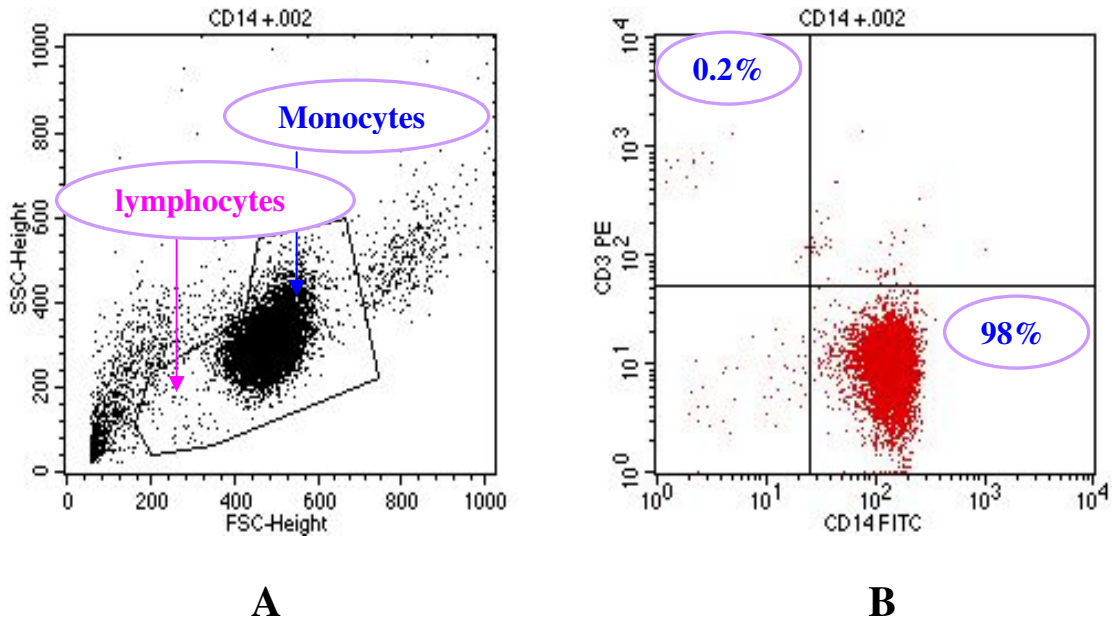


Figure 10: Dot-Plot flow cytometric analysis of positively selected CD14⁺ cell fraction: (A) forward scatter and side scatter (B) anti-CD14 FITC and anti- CD3 PE. Numbers in dot plot panels represent the percentage of CD14⁺ cells. This is a representative plot from 17 independent experiments.

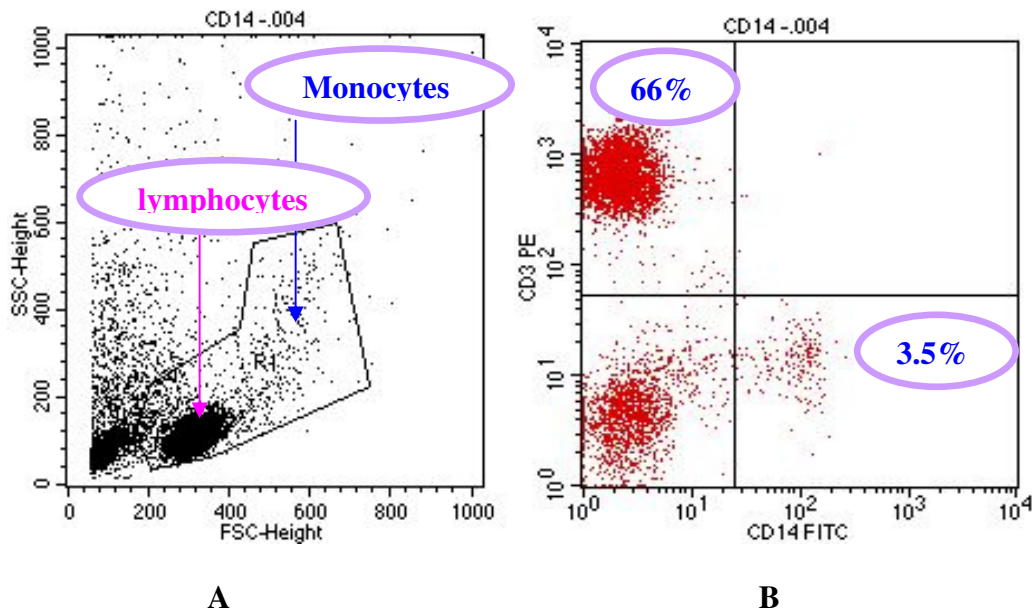


Figure 11: Dot-Plot flow cytometric analysis of CD14 depleted cell fraction: (A) forward scatter and side scatter (B) anti CD14 FITC and anti CD3 PE. Numbers in dot plot panels represent the percentage of CD14⁺ and CD3⁺ cells. These are a set of representative plots from 17 independent experiments.

Table 2: Proportion and purity of CD14⁺ cells from total PBMC isolated in each experiment

No	Blood Volume collected (ml)	PBMC obtained (10 ⁶)	CD 14+ Cells obtained (10 ⁶)	Immature DC obtained (10 ⁶)	CD14+ cells Purity obtained (%)	Yield of PBMC/ml of blood (10 ⁶)	Yield of CD14+ cells per 10 ⁶ PBMC (10 ⁶)	Yield of iDC per 10 ⁶ CD14+ cells (10 ⁶)
DC01	50	72	10.1	4.1	97	1.4	0.14	0.41
DC02	45	62	5.7	1.4	96	1.4	0.09	0.25
DC03	45	42.6	4.1	1.8	94	0.9	0.10	0.44
DC04	40	49	8.7	3.1	95	1.2	0.18	0.36
DC05	50	63.4	11.4	4.56	98	1.3	0.18	0.39
DC06	50	61	10	5.4	97	1.2	0.16	0.54
DC07	50	71	8.2	3.9	96	1.4	0.12	0.48
DC08	50	67.2	11	6.2	95	1.3	0.16	0.56
DC09	50	63	7.7	4.3	98	1.3	0.12	0.56
DC10	50	54	5.6	2.3	96	1.1	0.10	0.41
DC11	50	74.2	8.7	3.8	95	1.5	0.12	0.44
DC12	50	46	4.8	3.5	98	0.9	0.10	0.73
DC13	50	74.6	7.8	4.8	96	1.5	0.10	0.62
DC14	45	56	6.2	4.5	97	1.2	0.11	0.73
DC15	40	42.6	4.6	2.1	94	1.1	0.11	0.46
DC16	45	51	5.3	3.6	98	1.1	0.10	0.68
DC17	40	62.4	7.6	3.8	98	1.6	0.12	0.50
Mean	47	60	8	4	96	1.26	0.13	0.50
SEM	0.97	2.58	0.56	0.34	0.05	0.03	0.01	0.31

3.1.2 Generation of Immature Dendritic cells

Immature monocyte derived DC were generated *in vitro* after culturing positively selected CD14⁺ monocytes in R10 with 50 μ M 2-mercaptoethanol, 50ng/ml GM-CSF and 50ng/ml IL4. Examination of cultures using conventional phase contrast light microscopy revealed that immature DC exhibited a dendritic morphology featuring a prominent cytoplasmic processes or ruffled membranes (Fig. 12) and 69-78% (74%) of immature DC displayed CD 14⁻ and CD 54⁺ phenotype on cell surface (Fig.13).

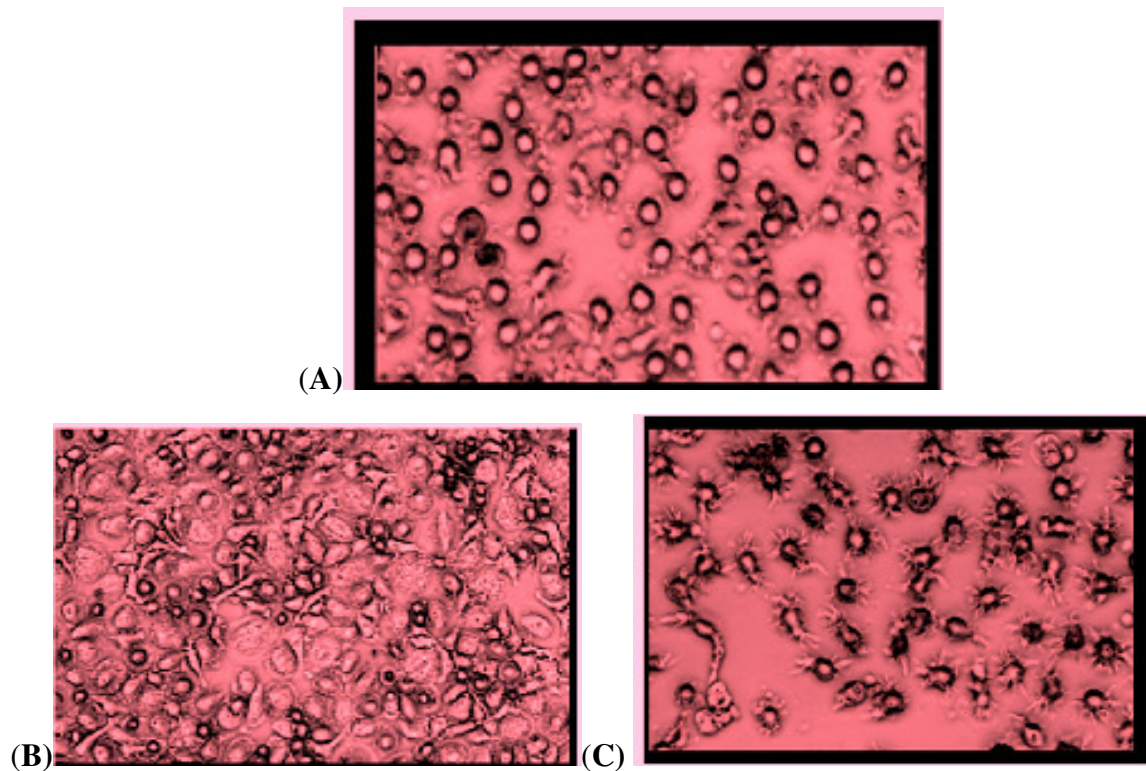


Figure 12: Micrograph of DC by phase contrast microscopy (40x): (a) CD14⁺ cells at day 1, (b) Immature dendritic cells at day 7 (c) mature dendritic cells. The CD14⁺ cells are round whereas the immature and mature DC are exhibited prominent cytoplasmic process.

