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**Involvement of Endothelial Cells and Macrophages in  
Mycobacterial Infections.**

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**Senate Nkhahle**

March 2002

“The Lord is my shepherd, I shall not want...”

**-Psalm 23**

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# Abbreviations

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AIDS	acquired immuno-deficiency syndrome
APC	antigen presenting cell
BALF	bronchial alveolar lavage fluid
BCG	bacillus calmette guerin
BMD	bone marrow derived
CD	cluster of differentiation
cfu	colony forming units
CR	complement receptor
DMEM	dulbecco's modified enriched medium
<i>E. coli</i>	<i>Escherichia coli</i>
EC	endothelial cell
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FACS	fluorescent activated cells sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HUVEC	human umbilical vein endothelial cells
ICAM	intracellular cell adhesion molecule
IFN	interferon
H. Serum	heated serum
Ig	immunoglobulin
IL	interleukin
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
<i>M. Smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
M199	medium 199
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex

MOI	multiplicity of infection
N. Serum	normal serum
OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
PBS	phosphate buffered saline
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PNPP	P-nitrophenyl-phosphate
RPMI	Rosewall Park Memorial Institute
SD	standard deviation
TNF	tumour necrosis factor
UV	ultra violet
VCAM	vascular cell adhesion molecule
WT	wild type
ZN	Ziehl Neelson

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# Abstract

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Tuberculosis remains to be a leading infectious cause of death worldwide. This is in spite of the BCG vaccine against tuberculosis that has been in use for over 80 years as well as several chemotherapies. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is a facultative intracellular pathogen targeting host cells, predominantly macrophages, to establish an infection. Endothelial cells form a barrier that has to be crossed by the bacilli in the establishment, and subsequent dissemination of mycobacterial infection. The present study was undertaken to investigate the phagocytosis of mycobacteria by endothelial cells and macrophages and the subsequent activation of these host cells after infection with the bacilli.

Endothelial cells, obtained from the human umbilical vein (HUVEC) were infected with BCG-GFP under various conditions and uptake determined by means of flow cytometry. Endothelial cells phagocytised mycobacteria in a dose- and a time-dependant manner. Exposure of mycobacteria to serum opsonins enhanced the uptake of the bacilli by endothelial cells. However, heat killing of mycobacteria inhibited its uptake by endothelial cells. Data from the fluorescent microscope showed the association of BCG-GFP signal with endothelial cells detected on the FACS caliber. Analysis by confocal microscopy confirmed internalisation of endothelial cells by mycobacteria. Endothelial cells were further investigated for an acquired phenotype following infection with mycobacteria. CD31, a marker for endothelial cells, was neither down regulated nor up regulated. However, ICAM-1 expression, one of the adhesion molecules was down regulated upon infection of endothelial cells with mycobacteria. No TNF- $\alpha$  and IL-6 were detected in culture supernatants of infected endothelial cells.

Macrophages obtained from murine bone marrow, phagocytosed mycobacteria in both a dose- and a time-dependant manner. Unlike endothelial cells, heat killing of mycobacteria did not obliterate their uptake by macrophages. However, macrophages preferentially phagocytosed viable mycobacteria in a 3h infection period, but not in an 18h period. Macrophages from the Mac-1 mouse strain, lacking a phagocytic receptor 3 (CR3), were included in this study. The uptake of pathogenic mycobacteria, H37Rv by macrophages from Mac-1, was reduced in cell cultures infected for 4 hours but not those infected at 1

and 2 hours. Similarly, reduced uptake of avirulent mycobacteria strains H37Ra and BCG in the absence of CR3 was pronounced in cell cultures infected for longer periods.

The activation state macrophages acquire after infection with mycobacteria was investigated with respect to the expression of MHC glycoproteins, and secretion of IL-10 and IL-12. The activation state of macrophages with respect to these parameters studied is critical in the interaction with T-lymphocytes, for subsequent containment of mycobacteria infection. Production of IL-12, a critical Th-1 cytokine, was proportional to MOI, and enhanced by viability of pathogenic mycobacteria. Furthermore, prior exposure of mycobacteria to serum opsonins inhibited the secretion of IL-12, while exposure of the bacilli to bronchoalveolar factors greatly enhanced it. IL-10 production by infected macrophages was on the other hand inhibited by prior exposure of mycobacteria to both serum and bronchoalveolar fluid factors.

Macrophages constitutively expressed MHC I. After infection with pathogenic mycobacteria, cells positive for MHC I, were hardly detected in macrophage cultures infected with mycobacteria that had been exposed to fresh serum and bronchoalveolar fluid opsonins. MHC II on the other hand, was not constitutively expressed on macrophages. Following infection with pathogenic mycobacteria, the highest percentage of cells positive for the antibody against MHC II were observed in macrophage cultures infected both without any opsonin and in the presence of bronchoalveolar fluid factors.

In conclusion, the present study demonstrates that endothelial cells bind and internalise mycobacteria. That they get activated as evidenced in the down-regulation of ICAM-1 following infection with mycobacteria. Thus endothelial cells may not just be a passive, physical barrier but host cells that may have an active role in mycobacterial infection. Macrophages in comparison to endothelial cells were more effective in phagocytosis of mycobacteria in a time- and dose-dependant manner, differentiating themselves as professional phagocytes in the internalisation of heat-killed bacteria where the endothelial cells lacked the ability for the uptake of mycobacteria.

# 1 INTRODUCTION

---

## 1.1 Global burden of Tuberculosis

Tuberculosis is a bacterial infectious disease caused by the obligate human pathogen, *Mycobacterium tuberculosis*. The global burden of *M. tuberculosis* infection is overwhelming. In addition, bovine tuberculosis (caused by *Mycobacterium bovis*) and leprosy (caused by *Mycobacterium leprae*) remain significant global health problems (Glickman and Jacobs 2001). Recent estimates of tuberculosis infection burden documented approximately 1.86 billion people infected with *M. tuberculosis* with 16.2 million cases of active disease. These active cases resulted in approximately 2 million deaths in 1997, an average global case fatality rate of 23% (Dye et. al. 1999). These unfortunate statistics are even more daunting when compared to the relative rarity of lethal tuberculosis in the industrialised world due to effective medical care and antibiotic therapy. The emergence of multi-drug resistant strains of *M. tuberculosis*, and the occurrence of AIDS by HIV, has complicated the global fight against tuberculosis. Tuberculosis has been declared “A GLOBAL EMERGENCY” by the World Health Organisation (WHO 1993).

## 1.2 History of Tuberculosis

This infection has been a leading cause of death in the world for centuries (Wigginton and Kirschener 2001). The name tuberculosis is derived from the formation by the body of characteristic cellular structures called tubercles, in which the bacilli are trapped and walled off. In 1882, the etiology of tuberculosis got a tremendous boost with the isolation of the causative organism, tubercle bacilli, by the German scientist Robert Koch. Eight years later, the same scientist came up with the diagnostic test for tuberculosis, the so-called tuberculin test. In 1924, the French scientists Albert Leon Calmette and Alphonse F. M. Guerin developed a vaccine against tuberculosis, which is still known today as the BCG (Bacillus

Calmette-Guerin) vaccine. Despite the fact that BCG vaccine has been in place for over many years, tuberculosis is still a problem.