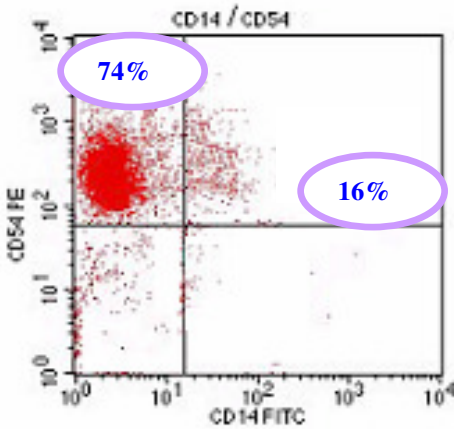


Figure 13: Dot-Plot flow cytometric analysis of analysis of immature DC stained with anti CD14 FITC and anti CD54PE. Monocytes lose their surface marker and become CD14⁻ and CD54⁺ on day 7. This is a representative plot from 17 independent experiments.

3.1.3 Viability of Dendritic Cells Following Infection with H37Rv

Immature dendritic cells were infected with H37Rv at different multiplicity of infection (MOI). Twelve experiments were done to determine the optimum MOI that we should be used in subsequent experiments. The percentage of the cells infected at the various MOIs at the time of collection varied from 26 to 89 % (Fig 14). Forty-eight hours after infection cells were collected and viability was determined by staining with 7-AAD. Infected cells were stained for the presence of intracellular acid-fast bacteria (fig. 15). At the lowest MOI of 3, only $31.6 \pm 2.6\%$ of the cells stained positive for intracellular bacteria and 5.6% were positive for 7-AAD. At MOI of 5, the percentage of cells staining positive for intracellular bacteria increased to $60 \pm 4.6\%$ and the percentage of cells positive for 7-AAD was $11.1 \pm 4.5 \%$, whereas at an MOI of 10, $84.4 \pm 4.5 \%$ of cells stained positive for intracellular bacteria. At the highest MOI of 10, although the percentage of cells positive for intracellular bacteria was higher, significant proportions (18.2%) of the DC were not viable because of lysis by the *M. tuberculosis*. The mean percentage of viable immature DC was 97.7% ($96.8-98.6\%$) in the case of uninfected cells. On the basis of these data, a MOI of 5 was chosen as optimal for further experiments.

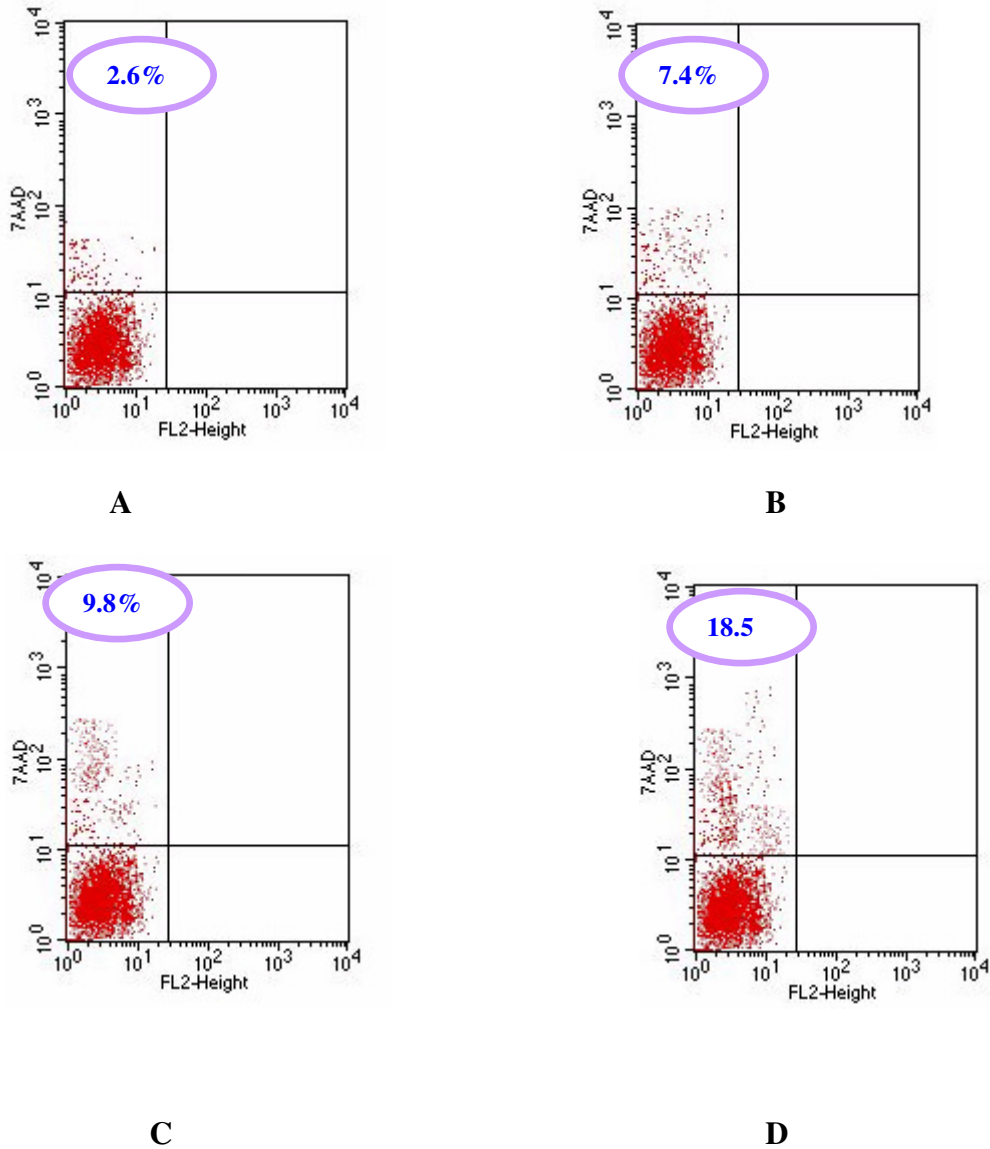


Figure 14: Viability of DC following *M. tuberculosis* infection from the FACScan analysis of DC: (A) Uninfected, (B) infected with MOI of 3, (C) infected with MOI of 5 and, (D) infected with MOI of 10. The 7AAD dull are live cells and the 7AAD bright cells are necrotic cells and numbers in the left upper quadrant represent the percentage of 7AAD bright cells. Quadrants set arbitrarily to separate dull and bright cells depending on the intensity of the fluorochrome. This is a representative set of dot plots from 12 independent experiments.

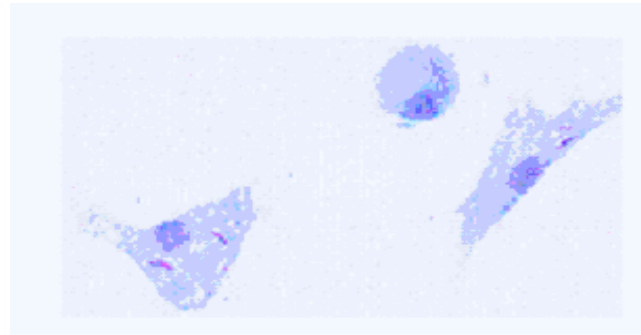


Figure 15: *M. tuberculosis* H37Rv wild type strain infected DC stained with Ziehl-Neelsen. DCs were cultured with live H37Rv for 48 hrs. DC were then allowed to adhere to glass slides and stained for acid-fast bacilli by the Ziehl-Neelsen method.

3.1.4 Maturation of Dendritic Cells

Functional activities of DC were determined by surface expression of adhesion and co-stimulatory molecules. To investigate the effect of *M. tuberculosis* H37Rv strain infection on the DC phenotype, the expression of the intracellular adhesion molecule (ICAM 1 and CD54), co-stimulatory surface molecules (B7-1 (CD80), B7-2 (CD86) and CD40) and MHC II by immature, H37Rv infected and LPS stimulated DC were assessed by flow cytometry. The cells were infected with H37Rv at a multiplicity of infection of 5 or stimulated with LPS at a concentration of 1 μ g/ml or immature DC treated with media alone and analyzed 48hr post treatment for the expression of the above-mentioned surface molecules. Significant ($P < 0.05$) upregulation in cell surface expression of co-stimulatory molecules, (CD40, CD80, CD86 and CD83), adhesion molecule (CD54), and MHC class II were observed both in LPS stimulated (Fig. 17) and H37Rv infected (Fig. 18) in comparison to negative control DC with media alone without any stimulation/infection (Fig. 16).

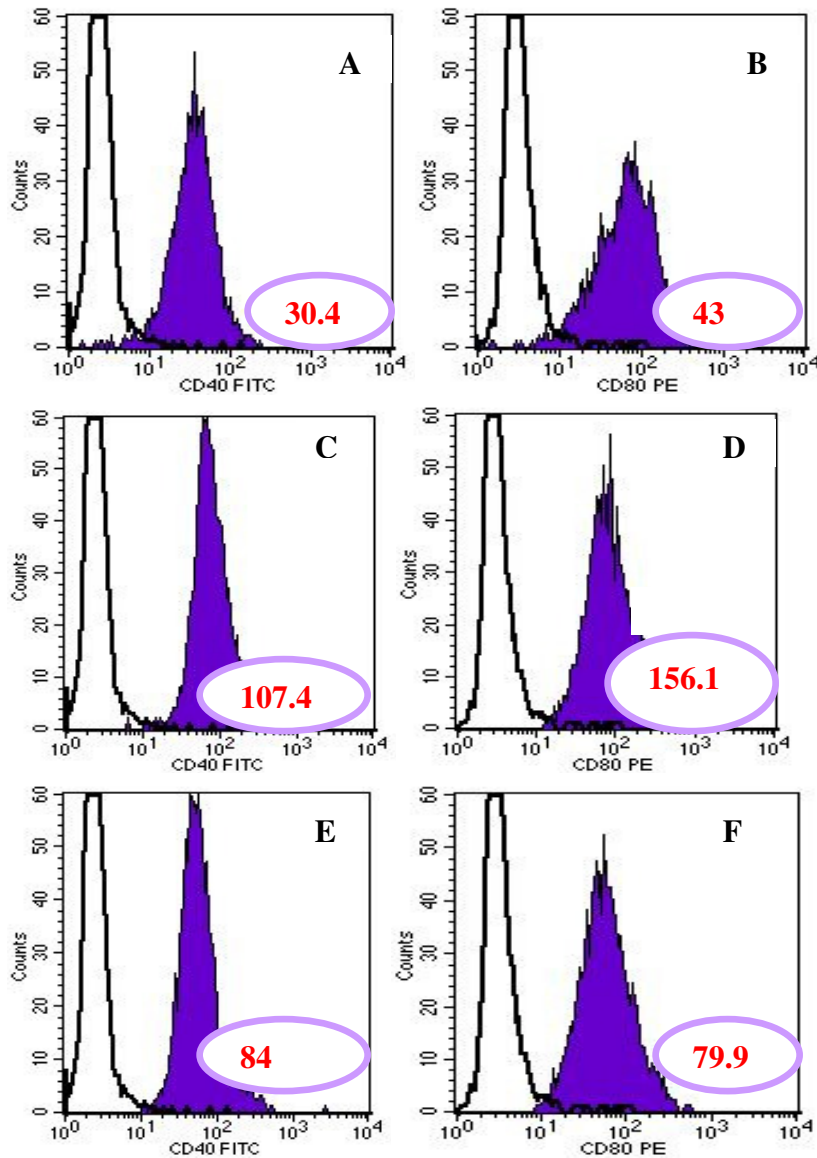


Figure16: Histogram Flow cytometric analysis of cell surface phenotype of immature dendritic cells (CD40 and CD80 when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*. Expression of the indicated marker is shown by solid histogram whereas cells stained with relevant isotype monoclonal antibody indicated by the open histogram. Indicated markers were set to exclude 95% of events recorded with the appropriate isotype control antibody. Numbers in histograms panels indicate the mean fluorescence intensity (MFI). These histograms are representative histograms of one of the 17 independent experiments.

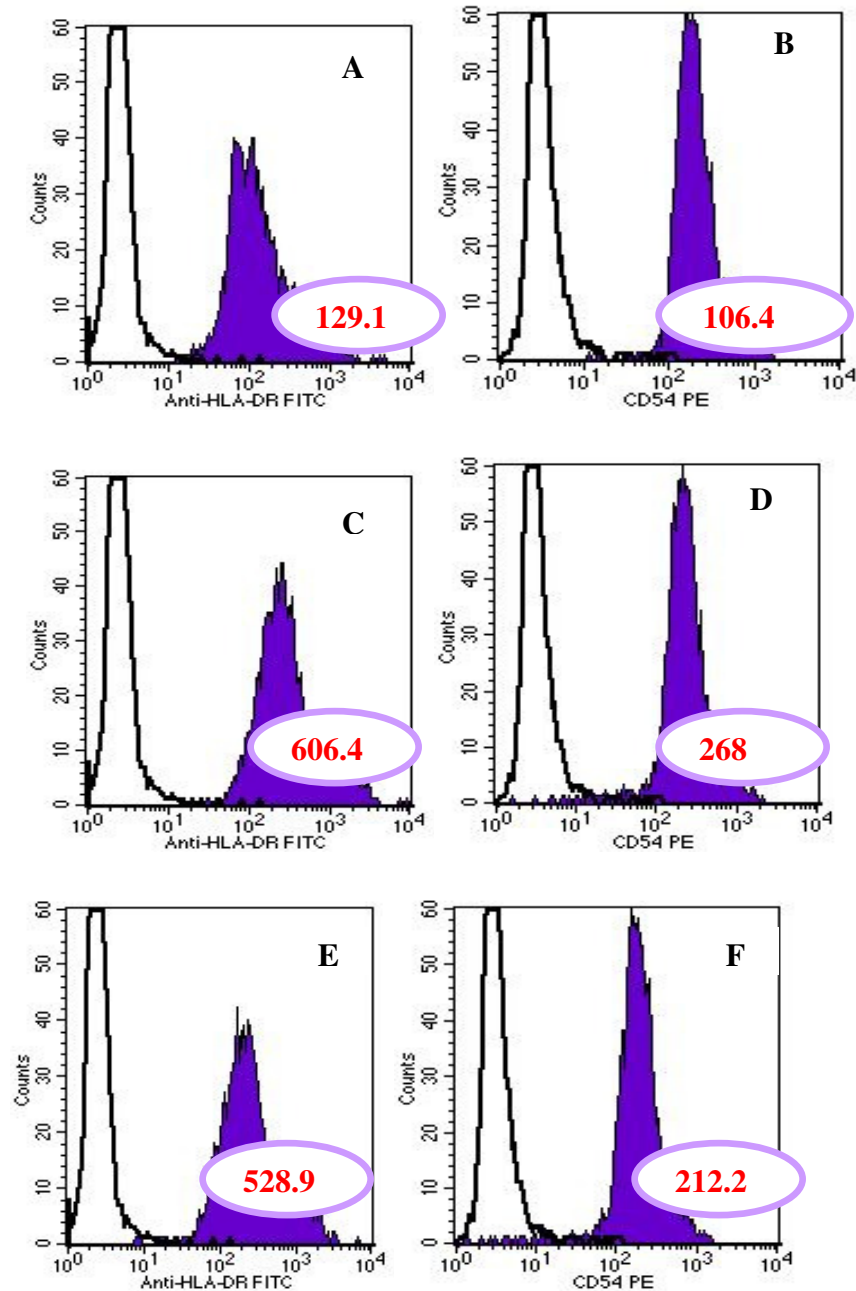


Figure 17: Histogram from flow cytometric analysis of cell surface phenotype of immature dendritic cells (HLA DR and CD54) when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*. Expression of the indicated marker is shown by solid histogram whereas cells stained with relevant isotype monoclonal antibody indicated by the open histogram. Indicated markers were set to exclude 95% of events recorded with the appropriate isotype control antibody. Numbers in histograms panels indicate the mean fluorescence intensity (MFI). These histograms are representative histograms of one of the 17 independent experiments.

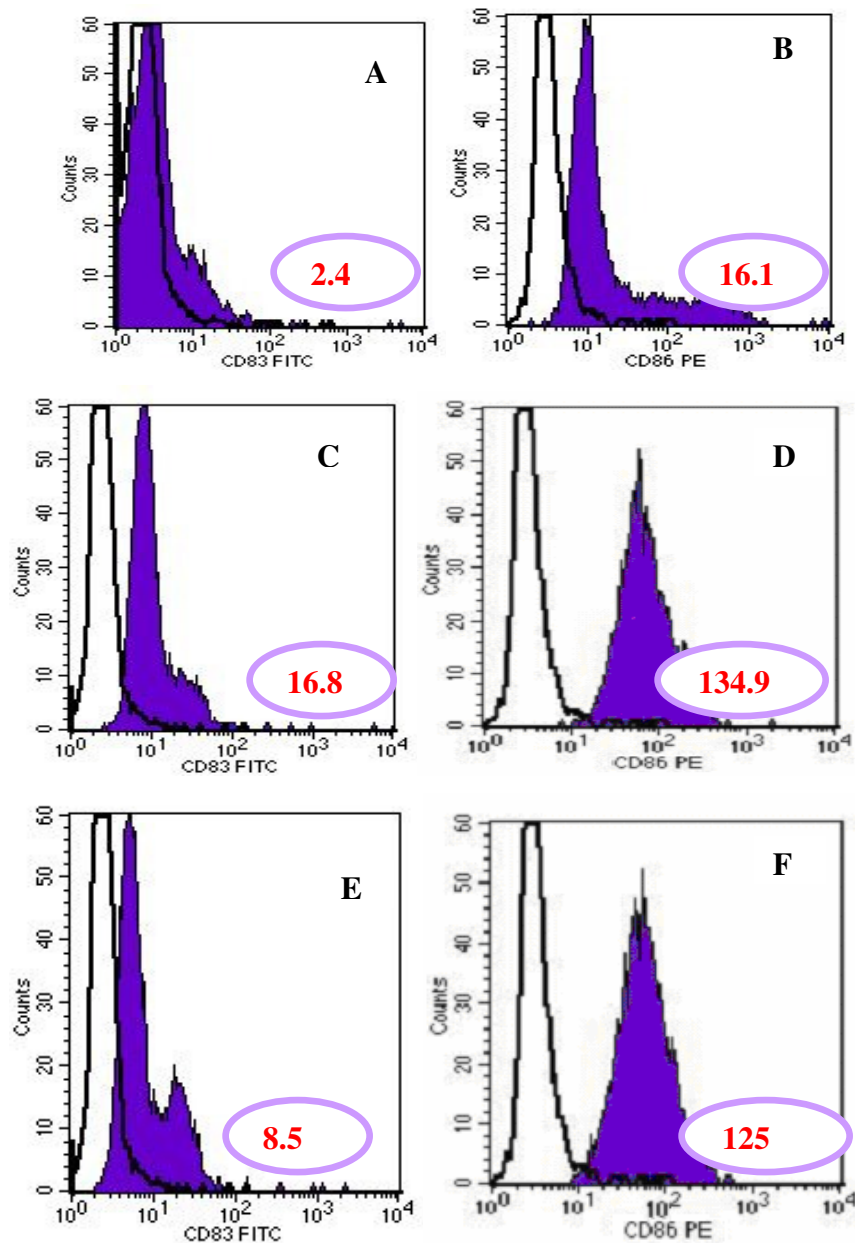


Figure 18: Histogram from flow cytometric analysis of cell surface phenotype of immature dendritic cells (CD83 and CD86) when exposed to (A, B) media alone, (C, D) LPS and (E, F) *M. tuberculosis*. Expression of the indicated marker is shown by solid histogram whereas cells stained with relevant isotype monoclonal antibody indicated by the open histogram. Indicated markers were set to exclude 95% of events recorded with the appropriate isotype control antibody. Numbers in histograms panels indicate the mean fluorescence intensity (MFI). These histograms are representative histograms of one of the 17 independent experiments.

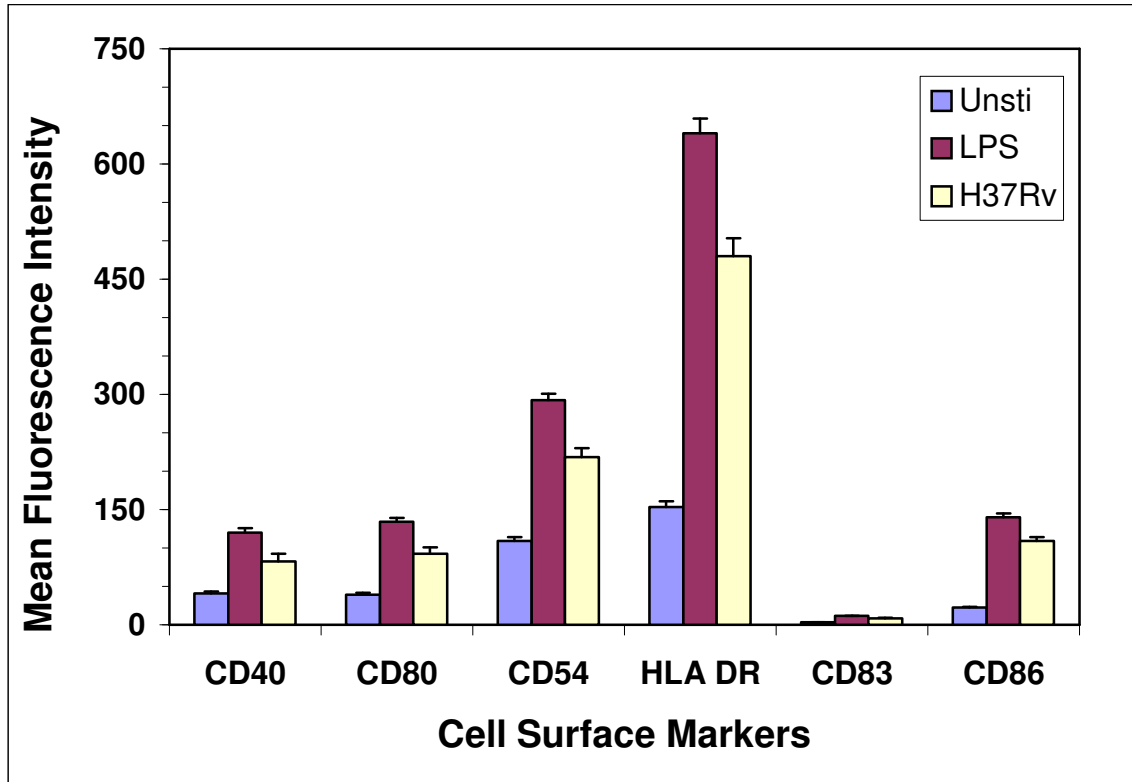


Figure 19: Effect of *M. tuberculosis* infection on surface phenotype of dendritic cells. Dendritic cell surface expression of multiple maturation markers was measured by flow cytometry 2 days after infection. Data are the Mean \pm SEM of the mean fluorescence intensity of maturation marker expression from 17 independent experiments.