### 1.3 Tubercle Bacilli

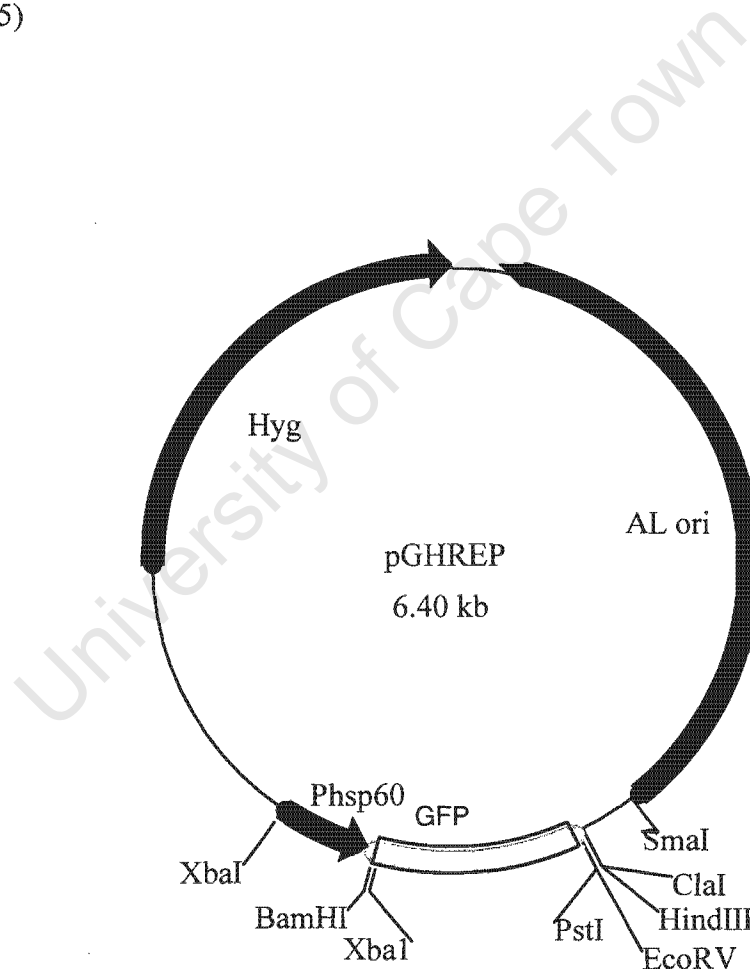
Mycobacteria are a distinctive rod shaped bacteria that share a common property of a lipid-rich cell wall that avidly retains carbol fuschin dye even in the presence of acidic alcohol, known as acid fast staining Ziehl Neelson. The tubercle bacillus is slender, straight or slightly curved, non-motile, non-encapsulated and does not form spores (Steyn 1998). It is aerobic and slow growing, dividing every 18-24 hours. It is sensitive to heat and UV light, but resistant to drying and chemical disinfectants (Steyn 1998). Mycobacterial cell wall contains unique hydrophobic compounds possessing mycolic acids (a long branched- chain molecular weight fatty acid) (Yano I. 1998) esterified to the cell wall, named the mycolyl arabinogalactan (MAG) (Glickman 2001). A variety of unique lipids and glycolipids are noncovalently associated with the cell envelope. These include trehalose dimycolate, phthiocerol dimycocerate and lipoarabinomannan among others. Many of these molecules are potent immunomodulators, suggesting a role in virulence (Glickman 2001).

### 1.4 BCG-Green Fluorescent Protein

The development of Green Fluorescent Protein (GFP), from the jellyfish *Aequorea victoria*, as a reporter gene in mycobacterium has opened the door to the use of fluorescent microscopy and flow cytometry to investigate *M. tuberculosis* infection. Snewin and colleagues (pers. comm.) developed a number of constructs which, when electroporated into different species of mycobacteria express GFP. These plasmids contain the *gfp* gene cloned under the control of the BCG *hsp60* promoter to generate pGHREP (Figure 1.1). This plasmid is a replicating shuttle vector in *E.coli* and mycobacteria and employs hygromycin (Hyg) as selectable marker. Non-pathogenic environmental strains (*M. smegmatis*) as well as the slow growing

*M. bovis* BCG and *M. tuberculosis* have all been successfully transformed with this GFP construct.

The GFP is the source of fluorescent-light emission in the jellyfish *Aequorea Victoria*. It absorbs UV light at 395nm and has a fluorescence emission maximum at 510nm (Kremer L et al. 1995). The active chromophore is generated by the spontaneous cyclization and oxidation of a serine-dehydrotyrosine-glycine trimer within a defined hexa-peptide sequence of the protein (Cody et al. 1993). Unlike other reporters, GFP generates fluorescence in the absence of any other proteins, substrates or co-factors, allowing expression to be monitored in real time (Kremer et al. 1995)



**Figure 1.1** The mycobacterial shuttle vector pGHREP

## 1.4 Tuberculosis

Tuberculosis is a disease that is almost exclusively transmitted by aerosolised droplets containing infectious *M. tuberculosis* bacilli. These droplets are generated by the cough of a person with *M. tuberculosis* lung infection and are infective via inhalation to other people. *M. tuberculosis* bacilli are inhaled into the lung, eventually reaching the alveoli, where the organisms may be ingested by alveolar macrophages (Birkness et al. 1999). Bacilli not killed by the macrophages, are able to survive, replicate intracellularly, and spread to other alveolar macrophages and to the nonactivated blood-borne macrophages attracted to the site by the released bacterial cell debris and host chemotactic factors (Dannenberg and Rook 1994). Dissemination of viable organisms from these macrophages into the lymph or circulatory system is critical to the establishment of systemic infection (McDonough and Kress 1995, McDonough et al. 1993)

After infection with *M. tuberculosis*, the lifetime risk of developing tuberculosis is approximately 10%, while 90% of infected persons have latent infection with viable bacilli (Raviglione et al. 1995). This 10% rate of tuberculosis accounts for the 8 million persons reported annually with active tuberculosis, and the resultant 3 million deaths (Bonecini-Almeida et al. 1998), making tuberculosis the leading cause of death from any infectious agent.

## 1.5 Interaction with host cells

### 1.5.1 Macrophages

*M. tuberculosis* primarily infects macrophages. In contrast to other bacterial pathogens that avoid phagocytosis as a specific pathogenic strategy, *M. tuberculosis* is promiscuous in its use of multiple cell surface receptors to gain entry into macrophages (Ernst, 1998). These receptors include the mannose receptor, complement receptors and Fc receptors. Once inside the host macrophage, *M. tuberculosis* resides within a membrane-bound vacuole. It is clear that *M. tuberculosis* modifies the maturation of this phagosomal compartment in

order to enhance its own intracellular survival (Armstrong and Hart, 1971; Clemenz and Horwitz, 1995). This altered phagosomal maturation is associated with alterations in the protein content of the vacuole including altered Rab GTPase composition (Via et al., 1997; Clemens et al., 2000), exclusion of the vacuolar proton ATPase with consequent lack of acidification (Sturgill-Koszycki et al., 1994), and retention of a protein designated TACO (Ferrari et al., 1999). These experiments and others have yielded a relatively detailed view of the biochemical events that accompany mycobacterial vacuolar trafficking within the macrophage.

Anti-tuberculous immunity involves the critical interplay of T lymphocytes, macrophages, and cytokines (Chan and Kaufmann 1994, Ho and Riley 1997). This co-operation between macrophages and lymphocytes in containing *M. tuberculosis* infection was demonstrated in an in vitro experiment of Bonecini-Almeida et al. (1998) in which they showed that induction of human macrophage anti-*M. tuberculosis* activity required dual signalling from lymphocytes and gamma interferon. In order to effectively interact with primed lymphocytes, macrophages must be activated. This largely involves the expression of antigen presenting glycoproteins, certain co-stimulatory molecules and secretion of appropriate cytokines. Knowledge generated in this regard may be important in immunotherapeutic interventions in Tuberculosis.

In order to disseminate and establish an infection in the body, *M. tuberculosis* encounters and crosses epithelial and endothelial cell barriers. This is thought to be achieved either through infected macrophages crossing these barriers or the bacilli themselves penetrating the non-professional phagocytes (Russell 2001). However, little is known about the interaction between endothelial cells and mycobacteria.

### **1.5.2 Endothelial cells**

The endothelium has been, for a long time viewed as an inert cellophane like membrane that lines the circulatory system with its primary and essential function being the maintenance of vessel permeability. It was as early as 1628, that the first description of circulating blood was made by William Harvey. Shortly after this,

Malphigi described the existence of a network of vessels and outlined the physical separation between blood and tissue. In the 1800's, von Reckinghausen established that the vessels were not just tunnels bored through tissues but were lined by cells. In 1891, Heidenhahn described the endothelium as an active secretory cell system while five years later Starling through his extensive work and his law of capillary exchange strengthened the notion that the endothelium was principally a selective but static physical barrier (Cines et al. 1998).

Electron microscopic studies of the vessel wall by Palade (1953) and physiologic studies by Gowan (1959) which described the interaction between lymphocytes and endothelium were landmark studies. These studies led to the current view of the endothelium as a dynamic, heterogeneous, disseminated organ that possesses important secretory, synthetic, metabolic, and last but not least, immunologic functions (Fishman 1982.)