3.1.5 Mixed Leukocyte Reaction

The ability of bacterium infected DC to stimulate autologous T cells was assessed using a mixed DC -autologous T cell reaction. The T cell proliferation was quantified by CFSE dilution techniques described in the methodology part. The results were expressed as the mean Cell Division Index (CDI) from each experiment.

The DC stimulated with LPS and infected with H37Rv augments T cells proliferation both at 1:10 (Mean CDI=34.2 and 15) and 1:100 (Mean CDI=15.6 and 10.6) DC: T cell ratio respectively (Fig 20).

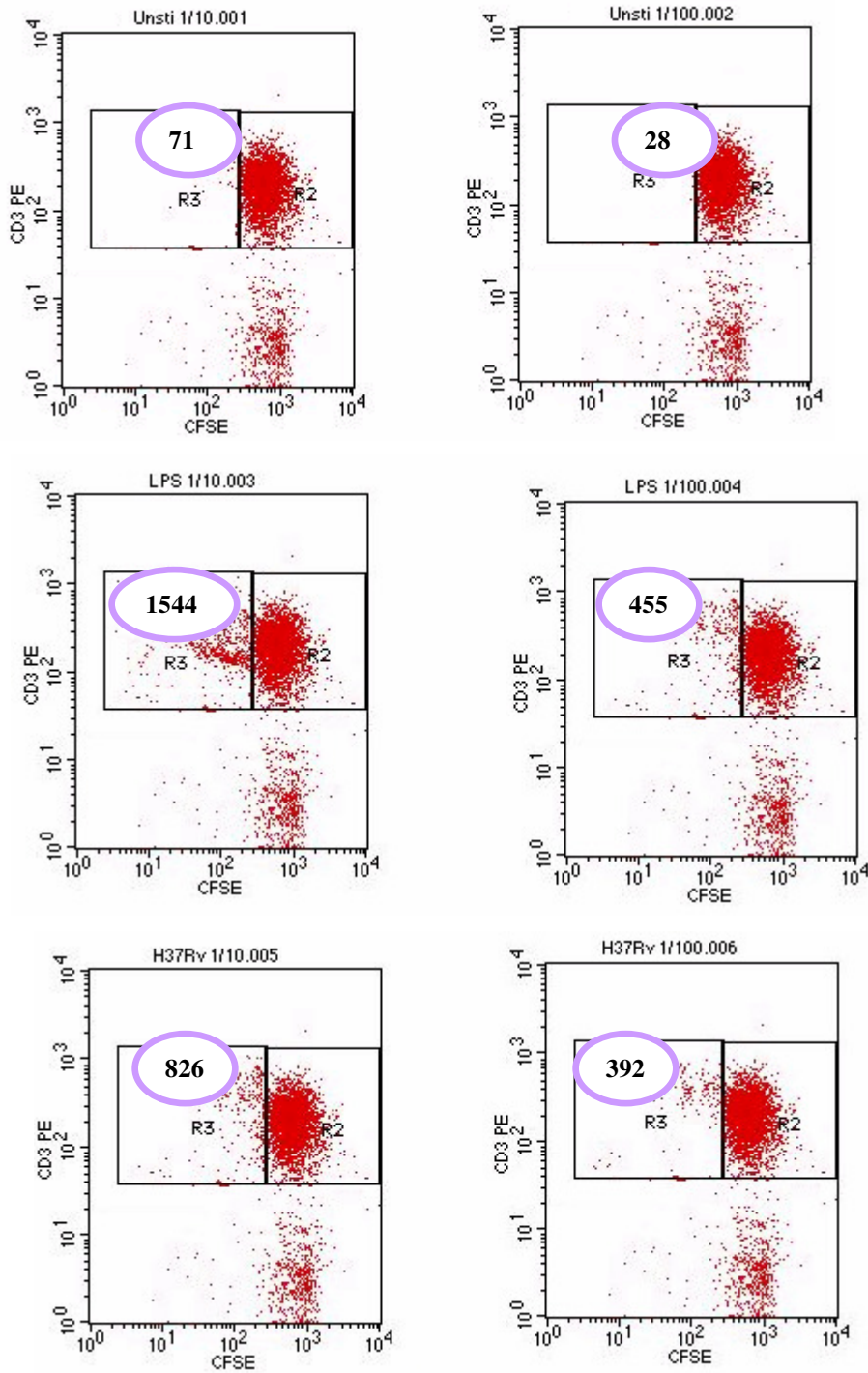


Figure 20: Autologous T cell proliferation at 1:10 and 1:100 of DC: T cell ratio by CFSE dilution technique. The cells were stained with antiCD3 PE and analysed with flow cytometry. In all cases 5000 CD3⁺ CFSE^{bright} events collected (Right Hand Box); the Number of CD3⁺ CFSE^{dim} events (Left Hand Box) was then determined. The cell division index (CDI) was calculated as described in methodology. These are a set of representative figures from 15 independent experiments.

3.2 Discussion

TB is responsible for the largest number of infections and is only second to HIV in the number of deaths per year from a single infectious agent. These high numbers of deaths occur despite effective chemotherapeutic regimens of DOTS, widespread immunisation with BCG, which is ineffective in adult TB, and the host's ability to mount a protective immune response. Individuals following recovery from TB disease are not protected from reinfection with *M. tuberculosis*. Hence it is crucial to understand the pathogenesis and protective immunity in efforts to develop improved interventions against this most resilient pathogen.

Numerous studies in humans and animal models over the years have shown that cell-mediated immunity is essential for protection against *M. tuberculosis* infection. The cellular factors of the immunological response, in particular antigen specific IFN- γ producing CD4⁺ T cells and cytotoxic CD8⁺ T cells producing perforin and granulysin, have been attributed as essential components of the protective immune response against *M. tuberculosis*. Antigens have to be effectively processed and presented by APCs in the context of MHC I or MHC II molecules. DC are unique among all APCs in the adult immune system in many critical ways as potent antigen presenting cells and play critical roles in many infections including eliciting T cell responses to air way pathogens. Dendritic cells can secrete cytokines, which favour a type 1 T cell response necessary for the control of *M. tuberculosis* infection.

DC based immunotherapies have been shown to mediate protection against a wide spectrum of infectious diseases caused by viral, bacterial, parasitic and fungal pathogens (Moll, 2003). Therapeutic vaccination with DC pulsed with inactivated simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) elicits effective cellular and humoral immune responses against SIV and HIV respectively, allowing the control of SIV and HIV replication and the reduction of viral DNA and RNA levels in SIV infected macaques and HIV infected humans respectively (Lu *et al.*, 2004; Lu *et al.*, 2003).

In two different studies, mice immunised with BCG or *M. tuberculosis* stimulated DC were shown to generate protective immune response and reduce the bacterial burden after challenge with *wild type M. tuberculosis* (Damangel *et al.*, 1999; Tascon *et al.*, 2000). Mycobacteria-infected DCs have an enhanced capacity to release pro-inflammatory cytokines and chemokines and are potent inducers of interferon-gamma-producing cells *in vivo*. Therefore,

DC manipulation for maximal antigen presentation and Th1 cytokine production may form the basis of a new generation of immunotherapeutic and/or vaccines, with improved efficacy against mycobacterial infections (Demangel and Brittol, 2000).

In humans, DC can be generated either from rare, proliferating CD34⁺ precursors by using GM-CSF and TNF α as key differentiating cytokines or from more frequent, but non-proliferating CD14⁺ precursor monocytes in PBMC under the influence of GM-CSF and IL-4. The latter approach has been used widely for experimental purposes since its introduction in 1994 (Romani *et al.*, 1994) including for immunotherapy. First, the CD14⁺ precursors are abundant so that pretreatment of patients with cytokines such as G-CSF used to increase CD34⁺ cells and more committed precursors in peripheral blood is unnecessary in most cases (Romani *et al.*, 1996). Secondly, the DC generated by this approach appears homogenous and fully differentiated and can be matured on stimulation with antigen(s). Therefore, we used CD14⁺ mononuclear cells as a source of DC in our study.

We show here that positively selected CD14⁺ monocytes can be induced *in vitro* to acquire typical DC characteristics in the absence of any detectable signs of proliferation. The cytokine combination used for the *in vitro* generation of DC from purified CD14⁺ peripheral blood monocytes (GM-CSF plus IL-4) is the cytokine combination used by Romani *et al.* (1996) for the *in vitro* development of DC from monocyte enriched mononuclear cell fractions. We used highly purified CD14⁺ peripheral blood monocyte preparations as the starting material to avoid “contaminating” subsets of DC progenitors known to be present in PBMC preparations. Other workers (Pickl *et al.* 1996) also showed that a highly purified CD14⁺ cell population represented a homogeneous population of equally sized, round cells which can be induced *in vitro* to acquire typical DC characteristics after culturing for 7 days in the presence of GM-CSF and IL-4.

Immature DC are specialized in capturing and processing antigens into peptides for effective presentation in the context of MHC for recognition by TCR. Microbial products or inflammatory cytokines can trigger DC maturation. Upon maturation, these cells function as initiators and modulators of the immune system. The DC activation process that results in a mature phenotype appears to be a crucial step in generating a specific immune response. It is therefore important to understand how DC are affected when they encounter pathogens. For

example, it has been shown that the pathogens *Plasmodium falciparum* (Urban, *et al.*, 1999), *Trypanosoma cruzi* (Van Overtvelt, *et al.*, 1999), *Herpes Simplex virus* (Salio, *et al.*, 1999) and *Mycobacterium leprae* (Hashimoto, *et al.*, 2002) prevent DC maturation *i.e.* downregulation of CD1a, CD40, CD54, CD80, CD86, HLA-A, HLA-B, HLA-C and HLA-DR and that *Helicobacter pylori* (Kranzer *et al.*, 2004), *Salmonella enterica serovar typhimurium* (Svensson, *et al.*, 2000) and *Chlamydia psitacci* (Ojcius, *et al.*, 1998) induce activation or maturation of DC *i.e.*, upregulated maturation marker molecules such as CD80, CD83, CD86 and HLA-DR. In the present study we investigated the effect of *M. tuberculosis* infection on maturation or activation of DC.

We used the virulent *M. tuberculosis* H37Rv strain to infect DC. A percentage of infected DC was proportional to the MOI. In our study, we used a MOI of 5 which resulted in 60 \pm 1.3% level of infection. Previous work (Henderson, *et al.*, 1997) reported a 50-70% infection rate of monocyte derived dendritic cells at a MOI of 5 ~ 10. It was also reported that human derived DC infected with *M. tuberculosis* at a MOI of 2.5 and 12.5 resulted in 63% and 93% of the cells being infected respectively. Higher MOI of 12.5 caused a large amount of necrosis (Fortsch *et al.*, 2000). In our study we have found 5.6%, 11.1% and 18.2% of cell death at a MOI of 3, 5 and 10 respectively, which was comparable with the above-mentioned previous studies.

So far, no single molecule has been identified to explain the efficacy of DC in T cell binding and activation, and the special effects of DC seem solely to relate to quantitative aspects and their regulation. For example, MHC products and MHC peptide complexes are 10-100 times higher on DC than on other APCs like B cells and monocytes. Mature DC resist the suppression by IL-10 but synthesize high levels of IL-12 that enhance both innate and acquired immunity (Banchereau and Steinman, 1998). DC also express many accessory molecules that interact with receptors on T cells to enhance adhesion and signalling (co-stimulation), for example ICAM-1 (CD54), B7-1/CD80, B7-2/CD86. All these surface expression (surface display of MHC molecules and MHC-peptide complexes available to bind TCR, expression of IL-12 and the expression of co-stimulatory molecules) are upregulated within a day of exposure to danger signals including microbial products. Inadequate stimulation, either through a poor fit of the T cell receptor (TCR) for the DC MHC/peptide

complex or lack of co-stimulation as a result of a low level of DC co stimulatory molecules or both, would lead to T cell anergy or programmed cell death (Banchereau and Steinman, 1998).

In this study we have shown that the exposure of monocyte-derived immature DC to H37Rv *M. tuberculosis* strain leads to activation or maturation of DC. Significant increases in cell surface expression of accessory molecules, CD40, CD80, CD86 and CD83, CD54), and MHC class II maturation marker ($p < 0.05$) observed (Fig 21).

The increase in the expression of CD40 may result in an increased capacity of DC to trigger proliferative responses and IFN- γ production by T cells against *M. tuberculosis* infection since ligation of CD40 with CD40L triggers the production of extremely high levels of bioactive IL-12. In addition, CD40-CD40L interaction is the most potent stimulus in upregulating the expression of CD54, CD80, and CD86 molecules on DC (Fontana, *et al.*, 2003).

The increase in expression of the CD80 and CD86 molecules on DC upon infection with *M. tuberculosis* would lead to activation of T cells since these co stimulatory molecules are important in delivering signals to the T cells that induce the expression of anti-apoptotic proteins, stimulate production of cytokines and promote T cell proliferation and differentiation. Moreover, the increment of expression of CD54 would help to increase the strength of adhesion between T cells and DC, thus allowing the TCR to be engaged by antigen to transduce the necessary signals since it is known that the affinity of TCR for peptide MHC complex is quite low and the off rate of this interaction is also rapid.

Although the precise function of CD83 is still unknown, several pathogens such as HSV-1 (Kruse, *et al.*, 2000) and Measles virus (Fugier-Vivier, *et al.*, 1997) have been shown to interfere with CD83-expression in infected DC, which then consequently also interfered with DC-mediated T-cell stimulation. Furthermore, in experiments where the CD83 mRNA transport from the nucleus into the cytoplasm was specifically inhibited, and thus CD83 expression was blocked, the T-cell stimulatory capacity of these DC was also inhibited (Kruse, *et al.*, 2000). All these reports clearly suggested an important role for CD83 during the induction of immune responses. The upregulation of HLA DR molecules on the surface of DC may also ensure that T cells will have the chance to recognize and respond to MHC associated peptides. In general, upregulation of these cell surface markers that are important in

antigen presentation and T cell stimulation indicates that the dendritic cell are maturing and preparing for the presentation of antigen to T lymphocytes.

Our study is in agreement with previous work (Henderson *et al.*, 1997) which indicated that DC generated from human peripheral blood by short term culture in medium containing GM-CSF plus IL-4 were capable of phagocytosing *M. tuberculosis*. Also, infection of DC with *M. tuberculosis* (however in that study Erdmann strain was used whereas we used H37Rv strain) resulted in increased surface expression of the co stimulatory molecules (CD54, CD40 and CD80 as well as MHC class I molecules). In addition, we didn't extend our studies to observe the level of cytokines reported in the other study demonstrating secretion of elevated levels of inflammatory cytokines, including TNF α , IL-1 and IL-12 by the infected DC (Henderson *et al.*, 1997).

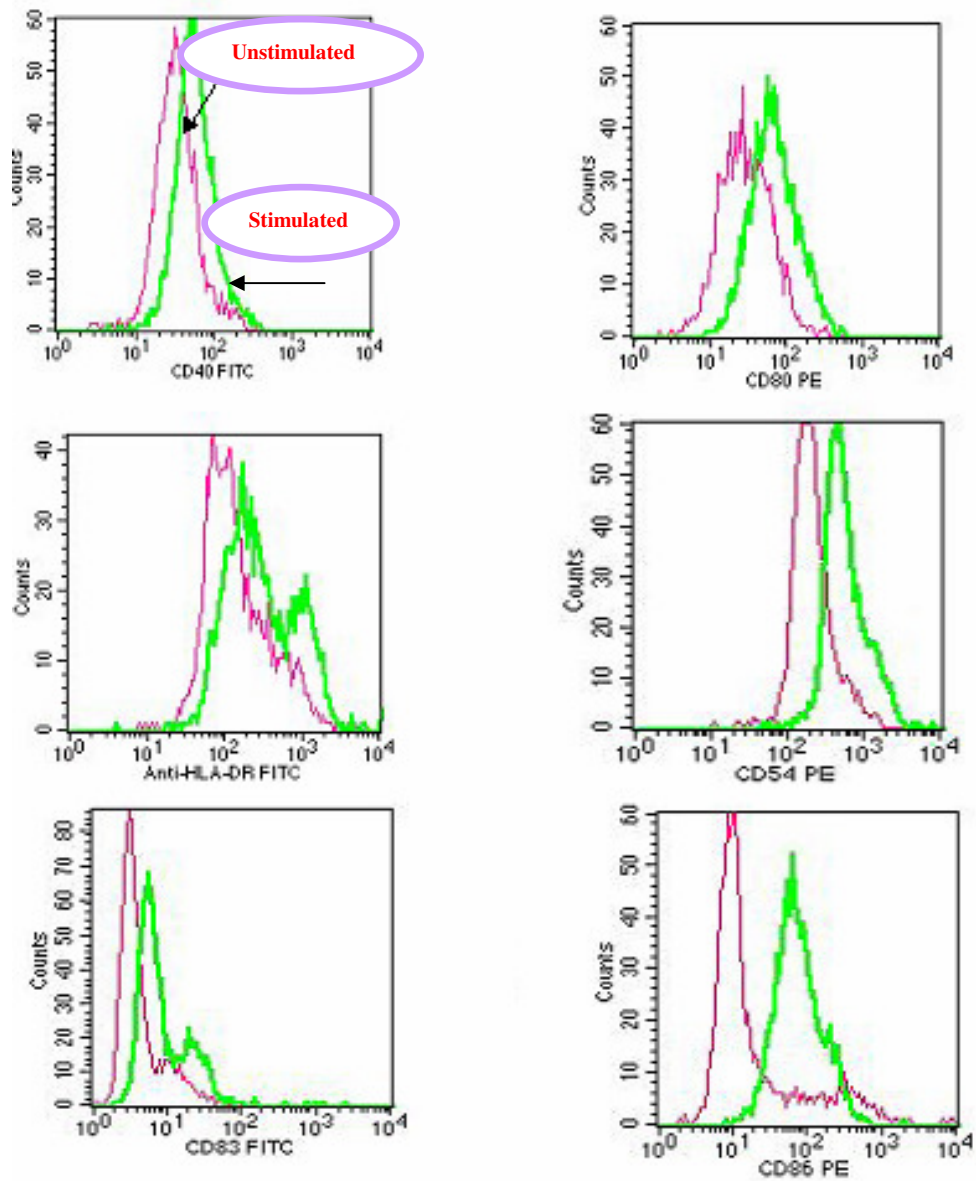


Figure 21: Overlay histogram of unstimulated and H37Rv stimulated dendritic Cells ($p < 0.05$).

Other workers also reported that DC readily internalized *M. tuberculosis* bacilli and subsequently displayed phenotypic changes including upregulation of various cell surface molecules important in initiating immune responses and downregulation of phagocytic activity, as well as producing inflammatory cytokines. In mouse model, these DC are superior to macrophages in stimulating antigen-specific cytokine production by both CD4⁺ and CD8⁺ T

cells (Serbina and Flynn, 1999 ; Tascon, *et al.*, 2000). In the mouse model, *M. tuberculosis* infected DC and macrophages have recently been suggested to differentially polarize naïve T cells. *M. tuberculosis*- infected DC biased a type 1 (IFN- γ producing) response (Th1) whereas macrophages did not (Hickman, *et al.*, 2002) . In order to develop a protective immune response to *M. tuberculosis*, type 1 T cell response must be initiated; therefore our data is in agreement with the previous studies suggesting that the DC activation observed in our *in vitro* experiment favours Th₁ polarization.

Other studies also clearly indicated that alveolar macrophages have also been shown to be poor APCs and even demonstrated to be suppressive to T cells whereas DCs in the lungs were shown to be efficient APCs. There are also data suggesting that mice in which the alveolar macrophages were depleted are protected during pulmonary TB (Leemans, *et al.*, 2001) probably indicating that DC can compensate well for the alveolar M ϕ s.

In a study where DC and alveolar macrophages were injected intratracheally into rats, the DC could migrate to the lymph nodes whereas the alveolar macrophages appeared to be unable to pass through the epithelium (Havenith, 1993). Others workers have also shown that pulmonary DC traffic from the lungs to the draining lymph nodes to present inhaled antigens to T cells after bacterial infection (Xia, *et al.*, 1995). In response to *M. tuberculosis* infection DC shift to an antigen presenting phenotype and can stimulate T cells from the spleens and lungs of infected mice *in vitro* (Bodnar, *et al.*, 2001). *M. tuberculosis* infected DCs have been observed *in-vivo* in infected mice (Gonzalez-Juarrero, 2001).