The endothelial cell surface in an adult human is made up of approximately  $10^{13}$  cells, weighs approximately 1kg and covers a surface area of 1 to 7m<sup>2</sup> (Augustine et al. 1994). Endothelial cells line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules as well as the blood cells themselves. This gate-keeping role of endothelium is effected through the presence of membrane-bound receptors for numerous molecules including proteins, lipid transporting particles, metabolites, and hormones as well as through specific junctional proteins and receptors that govern cell-cell matrix interactions (Cines et al. 1998).

Interactions between vascular endothelial cells and pathogenic bacteria are common events in many infectious diseases and often result in endothelial cell stimulation and enhance leukocyte adhesion to infected cells (Beilke 1989). Such interactions are comprised of two components: endothelial cell stimulation by bacterial products and direct microbial infection of the endothelial cell. Bacterial products can stimulate endothelial cells in the absence of cellular infection, or the two processes can act in concert when bacteria invade endothelial cells (Drevets 1998). Several different pathogenic bacteria have been shown to bind or invade endothelial cells and to stimulate them in the process (Drevets 1997, Sellati et al.

1995, Yao et al. 1995). Examples of such bacteria are *Listeria monocytogenes*, *Staphylococcus aureus* and *Candida albicans* (Drevets 1997, Greiffenberg et al. 1998, Beekhuizen et al. 1997).

Endothelial cells have not been known to be “professional phagocytes”, but we hypothesised that they might be involved in the host response against bacterial mycobacterial infection. It has not been explicitly stated as such, but some of the earlier literature could be interpreted as implying that phagocytic responses are inducible rather than constitutive properties of endothelium. There are also several reports of its phagocytic activity in the recent literature, many of these involving in vitro experiments. Ryan and colleagues (Ryan et al. 1987 and Gonzalez et al. 1987) showed that uptake of bacteria by endothelial cells is selective, stimulates an oxidative burst (Ryan and Vann 1988), results in the unmasking of Fc receptors on the endothelial surface (Ryan et al. 1981). It also causes an increase in the rates of division, migration, and propensity for further phagocytosis (Ryan et al. 1987). These consequences of phagocytosis have been collectively referred to as “activation” responses of endothelial cells (Ryan et al. 1987)

Endothelial cells and fibroblasts infected by *M. tuberculosis* exhibit increased sensitivity to the cytolytic effect of TNF, which suggests that this cytokine plays an important role in the immunopathology of tuberculosis (Filley and Rook, 1991). The enhanced susceptibility of these non-phagocytic cells to TNF upon mycobacterial infection may explain the difficulties encountered in identifying such target cells in vivo. It is also possible that these non-phagocytic cells serve as a reservoir for bacterial multiplication and thus aid in disease dissemination upon lysis by TNF (Chan and Kaufman, 1994).

Bacterial infection coincides with recruitment of leukocytes. It has been shown that during an inflammatory reaction various stimuli including gram-negative bacteria and host cell derived chemokines or other cytokines induce adherence of leukocytes to vascular endothelial cells through modulation of the surface expression of endothelial adhesion molecules such as ICAM-1, VCAM-1, P- and E-selectin (Smith 1993, Beekhuizen et al. 1993, and Carlos et al. 1994). However,

as to whether mycobacteria could contribute to the increased hyper-adhesiveness of endothelial cells remains to be established.

### 1.5.3 Aims

The present study was undertaken to ascertain whether mycobacteria is taken up by endothelial cells and to determine the consequences of the internalisation of mycobacteria by endothelial cells, with respect to the phenotype they acquire. In the first part of the study, endothelial cells obtained from the human umbilical vein are infected with live *Mycobacterium bovis* BCG encoded with the green fluorescent protein (BCG-GFP). Endothelial cells are also infected with killed BCG-GFP to study the phagocytic properties of these cells or invasiveness of mycobacteria. Interaction between the endothelial cells and mycobacteria was also looked at in the presence of serum opsonins.

The second part of the study looks at the interaction of mycobacteria with the primary phagocytes, macrophages. Various aspects on the uptake of mycobacteria by macrophages are looked at. The subsequent activation state of macrophages following infection with mycobacteria is also investigated.

## 2 MATERIALS AND METHODS

---

### 2.1 Mycobacteria

*Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Rv and H37Ra were obtained from the Trudeau Mycobacterial Culture collection. Mycobacterial strains were grown in Difco Middlebrook 7H9 medium containing 0.5% glycerol and enriched with 10% OADC. Cultures were incubated at 37<sup>0</sup>C and grown until log phase. Sample aliquots (1ml) were frozen and stored at -80<sup>0</sup>C in screw cap vials (Nalge Nunc International, Naperville, IL, USA). To determine the mycobacterial concentrations (defined as cfu/ml) of frozen stocks, an aliquot was thawed, passed 30 times through a 29G needle (B.Braun, Melsungen, Germany) to obtain a single cell suspension. 100µl of sample was plated in duplicate on Difco Middlebrook 7H10 agar plates in 10-fold serial dilutions, plates semi-sealed in bags and incubated for 21 days at 37<sup>0</sup>C after which the number of mycobacterial colonies were counted and the batch concentration was calculated. To confirm the batch concentration, this procedure was repeated independently by at least two individuals.

Valerie Snewin (Department of Infectious Diseases and Microbiology, Imperial College School of Medicine, St. Mary's Campus, London, UK) kindly provided *Mycobacterium bovis* transfected with a reporter gene from the jellyfish *Aequorea victoria* (BCG-GFP). Fluorescent colonies were picked from agar plates and their fluorescence verified using a fluorescent lamp. The colony was suspended in approximately 10ml 7H9 Middlebrook broth supplemented with 10% OADC, 0.1% Hygromycin and 5% Glycerol. The culture was then incubated at 37<sup>0</sup>C until confluence, subcultured and stored at -80<sup>0</sup>C in 1ml aliquots. The batch concentration was verified as above.

## **2.2 Mice**

Mice were housed and bred under specific pathogen free conditions in the animal unit of the University of Cape Town. Mice between 8-12 weeks were used in experiments. Genotypes of mice were confirmed by PCR analysis of tail biopsies. Mac-1 mice generated on a C57/BL6 background were used in the experiments and these were obtained from the animal unit of the University of Cape Town.

## **2.3 Human Umbilical Vein Endothelial cells**

Human umbilical veins were obtained from the Labour ward theatre of the Grootte Schuur Hospital, with approval from Prof J. Anthony, who is Head of Department of Obstetrics and Gynaecology at the University of Cape Town. Consent from the donor patients was given. As soon as the baby was delivered, 15 – 20cm of umbilical vein was collated aseptically, placed in a bottle with sterile cord buffer, and taken back to the lab. The vein was then used immediately for isolation of endothelial cells or placed at 4<sup>0</sup>C for a maximum of 1 hour.

### **2.3.1 Isolation and culture of endothelial cells**

Endothelial cells were obtained from human umbilical veins obtained at delivery by caesarean sections. After the umbilical vein was brought from theatre, 1.5cm was cut off from either end of the vein to remove the traumatised bits, which may also carry contaminants. It was also ensured that there were not any other areas along the length of the vein that were traumatised in any way. The vein was then transferred to a 500ml beaker with cord buffer at 37<sup>0</sup>C. The needle part of a butterfly cannulen (Surgiprod Pty. Ltd, Johannesburg) cut off and about 2cm of the same end inserted into the vein. With the piece of tubing firmly held in place, the remaining blood in the vein was flushed out with sterile cord buffer injected into the vein using a 20ml syringe. About 20ml of warm 0.1% collagenase type II (Worthington Biochemical Corporation, New Jersey, USA) would then be injected into the vein, shutting off the other end with a clamp after 2 to 3 drops of

collagenase have come out. The vein would then be left for 12-15 minutes in the warm cord buffer, in a 37<sup>0</sup>C water bath completely immersed and with both ends sealed off. The contents of the vein would then be emptied into a 50ml Falcon tube with approximately 20ml cold 20% FCS in M199, gently flushing the vein twice afterwards with cord buffer to make sure all the cells are rinsed out. The cells in the Falcon tube were spun at 1200rpm for 10 minutes, re-suspended in 5ml complete medium and transferred into a 25cm<sup>2</sup> fibronectin-coated tissue culture flask. The flask was placed in a 5% incubator at 37<sup>0</sup>C with a total volume of 8ml complete medium for 5-8 days until cells were confluent. Cells were harvested with 0.05% trypsin and 0.01% EDTA in PBS and subcultured in fibronectin-coated (20µg/ml) 6-well plates or tissue culture chamber slides. The cell density in confluent secondary cultures was 1.5 – 2.0 x 10<sup>5</sup> cells/ cm<sup>2</sup>.