Moreover, in their recent work using nonhuman primate experiments and mathematical model of draining lymph node dynamics, Marino *et al.* (2004) have indicated that DC are necessary in establishing protective immunity and in containing infection. A delay either in DC migration to the draining lymph node or T cell trafficking to the site of infection could alter the out come of *M. tuberculosis* infection or define a progression from latency to active TB or a possible reactivation scenario. A fast DC turnover at the site of infection, as well as strong activation of DCs leading to maximal antigen presentation and production of key cytokines induce the most protective T cell responses.

In contrast to our findings, other workers reported that *M. tuberculosis* targets DC-specific C-type lectin intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) both to infect

DC and to down-regulate maturation of human monocyte-derived dendritic cells. *M. tuberculosis* induced minimal upregulation of the chemokine receptor CCR7, the co stimulatory molecules CD40, CD80, and CD86 and the antigen presenting molecules MHC class I and MHC class II (Hanekom *et al.*, 2003; (Geijtenbeek, 2003).

The discrepancy in the previous works with this study may arise from two points: multiplicity of infection and the positive Control that they chose to use. The MOI that they used was 3 with an infection of approximately 25%. This indicates a very low proportion of infection and with a possibility of very few bacilli per cell, which consequently could lead to a false negative or low maturation of DC. The other point is that they compared the state of maturation or MFI of *M. tuberculosis* infected DC with DC stimulated with maturation cocktail and this optimal positive control would give a higher MFI as compared to *M. tuberculosis*. However, we used a MOI of 5 that would give relatively high number of bacilli per cell and we used a bacterial cell wall component (LPS) as a positive control, which would give us a picture comparable to natural pathogens.

On the other hand, a recent study by Gagliardi,*et al.*(2005) have demonstrated that DC-SIGN (-ve) DC cultured in GM-CSF and IFN α are able to phagocytose BCG and to undergo a maturation program as well as DC-SIGN (+ve) DC cultured in IL-4 and GM-CSF. The capacity to stimulate a mixed reaction of naive T lymphocytes was not altered by the treatment of both DC populations with BCG. Therefore, their findings suggested that DC-SIGN couldn't be considered as the unique DC receptor for internalisation of mycobacteria.

We also examined the ability of *M. tuberculosis* infected DC to induce T cell proliferation. Maturation doesn't necessarily mean that the cell is capable of presenting antigen to T cells. The ability to measure autologous T cell proliferation in response to antigen was assessed by CFSE dilution technique. Both at 1:10 and 1:100 of DC: T cell ratio, infected DC induced positive T cell proliferation (CDI > 2), although T cell proliferation was stronger in a 1:10 than 1:100 DC: T cell ratio, which could in fact be due to the higher number of dendritic cells.

In agreement with our work, studies indicated that DCs are the most potent antigen-presenting cells and one dendritic cell stimulates 100 to 3000 T cells (Banchereau *et al.*, 2000).

3.3 Conclusion and Recommendation

Numerous studies in humans and animal models over the years have shown that cell-mediated immunity is key to protection against *M. tuberculosis* infection. In cell-mediated immunity, antigens should be presented by APCs in context with MHC Class I or II molecules should present antigens to T cells for generation of an effective immune response. DC are unique among all APCs in the adult immune system as potent antigen presenting cells and play critical roles in many infections. Dendritic cells can secrete cytokines, which favours a type 1 T cell response necessary for the control of *M. tuberculosis* infection.

However, whether the interaction between human dendritic cells and *M. tuberculosis* represents a defence mechanism by the host, or a smoke screen, masking the presence of an invader has not been clearly elucidated. In this study, we have clearly demonstrated that *M. tuberculosis* infection resulted in a phenotype consistent with activation of the dendritic cells, suggesting that infected dendritic cells produce cytokines that lead to maturation and possibly to migration and effective antigen processing and presentation. Moreover, we have demonstrated that *M. tuberculosis*-infected dendritic cells are capable of inducing T cells proliferation. In general, dendritic cells could be the basis for the initiation and modulation of the immune response leading to protection in the majority of *M. tuberculosis*-infected people. However, this needs further investigation.

DC-based immunotherapeutic protocols have been shown to mediate protection against a wide spectrum of infectious diseases caused by viral, bacterial, parasitic and fungal pathogens as well as in cancer. It is speculated that targeting DC *in vivo* could be an additional tool for drug and vaccine development against *M. tuberculosis* infection in the future. DC-based immunotherapy could be potential intervention strategies for complicated TB such as multi drug resistant tuberculosis (MDR-TB).

4 REFERENCES

- Albert, ML., SF. Pearce, LM. Francisco, B. Sauter, P. Roy, RL. Silverstein, and N. Bhardwaj.** 1998. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J Exp Med* **188**:1359.
- Andreasen, SO., JE. Christensen, O. Marker, and AR. Thomsen.** 2000. Role of CD40 ligand and CD28 in induction and maintenance of antiviral CD8+ effector T cell responses. *J Immunol* **164**:3689.
- Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, YJ., Pulendran, B., and K. Palucka.** 2000. Immunobiology of dendritic cells. *Annu Rev Immunol* **18**:767.
- Banchereau, J., and RM. Steinman.** 1998. Dendritic cells and the control of immunity. *Nature* **392**:245.
- Bean, AG., DR. Roach, H. Briscoe, MP. France, H. Korner, JD. Sedgwick, and WJ. Britton.** 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J Immunol* **162**:3504.
- Bergeron, A., M. Bonay, M. Kambouchner, D. Lecossier, M. Riquet, P., and AH. Soler, and A. Tazi.** 1997. Cytokine patterns in tuberculous and sarcoid granulomas: correlations with histopathologic features of the granulomatous response. *J. Immunol* **159**:3034.
- Bodnar, KA., NV. Serbina, and JL. Flynn.** 2001. Fate of *Mycobacterium tuberculosis* within murine dendritic cells. *Infect Immun* **69**:800.
- Boom, WH., DH. Canaday, SA. Fulton, AJ. Gehring, RE. Rojas, and M. Torres.** 2003. Human immunity to *Mycobacterium tuberculosis*: T cell subsets and antigen processing. *Tuberculosis (Edinb)* **83**:98.
- Brennan PJ., and H. Nikaido.** . 1995. The envelope of mycobacteria. *Annu. Rev. Biochem Biophys Res Commun* **64**:29.

- Brocker, T.** 1999. The role of dendritic cells in T cell selection and survival. *J Leukoc Biol* **66**:331.
- Chensue, SW., KS. Warmington, EJ. Allenspach, B. Lu, C. Gerard, SL. Kunkel, and NW. Lukacs.** 1999. Differential expression and cross-regulatory function of RANTES during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granulomatous inflammation. *J Immunol* **163**:165.
- Cooper, AM., J. Magram, J. Ferrante, and IM. Orme.** 1997. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. *J Exp Med* **186**:39.
- Degli-Esposti, M., and M. Smyth.** 2005. Close encounters of different kinds: dendritic cells and NK cells take centre stage. *Nat Rev Immunol* **5**:112.
- Delamarre L, H. Holcombe, and I. Mellman.** 2003. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J. Exp. Med* **198**:111.
- Demangel, C., AG. Bean, E. Martin, CG. Feng, AT. Kamath, and WJ. Britton.** 1999. Protection against aerosol *Mycobacterium tuberculosis* infection using *Mycobacterium bovis* Bacillus Calmette Guerin-infected dendritic cells. *Eur J Immunol* **29**:1972.
- Demangel, C., and WJ. Britton.** 2000. Interaction of dendritic cells with mycobacteria: where the action starts. *Immunol Cell Biol* **78**:318.
- Dubois, B., JM. Bridon, J. Fayette, C. Barthelemy, J. Banchereau, C. Caux, and F. Briere.** 1999. Dendritic cells directly modulate B cell growth and differentiation. *J Leukoc Biol* **66**:224.
- Erb, KJ., J. Kirman, B. Delahunt, W. Chen, and G. Le Gros.** 1998. IL-4, IL-5 and IL-10 are not required for the control of *M. bovis*-BCG infection in mice. *Immunol. Cell Biol.* **76**:41.
- Fenhalls, G., A. Wong, J. Bezuidenhout, P. van Helden, P. Bardin, and PT. Lukey.** 2000. *In situ* production of gamma interferon, interleukin-4, and tumor necrosis factor alpha mRNA in human lung tuberculous granuloma. *Infect. Immun* **68**:2827.

- Fine, P.** 1995. Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* **346**:1339.
- Flynn, JL., and J. Chan.** 2003. Immune evasion by *Mycobacterium tuberculosis*: living with the enemy. *Curr Opin Immunol* **15**:450.
- Flynn, JL., and J. Chan.** 2001. Immunology of tuberculosis. *Annu Rev Immunol* **19**:93-129.
- Flynn, JL., J. Chan, K. J. Triebold, DK. Dalton, TA. Stewart, and BR. Bloom.** 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **178**:2249.
- Flynn, JL., MM. Goldstein, KJ. Triebold, J. Sypek, S. Wolf, and BR. Bloom.** 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *J Immunol* **155**:2515.
- Flynn, JL., and JD. Ernst.** 2000. Immune responses in tuberculosis. *Curr. Opin. Immunol.* **12**:432.
- Fontana, S., D. Moratto, S. Mangal, M. De Francesco, W. Vermi, S. Ferrari, F. Facchetti, N. Kutukculer, C. Fiorini, M. Duse, P. Das, L. Notarangelo, A. Plebani, and R. Badolato.** 2003. Functional defects of dendritic cells in patients with CD40 deficiency. *Blood.* **12**:4099.
- Fortsch, D., , M. Rollinghoff and S. Stenger.** 2000. IL-10 Converts human dendritic cells into macrophage-like Cells with increased anti-bacterial activity against virulent *Mycobacterium tuberculosis*. *Journal of Immunology* **165**:978.
- Fugier-Vivier, I., C. Servet-Delprat, P. Rivaller, M. Rissoan, Y. Liu , and C. Roubourdin-Combe.** 1997. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. *J Exp Med* **186**:813.
- Gagliardi, M., R. Teloni, F. Giannoni, M. Pardini, V. Sargentini, L. Brunori, L. Fattorini, and R. Nisini.** 2005. *Mycobacterium bovis* Bacillus Calmette-Guerin infects DC-SIGN- dendritic cell and causes the inhibition of IL-12 and the enhancement of IL-10 production. *J Leukoc Biol.* **in press**.

- Geijtenbeek, T., SJ. Van Vliet, EA. Koppel, M. Sanchez-Hernandez, CM .Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk.** 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J Exp Med* **197**:7.
- Geijtenbeek, TB., DJ. Krooshoop, DA. Bleijs, SJ. van Vliet, GC. van Duijnhoven, V. Grabovsky, R. Alon, CG. Figdor, and Y. van Kooyk.** 2000. DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. *Nat Immunol* **1**:353.
- Gong JH, M. Zhang, RL. Modlin, PS. Linsley, D. Iyer, Y. Lin, and PF. Barnes.** 1996. Interleukin-10 downregulates *Mycobacterium tuberculosis*-induced Th1 responses and CTLA4 expression. *Infect. Immun* **64**:913.
- Gonzalez-Juarrero, M., O.C. Turner, J. Turner, P. Marietta, J.V.Brooks, and I.M. Orme.** 2001. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with *Mycobacterium tuberculosis*. *Infect. Immun* **69**:1722.
- Grotzke, J., and D. Lewinsohn.** 2005. Role of CD8(+) T lymphocytes in control of *Mycobacterium tuberculosis* infection. *Microbes Infect* **in Press**.
- Hanekom, WA., M. Mendillo, C. Manca, PA. Haslett, MR. Siddiqui, C. Barry, and G. Kaplan.** 2003. *Mycobacterium tuberculosis* inhibits maturation of human monocyte-derived dendritic cells in vitro. *J Infect Dis* **188**:257.
- Hart, D. N.** 1997. Dendritic cells: unique populations which control the primary immune response. *Blood* **90**:3245.
- Hashimoto, K., Y. Maeda, H. Kimura, K. Suzuki, A. Masuda, M. Matsuoka, and M. Makino.** 2002. *Mycobacterium leprae* infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. *Infect Immun* **70**:5167.
- Havenith, C., P. Miert, A. Breedijk, H. Beelen, and E. Hoefsmit.** 1993. Migration of dendritic cells into the draining lymph nodes of the lung after intratracheal instillation. *Am. Res. Cell. Mol. Biol.* **9**:484.
- Henderson, R. A., SC. Watkins, and JL. Flynn.** 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J Immunol* **159**:635.

- Hernandez Pando, R., H. Orozco, A. Sampieri, L. Pavon, C. Velasquillo, J., and JM. A. Larriva Sahd, and MV. Madrid.** 1996. Correlation between the kinetics of Th1/Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Immunology* **89**:26.
- Hickman, SP., J. Chan, and P. Salgame.** 2002. *Mycobacterium tuberculosis* induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization. *J Immunol* **168**:4636.
- Howard, AD., and BS. Zwilling.** 1999. Reactivation of tuberculosis is associated with a shift from type 1 to type 2 cytokines. *Clin. Exp. Immunol.* **115**: 428.
- <http://www-ermm.cbcu.cam.ac.uk/figures.htm>. The interaction between dendritic cells (DC) and T cells involves three signals. Accessed on 24/05/2005.
- Itano, AA., SJ. McSorley, RL. Reinhardt, BD. Ehst, E. Ingulli, AY. Rudensky, and MK. Jenkins.** 2003. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* **19**:47-57.
- Jacob, J., and D. Baltimore.** 1999. Modelling T-cell memory by genetic marking of memory T cells *in vivo*. *Nature* **399**:593.
- Jameson SC., and MJ, Brevan.** 1995. T cell receptor antagonists and partial agonists. *Immunity* **2**:1.
- Jouanguy, E., R. Doffinger, S. Dupuis, A. Pallier, F. Altare, and JL Casanova.** 1999. IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. *Curr Opin Immunol* **11**:346.
- Juffermans, N P., A. Verbon, S J. van Deventer, H. van Deutekom, JT. Belisle, ME. Ellis, P. Speelman, and T. van der Poll.** 1999. Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan. *Infect Immun* **67**:4295.
- Kaufmann, S. H.** 2001. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* **1**:20.

- Kranzer K., EA., M. Aigner, G. Knoll, L. Deml, C. Speth, N. Lehn, M. Rehli, and W. Schneider-Brachert.** 2004. Induction of Maturation and Cytokine Release of Human Dendritic Cells by *Helicobacter pylori*. *Infection and Immunity* **72**:4416.
- Kruse, M., O. Rosorius, F. Kratzer, D. Bevec, C. Kuhnt, A. Steinkasserer, G. Schuler, and J. Hauber.** 2000. Inhibition of CD83 cell surface expression during dendritic cell maturation by interference with nuclear export of CD83 mRNA. *J Exp Med* **9**:1581.
- Kruse, M., O. Rosorius, F. Kratzer, G. Stelz, C. Kuhnt, G. Schuler, J. Hauber, and A. Steinkasserer.** 2000. Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J Virol* **74**:7127.
- Lalvani, A., R. Brookes, R J. Wilkinson, AS. Malin, AA. Pathan, P. Andersen, H. Dockrell, G. Pasvol, and AV. Hill.** 1998. Human cytolytic and interferon gamma-secreting CD8+ T lymphocytes specific for *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **95**:270.
- Lanzavecchia, A., and F. Sallusto.** 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* **290**:92.
- Leemans, JC., NP. Juffermans, S. Florquin, N. van Rooijen, M. J. Vervoordeldonk, A. Verbon, SJ. van Deventer, and T. van der Poll.** 2001. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. *J Immunol* **166**:4604.
- Lipscomb, MF. and BJ. Masten.** 2002. Dendritic cells: immune regulators in health and disease. *Physiol Rev* **82**:97.
- Lowrie, DB.** 1999. DNA vaccines against tuberculosis. *Curr Opin Mol Ther* **1**:30-33.
- Lu, B., BJ. Rutledge, L. Gu, J. Fiorillo, NW. Lukacs, SL. Kunkel, R. North C.Gerard, and BJ. Rollins.** 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J. Exp. Med* **187**:601.
- Lu, W., L. Arraes, W. Ferreira, and J. Andrieu.** 2004. Therapeutic dendritic-cell vaccine for chronic HIV-1 infection. *Nat Med* **10**:1359-1365.

- Lu, W., X. Wu, Y. Lu, W. Guo, and JM. Andrieu.** 2003. Therapeutic dendritic-cell vaccine for simian AIDS. *Nat Med* **9**:27.
- Lucey, DR., M. Clerici, and GM. Shearer.** 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin. Microbiol. Rev* **9**:532.
- Lukacs, NW., CL. Addison, J. Gauldie, F. Graham, K. Simpson, RM., and KW. Strieter, SW. Chensue, and SL. Kunkel.** 1997. Transgene induced production of IL-4 alters the development and collagen expression of T helper cell 1-type pulmonary granulomas. *J. Immunol* **158**:4478.
- MacMicking, JD., RJ. North, R. LaCourse, JS. Mudgett, SK. Shah, and CF. Nathan.** 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A* **94**:5243.
- Mannering, SI., JS. Morris, KP. Jensen, AW. Purcell, MC. Honeyman, PM. van Ender and LC. Harrison.** 2003. A sensitive method for detecting proliferation of rare autoantigen-specific human T cells. *J Immunol Methods* **283**:173.
- Marino, S., S. Pawar, CL. Fuller, TA. Reinhart, JL. Flynn, and DE. Kirschner.** 2004. Dendritic cell trafficking and antigen presentation in the human immune response to *Mycobacterium tuberculosis*. *J Immunol* **173**:494.
- Modlin, RL.** 2001. Activation of toll-like receptors by microbial lipoproteins: role in host defense. *J Allergy Clin Immunol* **108**:S104.
- Moll, H.** 2003. Dendritic cells as a tool to combat infectious diseases. *Immunol Lett* **85**:153
- Nigou J., M. Gilleron, and G. Puzo.** 2003. Lipoarabinomannans: from structure to biosynthesis. *Biochimie* **85**:153.
- Oddo, M., T. Renno, A. Attinger, T. Bakker, HR. MacDonald, and PR. Meylan.** 1998. Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*. *J Immunol* **160**:5448.

- Ojcius, D., Y. Bravo de Alba, J. Kanellopoulos, R. Hawkins, K. Kelly, R. Rank, and A. Dautry-Varsat.** 1998. Internalization of Chlamydia by dendritic cells and stimulation of Chlamydia-specific T cells. *J Immunol* **160**:1297.
- Peters, W., HM. Scott, HF. Chambers, JL. Flynn, IF. Charo, and JD. Ernst.** 2001. Chemokine receptor 2 serves an early and essential role in resistance to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **98**:7958.
- Pickl, WF., O. Majdic, P. Kohl, J. Stockl, E. Riedl, Schei, and C. necker, C. Bello-Fernandez, and W. Knapp.** 1996. Molecular and functional characteristics of dendritic cells generated from highly purified CD14⁺ peripheral blood monocytes. *J. Immunol. Methods* **157**:3850.
- Porcelli, SA., and RL. Modlin.** 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu Rev Immunol* **17**:297.
- Quesniaux, V., C. Fremont, M. Jacobs, S. Parida, D. Nicolle, V. Yermeev, F. Bihl, F. Erard, T. Botha, M. Drennan, M. Soler, M. Le Bert, B. Schnyder, and B. Ryffel.** 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect* **6**:946.
- Ragno, S., M. Romano, S. Howell, DJ. Pappin, PJ. Jenner, and MJ. Colston.** 2001. Changes in gene expression in macrophages infected with *Mycobacterium tuberculosis*: a combined transcriptomic and proteomic approach. *Immunology* **104**:99.
- Rescigno, M., F. Granucci, and P. Ricciardi-Castagnoli.** 2000. Molecular events of bacterial-induced maturation of dendritic cells. *J Clin Immunol* **20**:161.
- Rojas, RE., KN. Balaji, A. Subramanian, and WH. Boom.** 1999. Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to *Mycobacterium tuberculosis* by interleukin-10 and transforming growth factor beta. *Infect Immun* **67**:6461.
- Romani N., GS., Brang D, E. Kampgen, A. Lenz, B. Trockenbacher, G. Konwalinka, PO. Fritsch, RM. Steinman, and G. Schuler.** 1994. Proliferating dendritic cell progenitors in human blood. *J Exp Med* **180**:83.

- Romani, N., D. Reider, M. Heuer, S. Ebner, E. Kampgen, B. Eibl, D. Niederwieser, and G. Schuler.** 1996. Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods* **196**:137.
- Russell, D.** 2001. *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol.* **2**:569.
- Salio, M., M. Cella, M. Suter, and A. Lanzavecchia.** 1999. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur J Immunol* **29**:3245.
- Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia.** 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182**:389.
- Sallusto F, LD., R. Forster, M. Lipp, and A. Lanzavecchia.** 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**:708.
- Salyers, AA., and DD. Whitt.** 1994. Tuberculosis. In: *Bacterial Pathogenesis: A Molecular approach*. As press. Washington DC.:PP. 307.
- Schmid, I., CH Uittenbogaart, B Keld and JV Giorgi.** 1994. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Meth* **170**:145.
- Selwyn PA, HD., VA. Lewis, EE. Schoenbaum, SH. Vermund, RS, Klein, AT. Walker, and GH. Friedland.** 1989. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *N Engl J Med* **321**:545.
- Serbina, NV., and JL. Flynn.** 1999. Early emergence of CD8(+) T cells primed for production of type 1 cytokines in the lungs of *Mycobacterium tuberculosis*-infected mice. *Infect Immun* **67**:3980.
- Serbina, NV., CC. Liu, CA. Scanga, and JL. Flynn.** 2000. CD8+ CTL from lungs of *Mycobacterium tuberculosis*-infected mice express perforin *in vivo* and lyse infected macrophages. *J Immunol* **165**:353.