### **2.3.2 Incubation of Endothelial Cells with BCG-GFP**

Medium from cultures of human umbilical vein endothelial cells (HUVEC) grown to confluence was removed and replaced with medium lacking antibiotics, but containing three different ratios of bacteria to endothelial cells; 1:1, 10:1 and 30:1. These were carefully labelled and incubated at 37<sup>0</sup>C with 5% CO<sub>2</sub>. The cells were harvested at 3 and 18 hours for each of the multiplicities of infection (MOI's). Before harvesting the cells, the supernatants were collected and stored –80<sup>0</sup>C for cytokine analysis. The endothelial cells were then used for microscopy and flow cytometry.

### **2.3.3 Staining of Endothelial Cells for Fluorescent and Confocal Microscopy**

HUVEC cultured on chambered slides were stained with CD31 and observed under the fluorescent microscope to confirm the identity of the endothelial cells. Observations were also made to see whether the endothelial cells had taken up any BCG-GFP or what kind of association existed between the non-phagocytic cells and mycobacteria. Cytopins of HUVEC that had been infected in 6-well plates

were made. These were made on teflon coated slides with demarcated wells. These were covered with a piece of self-adhesive filter paper with holes cut in it to correspond with the wells on the slide. Cells were then placed in the wells, excess medium absorbed by the filter paper and the cells left to dry overnight. The cells were then fixed 10 minutes in acetone alcohol and glass coverslips fixed with fluorescent mounting medium (Dako Corporation, Carpinteria, USA). Slides were then examined for internalisation of BCG-GFP using confocal microscopy (Leica TCS/SP Confocal Laser Scanning Microscope connected to a Leica light microscope).

#### **2.3.4 Preparation of Endothelial cells for Transmission Electron Microscopy**

Endothelial cells were grown to confluence, infected at 10:1 and 30:1 in triplicate and incubated at 37<sup>0</sup>C with 5% CO<sub>2</sub> for 18h. They were then harvested by trypsinisation, and the cells infected at the same MOI pooled. Uninfected cells served as a negative control. The cells were then pelleted then warmed to 50<sup>0</sup>C in a water bath. A 2% solution of agar-agar was made and left to warm in the water bath. Warm agar was added to each pellet and the cells gently re-suspended. They were then left for about 5 minutes in the water bath to encapsulate, then spun for 1 minute in a micro-centrifuge to rapidly pellet the cells. After centrifugation, the cells were solidified by plunging the tubes into crushed ice. The encapsulated cell pellets were then removed and cut into small slices less than 2mm, with a razor blade and left in PBS. The cells were fixed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer (fixative 1), then further in osmium tetroxide in cacodylate buffer (fixative 2). The cells were post-fixed in uranyl acetate in veronal buffer, then dehydrated in increasing concentrations of ethanol until absolute ethanol, then 100% acetone and embedded in SPURR resin in moulds. The blocks were then cut into ultra-thin (100µm) sections, which were stained with uranyl acetate and lead citrate. Sections were viewed under the electron microscope (Jeol 100S).

### 2.3.5 Preparation and Analysis of Endothelial Cells for Flow Cytometry

Flow cytometry analysis was used to investigate the uptake of BCG-GFP by endothelial cells. It was also used to look at the regulation of adhesion molecules, namely ICAM-1 and PECAM-1 (CD31), upon infection. This was to see whether the endothelial cells acquire a different phenotype after they had been infected. For uptake studies, the cells were infected at three different MOI's, 1:1, 10:1 and 30:1. They were also infected under various conditions, in the presence and in the absence of serum factors, with viable and with heat-killed mycobacteria. The controls were the uninfected endothelial cells.

The surface of a pathogen may be coated to enhance phagocytosis, and this process is termed opsonisation. Pathogens may be coated with antibodies or complement proteins. Antibodies binding to the surface of a pathogen may activate the proteins of the complement system. Complement proteins bound to the pathogen also opsonise it by binding complement receptors on the phagocytes.

In this study, the BCG-GFP was opsonised by incubating it with FCS that had not been heat inactivated for 30 minutes at 37<sup>0</sup>C. The bacteria was then spun at 1800rpm for 10 minutes to wash off the FCS and re-suspended in 1ml M199 medium free of antibiotics. The bacteria were then used to infect endothelial cells at 1:1, 10:1 and 30:1. These were incubated at 37<sup>0</sup>C with 5% CO<sub>2</sub> and harvested at 3 and 18 hours.

BCG-GFP was heat-killed to find out whether bacterial components that are responsible for adhesion to and/or internalisation by HUVEC are inactivated or altered by heat. BCG-GFP was heated at 80<sup>0</sup>C in a water bath for 20 minutes. The same MOI's, incubation periods and harvesting procedures were the same as were carried out with opsonised BCG-GFP, the only difference being that the one group of endothelial cells was infected with opsonised BCG-GFP, and the other group of endothelial cells infected with heat-killed BCG-GFP.

Endothelial cells were also infected at 1:1, 10:1 and 30:1 with viable BCG-GFP that had not been exposed to any opsonins. Uptake of BCG-GFP by the endothelial

cells was analysed by flow cytometry using the FACS caliber and the (gating of endothelial cells with BCG-GFP established by the uninfected endothelial cells).

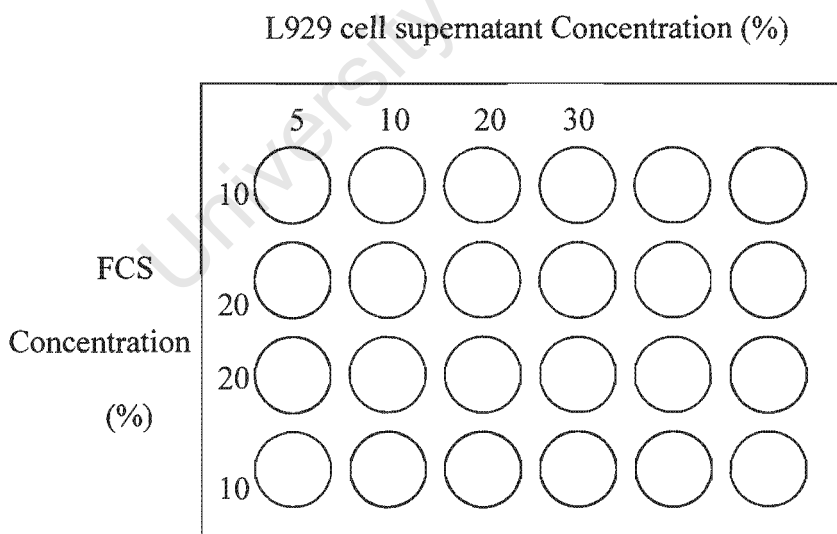
#### **2.4 Preparation of L929 supernatant and establishing the optimal working concentrations for macrophage cultures.**

In order to grow bone marrow-derived macrophages (BMD), it is essential to have the macrophage colony-stimulating factor (M-CSF) also known as CSF-1 supplemented in the growth medium. This growth factor induces proliferation of monocyte/ macrophage progenitor cells and can also activate some functions of mature cells and is produced by fibroblasts and macrophages (Metcalf 1989). To obtain the M-CSF, L929 fibroblasts were cultured for 6 days following the standard protocol.

Briefly, 1ml of complete medium was placed in the first 6 wells of a Falcon 24-well tissue culture plate. One cryotube of L-cells at  $2 \times 10^6$  cells/ml, was taken from its storage in liquid nitrogen and thawed immediately in warm water at  $37^\circ\text{C}$ . This was to minimise chances of taking up DMSO by the L929 cells. The contents of the cryotube were put in the first well, and five successive two-fold dilutions made in the next 5 wells to dilute out DMSO. The cells were left to grow for 48 hours at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator. At 48 hours cells from the last 3 wells were trypsinised with trypsin-EDTA, adding  $250\mu\text{l}$  per well and incubating for 3 minutes at  $37^\circ\text{C}$ . The cells of the 3 wells were then pooled into a centrifuge tube containing 10ml of ice-cold RPMI supplemented with 10% FCS to stop the reaction, spun at 1200rpm for 3 minutes at  $4^\circ\text{C}$ . The pellet was then re-suspended in 5ml of complete medium, then placed in a  $25\text{cm}^2$  Falcon tissue culture flask and grown for 2 days at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Spindle shaped and almost confluent, cells were trypsinised, counted, then re-plated at  $5 \times 10^4$  cells/ml in  $25\text{cm}^2$  flasks and left to grow for 2 days. These were then harvested, pooled, counted and finally seeded at a concentration of  $2.5 \times 10^4$  cells/ml in  $75\text{cm}^2$  Falcon flasks. At day 6, the L929-cells had attained confluence and produced maximal amounts of M-CSF. At that

point, the supernatants were harvested, filtered through 0.45µm, aliquoted and stored at -20°C, ready for use.

To establish the optimum working concentration for the L-cell conditioned medium; an L929 supernatant checkerboard was set up in a 24-well plate. Here, serial dilutions of the L929 supernatant were checked against two different concentrations of FCS, 10 and 20% (Figure 2.1) in DMEM. BMD-macrophages were derived from naïve C57/BL6 mice, counted and plated at  $3.0 \times 10^5$  in each of the 24 wells. They were then incubated at 37°C with 5% CO<sub>2</sub>. After 4 days, the cells were re-fed with the variously constituted complete medium as before and incubated for a further two days. On day seven, the cells were observed for 'outlook'. They were subsequently quantified to establish at which concentration of L929 supernatant and percentage of FCS the proliferation of bone marrow naïve cells into macrophages was most pronounced. This would be the optimum concentration that was also responsible for the maintenance of the spindle shaped appearance of the mature macrophages, characteristic of a good macrophage culture.



**Figure 2.1. L929 cell supernatant and FCS checkerboard.** BMD-macrophages were grown in varying concentrations of L929 cell supernatant and FCS in DMEM, to optimise these fundamental constituents of macrophage culture medium.

## 2.5 Isolation and culture of Bone Marrow Derived Macrophages

C57/BL6 and Mac-1 female mice that were 8 weeks old were used. These were sacrificed by cervical dislocation. Under sterile conditions, femurs from hind legs were removed and the tissue on the bones removed clean. The flesh-free femur was quickly rinsed in 70% ethanol and placed in antibiotic-supplemented DMEM on ice. This was repeated with the second femur. The bones were then transported from the animal unit to the lab on ice. Still under sterile conditions, the femurs were placed on a petri dish with DMEM, the remaining flesh teased off, and the two ends (epiphysis) of the bone were cut to expose the bone marrow. The bone with its ends cut, was immediately placed into the second petri-dish and with a pair of forceps holding it in one hand, the bone marrow cells were flushed out with a gentle stream of DMEM applied through a 25G needle. The flushing of the bone marrow cells was done until the bone turned white. After the cells from the two femurs had been pooled together, they were counted in 2% acetic acid to exclude red blood cells. An average of  $1-2 \times 10^7$  cells was obtained each time. The cells were then diluted to a concentration of  $3 \times 10^5$  in 20% FCS and 20% L929 medium in DMEM supplemented with glutamine and 3.7g/L  $\text{NaHCO}_3$  and seeded 5ml per 100mm cell culture petri dish. On day four of cell culture, the cells were gently washed with 1x PBS and fed with fresh complete medium. On day seven the cells reach maturity and ready to be used in experiments. The macrophages were harvested and sub-cultured in 24-well plates or 8-well chambered slides as required for the experiment. Cells were sub-cultured at  $2 \times 10^5$  cells/ml, 0.5ml in 24-well plates, and 0.2ml in 8-well chambered slides, allowed to adhere overnight and infected the following day.

## 2.6 Infection of Macrophages with *Mycobacteria* for uptake studies

BMD-macrophages were derived and cultured for seven days and sub-cultured in 8-well chambered slides. Culture supernatants were removed and cells gently rinsed with 1 x PBS. The culture medium was then replaced with variously constituted inoculating medium. Macrophages from C57BL6 mice were infected with viable and heat-killed H37Rv at various ratios of 1:1, 2:1, 4:1 and 8:1,

mycobacteria to macrophage, and incubated at 37<sup>0</sup>C for 3 and 18 hours. This was to investigate uptake of H37Rv by macrophages infected at increasing multiplicities of infection (MOI's) at an early and later time-point. Heat killing of H37Rv was performed by incubating it at 80<sup>0</sup>C for 20 minutes prior to infection. These infections were carried out in duplicate.

Macrophages from WT and Mac-1 mice were infected at 5:1 (mycobacteria: macrophage) and incubated for 1, 2, 4 and 6 hours, using H37Rv, H37Ra and *Mycobacterium bovis* BCG to infect. These infections were also carried out in 8-well chambered slides. This experiment was to compare uptake of H37Rv, H37Ra and BCG by macrophages in the presence and absence of complement receptor type 3 (CR3). After the infection periods were over, extracellular mycobacteria were rinsed off with 1 x PBS, fixed overnight in 4% paraformaldehyde and stained using a Ziehl Neelson stain for visualisation of phagocytosed mycobacteria.

#### **2.6.1 Studying Uptake of Mycobacteria Using Ziehl Neelson staining.**

Cells were fixed on the slides in acetone after they were allowed to air dry. They were then immersed in a filtered Carbol Fuschin solution, flamed and cooled for 5 minutes (2 times) then rinsed in water. Slides were immersed in 1% acid alcohol for 30 seconds to remove excess stain on the cells and rinsed again in water. The slides were then submerged in 25% H<sub>2</sub>SO<sub>4</sub> for 20 minutes and washed in running water for 10 minutes, then counterstained with Loefflers' methylene blue for 1 minute and rapidly rinsed in water. Cells were dehydrated by immersion for 10 seconds in 70% alcohol, 96% alcohol and then xylol. Cells were mounted using Entellan (Merck). The number of cells containing acid-fast bacilli was evaluated using oil immersion light microscopy and infected cells expressed as a percentage of the total number of cells counted. At least 200 cells were counted for each treatment. And as each treatment was done in duplicate, the average of the two was used. Experiments were repeated to confirm the observations and the averages used.

## **2.7 Establishment of the optimal serum concentration for phagocytosis of mycobacteria.**

In order to find the optimal concentration of mouse serum required for mycobacterial uptake by macrophages, macrophages were infected at MOI 8:1 for 4 hours at 37<sup>0</sup>C, on 8-well chambered slides in DMEM supplemented with 1%, 2%, 3% and 4% serum. This was performed with both fresh and heat-inactivated serum. After 4 hours, extracellular mycobacteria was rinsed off, cells fixed and a Ziehl Neelson stain performed to assess uptake. The concentration of serum that elicited highest percentage uptake was used in the following experiments investigating cytokine induction and MHC regulation.

## **2.8 Bronchial Alveolar Lavage**

WT mice were killed by cervical dislocation. A 20-gauge Introcan catheter (B. Braun, Germany) was inserted into the exposed trachea. The lungs were lavaged 10x with 3ml of PBS. 2.5ml were routinely recovered. The bronchoalveolar fluid freshly obtained was used in subsequent studies at 250µg/ml of surfactant protein A (Wright et al. 1987) quantified using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

## **2.9 Infection of mycobacteria for cytokine induction and major histocompatibility expression.**

BMD-macrophages grown for seven days in complete medium were infected at 5:1 and 8:1 using viable and heat-killed H37Rv in a 24-well plate, in the presence of normal mouse serum, serum that had been heat-inactivated at 56<sup>0</sup>C for 1 hour, and bronchoalveolar fluid. Macrophages were also infected in the absence of opsonins, and others left uninfected to serve as a negative control. The macrophages were infected for 4 hours in antibiotic and growth factor-free DMEM, after which period the cells were washed to remove extracellular bacteria. The cells were then

incubated with complete medium overnight at 37<sup>0</sup>C and supernatants then harvested. Aliquots were made and stored at -80<sup>0</sup>C for the analysis of cytokines IL-10 and IL-12. Macrophages infected at 5:1 with viable H37Rv were then detached using ice-cold, cation-free PBS, labelled for antibodies against MHC classes I and II expression and analysed by flow cytometry.