- Shiloh, M., and CF. Nathan.** 2000. Reactive nitrogen intermediates and the pathogenesis of Salmonella and mycobacteria. *Curr. Opin Microbiol* **3**:35.
- Shinkai, K., and RM. Locksley.** 2000. CD1, tuberculosis, and the evolution of major histocompatibility complex molecules. *J Exp Med* **191**:907.
- Sieling, PA., D. Chatterjee, SA. Porcelli, TI. Prigozy, RJ. Mazzaccaro, T. Soriano, B. R. Bloom, MB. Brenner, M. Kronenberg, and PJ. Brennan, et al.** 1995. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science* **269**:227.
- Steinbrink, K., M. Wolfl, H. Jonuleit, J. Knop, and AH. Enk.** 1997. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* **159**:4772.
- Svensson, M., C. Johansson, and MJ. Wick.** 2000. *Salmonella enterica* serovar *typhimurium*-induced maturation of bone marrow-derived dendritic cells. *Infect Immun* **68**:6311.
- Tascon, RE., CS. Soares, S. Ragno, E. Stavropoulos, EM. Hirst, and MJ. Colston.** 2000. *Mycobacterium tuberculosis*-activated dendritic cells induce protective immunity in mice. *Immunology* **99**:473.
- Toossi, Z., and JJ. Ellner.** 1998. The role of TGF beta in the pathogenesis of human tuberculosis. *Clin. Immunol. Immunopathol.* **87**:107.
- Toossi, Z., P. Gogate, H. Shiratsuchi, T. Young, and JJ. Ellner.** 1995. Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions. *J. Immunol* **154**:465.
- Trombetta, E., and I. Mellman.** 2005. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu. Rev. Immunol.* **23**:975.
- Turley, SJ., K. Inaba, WS. Garrett, M. Ebersold, J. Unternaehrer, RM. Steinman, and I. Mellman.** 2000. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science* **288**:522.
- Urban, BC., DJ. Ferguson, A. Pain, N. Willcox, M. Plebanski, JM. Austyn, and DJ. Roberts.** 1999. *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* **400**:73.

- Valladeau J, O. Ravel, C. Dezutter-Dambuyant, K. Moore, M. Kleijmeer, Y. Liu, V. Duvert-Frances, C. Vincent, D. Schmitt, J. Davoust, C. Caux, S. Lebecque, and S. Saeland.** 2000. Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules. *Immunity*. **12**:71.
- van Crevel, R., TH. Ottenhoff, and JW. van der Meer.** 2002. Innate immunity to *Mycobacterium tuberculosis*. *Clin Microbiol Rev* **15**:294.
- Van Overtvelt, L., N. Vanderheyde, V. Verhasselt, J. Ismaili, L. De Vos, M. Goldman, F. Willems, and B. Vray.** 1999. *Trypanosoma cruzi* infects human dendritic cells and prevents their maturation: inhibition of cytokines, HLA-DR, and costimulatory molecules. *Infect Immun* **67**:4033.
- Visintin, A., A. Mazzone, J. H. Spitzer, DH. Wylie, SK. Dower, and DM. Segal.** 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J Immunol* **166**:249.
- WHO.** 1994. Framework for Effective Tuberculosis Control. World Health Organization, Geneva, mimeographed document WHO/TB/94:179.
- WHO.** 2005. Global tuberculosis control: surveillance, planning, financing. WHO report 2005. Geneva, World Health Organization (WHO/HTM/TB/2005.349).
- Wykes, M., A. Pombo, C. Jenkins, and GG. MacPherson.** 1998. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J Immunol* **161**:1313.
- Xia, W., C. Pinto, and R. Kradin.** 1995. The antigen-presenting activities of Ia⁺ dendritic cells shift dynamically from lung to lymph node after an airway challenge with soluble antigen. *J. Exp. Med* **181**:1275.
- Yamada, H., S. Mizumo, R. Horai, Y. Iwakura, and I. Sugawara.** 2000. Protective role of interleukin-1 in mycobacterial infection in IL-1 alpha/beta double-knockout mice. *Lab Invest* **80**:759
- Zhang, Y., M. Broser, H. Cohen, M. Bodkin, K. Law, J. Reibman, and WN. Rom.** 1995. Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacterium tuberculosis* and its components. *J Clin Invest* **95**:586.

Appendix A. Mean fluorescence intensity of different surface markers of dendritic cells

No	Unstimulated			LPS Stimulated							H37Rv infected							
	CD 40	CD 80	HLA DR	CD 54	CD 83	CD 86	CD 40	CD 80	HLA DR	CD54	CD83	CD86	CD 40	CD 80	HLA DR	CD54	CD83	CD86
01	48.0	31.3	156.4	126.0	1.1	29.8	171.0	138.0	599.5	305.1	15.5	128.7	126.7	90.7	400.3	203.5	11.5	102.3
02	71.9	59.1	126.5	89.4	3.8	17.5	111.4	125.7	630.6	257.1	8.7	133.4	79.0	93.6	567.4	269.3	7.2	86.0
03	29.7	40.8	141.3	79.9	1.9	26.1	98.8	119.6	593.5	289.4	6.8	145.5	53.3	92.7	385.4	225.2	4.6	129.4
04	47.0	37.6	175.7	112.9	3.6	24.1	132.5	87.4	578.5	226.7	8.7	120.9	83.5	97.2	397.2	156.8	6.9	114.3
05	44.8	49.3	169.1	74.3	2.5	17.0	100.9	107.8	552.3	265.5	7.0	143.7	76.4	82.0	475.2	211.4	4.7	126.2
06	21.4	31.1	151.2	102.3	1.0	17.1	84.9	91.4	421.1	198.1	4.5	126.8	48.6	68.9	443.6	182.3	6.2	86.9
07	24.6	38.0	84.5	92.3	2.3	15.8	103.8	103.8	508.6	239.7	6.3	132.6	69.0	67.9	395.0	201.1	4.8	133.9
08	38.0	45.0	170.9	101.8	4.7	12.7	160.6	143.8	788.9	298.3	13.1	154.9	97.5	86.8	487.3	201.2	8.9	131.5
09	30.4	43.0	129.1	106.4	2.4	16.1	107.4	156.1	606.4	268.0	16.8	134.9	84.0	79.9	528.9	212.2	8.5	125.0
10	34.6	25.3	169.0	128.3	4.9	25.1	195.5	220.3	687.0	314.5	14.5	138.7	124.8	115.5	476.8	234.6	9.1	88.0
11	49.1	35.2	101.6	119.9	3.7	23.1	95.2	154.6	698.7	291.0	14.7	153.3	89.5	139.8	419.7	277.7	8.4	132.5
12	36.9	29.1	121.9	125.3	3.2	22.0	88.1	124.1	691.2	276.1	16.0	124.8	63.0	80.0	485.8	184.3	9.5	97.3
13	37.0	35.1	198.3	99.8	4.8	26.1	83.1	101.8	633.7	295.4	17.2	142.1	59.7	78.3	666.0	213.9	12.2	108.0
14	49.5	35.7	160.9	153.3	2.9	24.0	97.5	157.7	709.2	341.4	9.8	120.9	98.8	113.1	534.9	204.1	7.7	78.6
15	36.4	35.1	193.2	113.8	4.9	26.3	80.5	119.6	708.0	378.1	16.9	146.7	58.4	77.3	553.7	217.3	12.2	111.2
16	53.8	48.9	175.4	117.0	2.8	29.5	197.2	178.9	699.2	353.5	8.5	138.7	115.0	115.4	541.8	218.8	8.2	104.1
17	38.9	46.8	182.6	119.3	3.3	28.3	129.5	155.9	772.6	376.2	9.5	197.9	80.3	91.43	399.1	293.1	7.5	105.4

Appendix B. Viability and proportion of DCs after infection with H37Rv

No	Uninfected		LPS Stimulated				H37Rv infected			
	DC: T cell ratio		DC: T cell ratio				DC: T cell ratio			
	1:10	1:100	1:10	CDI	1:100	CDI	1:10	CDI	1:100	CDI
1	41	32	1521	37.1	667	20.8	726	17.7	297	9.3
2	51	21	1335	26.2	569	27.1	687	13.5	248	11.2
3	38	24	1640	43.2	414	17.2	515	13.5	353	14.7
4	32	24	1324	41.4	465	19.4	667	20.8	389	16.2
5	82	32	1548	18.9	186	5.8	526	6.4	168	5.2
6	38	22	1484	39	508	23.1	557	14.6	370	16.8
7	47	36	1522	32.4	391	10.8	549	11.7	347	9.6
8	38	42	1469	38.6	357	8.5	514	13.5	265	6.3
9	71	28	1544	21.7	455	16.2	826	11.6	392	14
10	78	49	1534	19.7	475	9.7	622	7.8	368	7.5
11	34	26	1298	38.2	342	13.1	742	21.8	324	12.5
12	29	23	1367	47.1	412	17.9	679	23.4	297	12.9
13	39	27	1570	40.2	511	18.9	766	19.6	187	6.9
14	43	38	1654	38.5	435	11.4	546	12.7	237	6.2
15	42	36	1278	30.4	523	14.5	612	14.6	313	8.7

Appendix C. Cell division index values

	Cell infection (%)			7AAD Expression (%)			
	Multiplicity of Infection (MOI)						
	3 X	5 X	10 X	Uninfected	3 X	5 X	10 X
1	32.2	64	87.7	2	5	11.4	18
2	31.6	61.2	89.3	1.7	5.2	10.8	17.9
3	28.4	63.8	86.4	2.3	4.8	12.3	18.5
4	32.7	52.4	87.4	1.3	6.2	12.1	16.8
5	29.3	62.4	76	3	5.8	11	16.9
6	33.7	62.3	87.2	2.2	5.1	10.6	17.7
7	31.2	50	83.4	3.3	4.6	11.5	21.2
8	35.4	63.7	88.5	2	7.1	10.1	19.2
9	27	61.4	87.4	2.6	7.4	9.8	18.5
10	34.5	57.3	83.6	1.5	5.6	11.5	18.3
11	33.6	60.4	78.4	2.8	4.4	11.3	20.1
12	29.2	60.5	77.7	3.1	6.2	10.3	15.3