### **2.9.1 Staining of Activated Macrophages for major histocompatibility complex (MHC)**

For MHC staining, approximately 1 x 10<sup>6</sup> cells infected with H37Rv in the absence of serum opsonins, were incubated on ice for 10 minutes with 25µl blocking buffer (which was made of normal mouse serum, normal rat serum, and CD16/CD32 antibody). The cells were rinsed in 3ml FACS buffer, then double-stained with 2µg/ml anti-ICAM-1 and either 2µg/ml anti-H2-kb-FITC or 2µg/ml anti-M5/114-FITC antibodies for 30 minutes on ice in the dark. ICAM-1 is constitutively expressed on BMD-macrophages and therefore staining the cells with it helps to isolate the BMD-macrophage cell population. The cells were then rinsed in FACS buffer free of the antibodies, spun at 1000rpm for 5 minutes, fixed in FACS fixing buffer (1% paraformaldehyde) and stored overnight at 4<sup>0</sup>C. This was to fix the mycobacteria and stained cells before analysis. This procedure was repeated with cells infected in the presence of normal serum, heated serum and bronchoalveolar fluid. The isotype controls used were rat IgG2a-PE and rat IgG2a-FITC. The cells were then analysed by flow cytometry for expression of the cell surface molecules on a FACS caliber (Becton Dickinson) using Cell Quest software (Becton Dickinson).

### **2.10 Cytokine Immunoassays**

Supernatants from the infected HUVEC that had been collected and stored at -80<sup>0</sup>C were thawed and used for measuring TNF-α, IL-6. These supernatants were from uninfected endothelial cells, endothelial cells infected at 10:1 and 30:1, and

incubated for 3 and 18 hours. Investigating cytokine production at an early and late time point, and also production at a low and higher multiplicity of infection (MOI).

Sandwich ELISA's were also performed for *Mycobacterium tuberculosis* stimulated bone marrow-derived (BMD) macrophages. BMD-macrophages from C57/BL6 mice were infected with *Mycobacteria tuberculosis* at MOI 5:1 and 8:1. These infections were performed using viable and heat-killed bacteria, in the presence of normal and heated serum, as well as bronchoalveolar fluid. Uninfected macrophages served as negative controls.

96-well maxisorb microtiter plates (Nalge Nunc International, Naperville, IL, USA) were coated overnight for the respective cytokines, TNF- $\alpha$  and IL-6 of endothelial cell experiments and IL-10 and IL-12 respectively for macrophage experiments and placed at 4<sup>0</sup>C overnight. The coating solution was washed off and the plates incubated with 200 $\mu$ l blocking buffer overnight at 4<sup>0</sup>C. The blocking buffer was washed off and the samples and recombinant standards added and left overnight. Samples were added undiluted and the standards in three-fold dilutions ranging from 100ng/ml - 0.6pg/ml. The samples and standards were washed off and biotinylated, developing antibodies added and incubated at 37<sup>0</sup>C for 3 hours. Plates were washed 4 times and incubated with alkaline phosphatase labelled strepavidin (BD Pharmingen) diluted 1:1000 in dilution buffer and added to the ELISA plates for 1 hour at 37<sup>0</sup>C. The plates were washed free of any strepavidin and P-Nitrophenyl-phosphate (PNPP) substrate at 1mg/ml (Boehringer Mannheim, Germany) diluted in di-ethanolamine substrate buffer finally added. The ELISA plates with *Mycobacterium tuberculosis* infected samples were left for approximately 10 minutes. The substrate reaction was then stopped by adding 100 $\mu$ l of absolute alcohol. This also disinfected the ELISA plate rendering it safe to read outside the P3 lab. The cytokine assays were read at 405nm using a microplate spectrophotometer (Molecular Devices, spectra MaxGemini).

## 2.11 Statistical Analysis

Statistical analysis was performed using a paired *t* test, and a p-value of  $< 0.05$  was considered significant. Data is expressed as the mean  $\pm$  standard deviation. Values were calculated using the statistical package on Microsoft excel 97.

## 2.12 Reagents and Materials

Chemical reagents were purchased from the following companies unless otherwise stated: BDH Chemicals Ltd., Poole, England, Merck Laboratory supplies, South Africa, Sigma-Aldrich South Africa. Corning and costar products were used for tissue culture and other plastic ware from sterilin, Bibby Sterilin Limited, UK.

### General Reagents and solutions

#### Agar

Dissolve 19g Difco Middlebrook 7H10 agar in 900ml distilled H<sub>2</sub>O. Add 5ml glycerol. Autoclave at 121<sup>0</sup>C for 10 minutes. Cool to 55<sup>0</sup>C in water bath and add 100ml OADC (State Vaccine Institute, South Africa). Pour 7ml in each of a two-compartment 90mm petri dish (Bibby Sterilin, Staffordshire, England). Allow to set, and store at 4<sup>0</sup>C for up to 2 weeks.

#### Broth

Dissolve 4.7g Difco Middlebrook 7H9 broth in 900ml distilled H<sub>2</sub>O. Add 2ml glycerol. Autoclave at 121<sup>0</sup>C for 10 minutes. To 180ml, sterile medium, add 20ml ADC enrichment under aseptic conditions.

#### Phosphate Buffered Saline (PBS)

Dissolve 8g NaCl, 0.2gKCl, 1.44g Na<sub>2</sub>HPO<sub>4</sub> and 0.24g KH<sub>2</sub>PO<sub>4</sub> in 900ml distilled H<sub>2</sub>O. Adjust to pH 7.4 with HCl. Make up to 1L. Sterilise by autoclaving for 30 minutes at 121<sup>0</sup>C.

## **Tissue culture Medium and Solutions**

### **Medium 199**

Dissolve powder M199 (Gibco) in 900ml distilled H<sub>2</sub>O. Add 2.2g NaHCO<sub>2</sub>, 2mM Glutamin. Adjust pH to 7.55 and make up to 1L. Filter sterilise (Millipore Corporation, Bedford, USA) and store at 4<sup>0</sup>C. For cell culture, add 90µg/ml sodium heparin and 25µg/ml ECGF to 600ml of the above medium. Filter sterilise. Add 20% sterile heat inactivated FCS and store at 4<sup>0</sup>C.

### **Cord Buffer**

Dissolve 40.92g NaCl, 1.49g KCl, 19.52g HEPES, 9.91g Glucose, 100E penicillin, 100g/ml streptomycin to 4.5L distilled water. Adjust to pH 7.55 with 1M NaOH. Make up to 1L and filter sterilise. Store at 4<sup>0</sup>C.

### **RPMI**

Dissolve RPMI 1640 in 900ml distilled H<sub>2</sub>O, add 2mmol L-glutamin, pH to 7.4 and make up to 1L. Filter sterilise (Millipore Corporation, Bedford, USA) and store at 4<sup>0</sup>C. Add 10% heat-inactivated FCS just before working to make up complete medium.

### **DMEM**

Dissolve DMEM in 900ml distilled water, add 3.7g/l NaHCO<sub>3</sub> and pH to 7.4. Make up to 1L, and filter sterilise with 0.45µm filter (Millipore Corporation, Bedford, USA). Store at 4<sup>0</sup>C. Add 20% FCS and 20% L929 cell supernatant just before working to make up complete medium.

### **Trypsin/EDTA**

To make up trypsin/EDTA, 2xTD buffer and trypsin must first be prepared.

### **2x TD buffer**

Dissolve 16.00g NaCl, 0.76g KCl, 0.25g Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O and 6.00g TRIS to 900ml distilled water. Leave at room temperature exposed to air for 30 minutes. Adjust to pH 7.4 using 3N HCl. Leave for 30 minutes. Keep aliquots of 50ml at 4<sup>0</sup>C.

### **Trypsin**

Dissolve 2.5g trypsin in 500ml 0.001N HCl by stirring at room temperature for 2 hours. Sterile filter and make aliquots of 50ml. Store at  $-20^{\circ}\text{C}$ .

### **Trypsin/EDTA**

Add 50ml 2x TD buffer to 50ml Trypsin and 1ml 2% EDTA. Store at  $4^{\circ}\text{C}$ .

### **FACS Reagents**

#### **FACS buffer**

Dissolve 1g Bovine Serum Albumin (BSA) (Boehringer Mannheim, Germany) and 0.1g  $\text{NaN}_3$  in 900ml PBS (pH7.4). Make up to 1L and filter sterilise using a  $0.45\mu\text{m}$  filter (Millipore Corporation, Bedford, USA). Store at  $4^{\circ}\text{C}$ .

#### **Fixing Buffer**

Dissolve 4g NaOH in 100ml PBS. Add 20g paraformaldehyde and dissolve. Adjust pH to 7.2. Make up to 1L and filter sterilise using  $0.45\mu\text{m}$  filter (Millipore Corporation, Bedford, USA). Store at  $4^{\circ}\text{C}$  in the dark.

#### **FACS blocking solution**

Make up 2.5% normal rat serum, 2.5% normal mouse serum and 6.25g/ml anti-CD32/16c (anti- $\text{FC}\gamma\text{RIII/II}$ ) (Clone:2.4G2, Pharmingen) in FACS buffer.

### **ELISA Reagents**

**Table 1: Antibodies used in ELISA's for cytokine detection.**

<b>Cytokine</b>	<b>Capturing antibody</b>	<b>Standard</b>	<b>Detecting antibody</b>	<b>Source</b>
HIL-6	Purified rat anti-human IL-6	Recombinant human IL-6	Biotinylated rat anti human IL-6	Pharmingen
HTNF- $\alpha$	Purified mouse anti human TNF- $\alpha$ . Mab1	Recombinant human TNF $\alpha$ .	Biotinylated mouse anti human TNF- $\alpha$ . Mab11	Pharmingen
MIL-10	Purified rat anti-mouse IL-10.	Recombinant mouse IL-10.	Biotinylated rat anti mouse IL-10.	Pharmingen
MIL-12	Purified rat anti-mouse IL-12.	Recombinant human TNF $\alpha$ .	Biotinylated rat anti mouse IL-12	Pharmingen

### **Coating Buffer**

Dissolve 0.2g  $\text{NaN}_3$  in 900ml PBS (pH7.2). Make up to 1L and autoclave.

### **Blocking Buffer**

Dissolve 40g BSA and 0.2g  $\text{NaN}_3$  in 900ml PBS (pH7.4) and make up to 1L. Filter sterilise and store at 4°C.

### **Dilution Buffer**

Dissolve 10g bovine Albumin Serum BSA (Boehringer Mannheim, Germany) and 0.2g  $\text{NaN}_3$  in 900ml PBS (pH7.4). Make up to 1L and filter sterilise using a 0.45µm filter (Millipore Corporation, Bedford, USA). Store at 4°C.

### **20X Washing Buffer**

Dissolve 20g KCl, 20g  $\text{KH}_2\text{PO}_4$ , 144g  $\text{NaH}_2\text{PO}_4$  and 800g NaCl in 4.5L distilled water. Add 50ml Tween 20 and 100ml 10%  $\text{NaN}_3$  solution. Make up to 5L and sterilise through a 0.45µm Millipore filter. Dilute 1 in 20 for 1x working concentration.

### **Substrate Buffer**

Dissolve 0.2g  $\text{NaN}_3$ , and 0.8g  $\text{MgCl}_2$  in 700ml distilled water. Add 97ml liquefied diethanolamine. Adjust to pH 9.8 and make up to 1L. Filter sterilise through a 0.45µm Millipore filter and store at 4°C.

### **Cell Staining (Ziehl Neelson)**

#### **Carbol Fuchsin**

Add 10ml 6% Basic fuchsin in absolute alcohol to 90ml 5% carbolic acid solution. Filter solution through Whatmann filter paper no.1, and store at room temperature.

#### **Loeffler's methylene blue**

Add 30ml of 0.8% methylene blue (in absolute alcohol) to 99ml distilled water. Add 1ml 1% potassium hydroxide. Filter solution through Whatmann paper no.1 and store at room temperature.

## **(TEM) Transmission electron microscopy Reagents**

### **Fixative 1**

2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, adjust to pH 7.2, add 0.1M sucrose.

### **Fixative 2**

1% osmium tetroxide in 0.1M sodium cacodylate buffer, adjust to pH 7.2, add 5mM CaCl<sub>2</sub> and 5mM MgCl<sub>2</sub>. No sucrose.

### **1% Uranyl acetate**

Add 250mg uranyl acetate to 25ml veronal buffer.

### **Cacodylate Buffer**

5mM CaCl<sub>2</sub> and 5mM MgCl<sub>2</sub>, plus or minus 0.1M sucrose.

### **Veronal Buffer**

Solution A (500ml)

Dissolve 9.7 g sodium acetate, 14.7g sodium veronal, and 14.0g NaCl in 500ml distilled water.

### **Buffer**

Add 50ml solution A and 70ml 0.1N HCl to 130ml distilled water and adjust to pH 6.0.

### **SPURR Resin**

NSA 13g, ERL 5, DER 3g, S-1 0.2g

## 4 DISCUSSION

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Bacterial invasion of mammalian cells is a complex process that involves active participation by the microbe and the eukaryotic target. Current models indicate that pathogenic bacteria use several different mechanisms to sense their microenvironment and adjust expression of their virulence factors accordingly. In tuberculosis, dissemination of mycobacteria to the body organs involves a systemic pathway in which the endothelial cells barrier is crossed. This is achieved either through the bacilli themselves gaining entry in the endothelial cells or via infected phagocytes, most notably macrophages, crossing the endothelial cell barrier into the blood stream (Russell, 2001; Birkness, 1999). In this study, the interaction of mycobacteria with the non-professional phagocytes, the endothelial cells, and the professional ones, the macrophages was studied.

### **Uptake of Mycobacteria by endothelial cells**

Endothelial cells exhibited the ability to take up mycobacteria. Like with the macrophages, uptake of mycobacteria by endothelial cells was both dose- and time-dependent. Uptake of mycobacteria by endothelial cells was however relatively slow compared to the rates at which endothelial cells take up other pathogenic bacteria. Maximal adherence of *Borrelia burgdorferi* to HUVEC was observed at 4h (Thomas and Com-stock, 1989), for example, and uptake of *Rickettsia rickettsii* was maximal within 6h, infecting 80 to 90% of HUVEC (Silverman, 1984; Walker 1984). The differences in uptake may be due to slower phagocytosis or reduced adherence to endothelial cells resulting from variations in bacterial strains.

Serum labile factors enhanced uptake of mycobacteria. This suggested the presence of certain receptors on the endothelial cells that may recognise serum ligands coating the mycobacteria. Endothelial cells do not normally express receptors for the Fc (constant fraction) of the IgG antibody or for C3b (fraction of the third component of complement (Ryan et al. 1980). However certain types of injury, such as viral infection, white cell lysates, endotoxin and antibodies to

endothelial surface enzymes in the presence of complement, cause an unmasking of Fc receptors and, to a lesser extent C3b receptors on endothelial cell surface (Ryan et al. 1985). This would have the effect of rendering the endothelial surface adhesive for immune complexes and would promote phagocytosis where opsonisation with IgG serves as a recognition factor (Ryan 1985). It is therefore possible that opsonisation of mycobacteria (BCG-GFP) could have exploited some of the receptors on endothelial cells to bring about enhancement in phagocytosis. Alternatively serum opsonins on their own could facilitate penetration of endothelial cells by mycobacteria.

The uptake of heat-killed mycobacteria suggested the presence of an active mechanism for mycobacterial uptake by endothelial cells. The fact that no increment in phagocytosis of heat-killed mycobacteria was observed at higher MOI implied that uptake via this mechanism was independent of the infection dose. This suggests a limited ability of endothelial cells to phagocytose mycobacteria. In other words, the infection is not simply a consequence of the invasive capacity of the bacterium but an endothelial cell function, most likely phagocytosis.

Unlike heat-killed mycobacteria, uptake of viable mycobacteria was dependent on infection dose. Thus a profound difference existed between the uptake of heat-killed and the uptake of viable mycobacteria by endothelial cells. This suggested an additional mechanism by which viable mycobacteria are taken up by endothelial cells. Elsewhere, the ability of mycobacteria to enter non-professional phagocytes has been reported (Pethe et al. 2001). Mycobacteria actively secrete an adhesin that binds heparin (Menozzi et al. 1996). It is this adhesin that facilitates an active entry of mycobacteria into non-professional phagocytes. Thus, the secretion of this factor could explain the uptake of viable but not heat-killed mycobacteria observed at higher MOI's.

### **Phenotype of endothelial cells after infection with mycobacteria.**

The phenotype of endothelial cells after infection with mycobacteria was looked at. On the basis of the sensitivities of the ELISA's applied in this study, no detectable levels of TNF- $\alpha$  and IL-6 cytokines were detected in the supernatants of endothelial cells after infection with BCG. This suggested that either uptake of mycobacteria by endothelial cells does not trigger the release of these inflammatory cytokines or that in fact it does, except only in minute quantities which though may be of physiological significance, could not be detected by the ELISA's employed. IL-6 is not only produced by HUVEC but may also affect its proliferation. The ability of the vascular endothelium to rapidly secrete IL-6 in response to inflammation-associated cytokines is of strategic value since it generates circulatory signal which helps mobilise the acute phase plasma protein response and enlists the immune system (May, 1989). TNF has multiple actions on endothelial cells that serve to promote coagulation and inflammation (Pober and Cotran, 1990).

In addition to the release of inflammatory cytokines, activated endothelial cells express surface adhesion molecules such as E- and P-selectin as well as intercellular adhesion molecule ICAM-1 and VCAM-1 which are involved in the recruitment and activation of leukocytes (Pohlman and Harlan, 1992). Infection of endothelial cells with mycobacteria did not upregulate the levels of CD31 expressed on the cell surface. However, there was a down-regulation in the expression of ICAM-1 after infection of endothelial cells with mycobacteria. This down-regulation of ICAM-1 on endothelial cells could suggest a reduction in the adhesiveness of endothelial cells after exposure to mycobacteria. This combined with the observations that no inflammatory cytokines could be detected in the supernatants of infected endothelial cells suggests that mycobacteria may not be among those pathogenic organisms that transform endothelial cells into an "activated" state as was suggested by Ryan et al. (1987).

### **Uptake of mycobacteria by macrophages**

The phagocytosis of mycobacteria by macrophages has been extensively studied. While endothelial cells form a barrier *M. tuberculosis* cross in order to disseminate, macrophages act both as innate cells in the recognition of the infection as well as effector cells in containment of the infection.

Unlike endothelial cells, the uptake of heat-killed mycobacteria by macrophages was enhanced by increasing the infection doses. This paralleled the observation of Pethe et al. (2001), where a mutant mycobacteria unable to express heparin binding adhesin was equally taken up by macrophages, but not epithelial cells, the non-professional phagocytes. Thus the resultant modifications on the mycobacteria cell wall following exposure to heat did not interfere with the phagocytic mechanism of the macrophages.

Infection for shorter periods favoured uptake of viable mycobacteria by macrophages, possibly indicating the ease with which intact mycobacteria are taken up by the macrophages. This suggests that heat killing of mycobacteria distorts the conserved motifs on mycobacteria cell wall by which they are recognised and taken up by phagocytes. Furthermore, the ability of mycobacteria to secrete heparin, may be obliterated by heat killing, thus undermining a possible heparin-mediated uptake of mycobacteria by macrophages. The fact that a similar preference in the uptake of viable mycobacteria was not observed in macrophage cultures infected for longer periods is likely to be due to some of the macrophages dying during the long infection period.

### **Uptake of mycobacteria in the absence of Complement Receptor 3**

Complement receptors (CR) CR1, CR3 and CR4 have long been considered the main macrophage receptors for mycobacteria (Schlesinger et al., 1990; Schlesinger, 1993; Hirsch 1994). Results obtained from studying the uptake of mycobacteria in the absence of CR3 are in agreement with those reported previously which showed that in the absence CR3, uptake of *M. tuberculosis* is impaired (Hu, 2000).

However, in the present study, striking differences between macrophages from CR3<sup>-/-</sup> mice and those from WT was only evident at 4h and 6h of infection. This is in contrast to Hu's idea that the apparent deficiency in *M. tuberculosis* phagocytosis by CR3<sup>-/-</sup> macrophages is reversible with increased contact between macrophages and bacteria. In the early stages of infection, it was observed, that in the absence of CR3, *M. tuberculosis* could be said to gain entry into macrophages via alternative receptors. Similar trends were observed in the phagocytosis of H37Rv and the avirulent strains H37Ra and BCG.

### **Activation of macrophages after infection with mycobacteria**

In the activated form, macrophages can act as cells of innate immune system to prime T-lymphocytes, thereby triggering a specific immune response. Alternatively, activated macrophages serve as effector cells of the immune system that directly contain the multiplication of mycobacteria. In order to execute these two functions, macrophages must express the necessary accessory molecules as well as the secretion of important cytokines.

Among the key accessory molecules are the major histocompatibility (MHC) glycoproteins, while IL-12 is the critical cytokine. MHC glycoproteins are required for the recognition of T-lymphocytes as well as presentation of the processed antigenic peptides. The inhibition of the number of macrophages positive for MHC Class I and Class II after infection with mycobacteria in the presence of serum factors was striking. One of the key mycobacterial opsonins in both normal and heat-inactivated serum is cholesterol. Cholesterol mediated uptake of mycobacteria is thought help in the retention of TACO on the phagosome, contributing to altered phagosomal maturation (Gatfield and Pieters, 2000, and Ferrari wt al. 1999). Hence, it is tempting to suppose involvement of a cholesterol-mediated uptake of mycobacteria in the inhibition of MHC expression on infected macrophages.

The infectious-dose dependant secretion of IL-12 might have been a result of more infected macrophages at higher doses leading to enhanced secretion of the cytokine. Interestingly, presence of serum factors, greatly inhibited secretion of IL-12 in the

infected macrophage cultures, while bronchoalveolar fluid enhanced IL-12 production two-fold. This suggests that bronchoalveolar opsonins mediated uptake of mycobacteria by macrophages enhances the signalling for IL-12 production. Such opsonins in bronchoalveolar fluid include surfactant A protein (Ernst 1998). In addition to promoting resistance to infectious agents by enhancing T<sub>H</sub>1-type responses, IL-12 has been shown to increase resistance to intracellular pathogens by a T-cell-independent mechanism involving IL-12-induced IFN- $\gamma$  production by NK cells followed by IFN- $\gamma$ -mediated activation of macrophages to display enhanced microbicidal activity (Gazzinelli et al., 1993; Tripp et al., 1993). Consistent with my observation with regard to IL-12 production, IL-12 levels were significantly higher in pleural fluids from human patients with tuberculous pleuritis than in serum from the same patients or in malignant pleural effusions (Zhang et al., 1994). These results suggest that IL-12 may play a role in the immune response to *M. tuberculosis*.

IL-10 production in *M. tuberculosis* infected macrophage cultures was inhibited in the presence of serum factors. These observations show the critical role of serum factors on the subsequent activation states of macrophages. Since in vivo, interaction of macrophages with mycobacteria is often in the presence of body fluids endowed with most of the serum factors, these findings indicate the importance of these observations in understanding further the host-mycobacteria relationship. The most dramatic changes induced by IL-10 are its potent deactivating effects on monocytes/ macrophages, including downregulation of MHC class II molecules, accessory molecules, and cytokine and chemokine production, which lead to inhibition of antigen presentation/ accessory functions and result in abrogation of T-cell cytokines (Malefyt 1998).

## 5 CONCLUSION

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In conclusion, endothelial cells have the ability to bind and internalise mycobacteria. The rates of uptake are comparable to those of uptake by *Mycobacterium leprae* (Scollard DM. 2000). Endothelial cells did not seem to be activated upon infection showing almost no production of TNF- $\alpha$  and IL-6. ICAM-1 is constitutively expressed on endothelial cells and appears to be down regulated upon infection with mycobacteria.

Since macrophages are endowed with multiple phagocytic receptors, uptake of mycobacteria by the cells is more depictable in comparison to that of endothelial cells. Among these phagocytic receptors is CR3, which exhibits a major role in the uptake of mycobacteria. Following infection with mycobacteria, macrophages variously produced cytokines IL-12, IL-10 and expressed MHC glycoproteins differently according to the nature of opsonins mycobacteria had been exposed to prior to infection of macrophages.

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