Interactions between dendritic cells and *Mycobacterium tuberculosis*

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A thesis submitted in partial
Fulfilment of the requirements of
University College London for the degree of
Doctor of Philosophy

Division of Mycobacterial Research
National Institute for Medical Research
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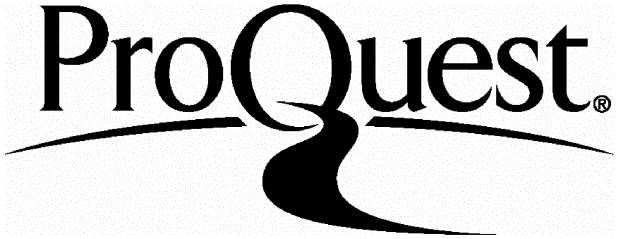
London

April 2001



Abstract

Mycobacterium tuberculosis (*Mtb*) is an intracellular pathogen that has a major impact on human health. Control of tuberculosis has proved extremely difficult, particularly in developing and underdeveloped countries; this has been exacerbated by the variable efficacy of BCG, the current vaccine, and by the increasing prevalence of drug resistant strains. Effective immunity against *Mtb* involves cell-mediated mechanisms. Dendritic cells (DC) are likely to play a critical role in the induction of a cellular response to *Mtb* since they are the most efficient antigen-presenting cells (APC) for priming naïve T cell lymphocytes. In this study we have investigated the interaction between *Mtb* and DC, and how that interaction might be used to generate protective immune responses.

Working with a dendritic cell line and using electron microscopy we have shown that coincubation of DC with *M.tuberculosis* results in the rapid internalisation of the mycobacteria. Twelve hours after infection the mycobacteria are found within membrane-bound phagosomes. By 96 hours we could see some lysis of the DC although there was no evidence of apoptosis; the presence of *Mtb* was more difficult to detect by this stage. Over a 5-day period, the viability of *M.tuberculosis* that had been phagocytosed by DC was found to decline slightly, whereas an identical inoculum was able to replicate in cultured macrophages. We therefore investigated the mechanisms associated with this growth suppression and found that both oxygen and nitrogen radicals where involved.

Changes in cytokine production by DC infected with *Mtb* were observed, with a significant up-regulation of cytokines involved in Th1 and Tc1 responses. Similar results were obtained when primary bone-marrow derived DC were infected with *Mtb*. Characterisation of surface molecules expressed by DC which had been infected with *Mtb* confirmed the maturation process of the cells with significant up-regulation of the costimulatory molecules B7-1 and B7-2 and increased expression of MHC class II molecules and ICAM-1. This response was found to be dependent on the rapid activation of the nuclear transcription factor NF-κB, and was independent of TNF-α release. We also demonstrated expression of c-Rel and Rel-B proteins in *Mtb*-activated DC.

In addition to these *in vitro* studies, we have also demonstrated that *Mtb*-activated DC are extremely efficient in priming naïve murine T cells and that this immune response does not require T cell help. *Mtb*-activation of DC also results in efficient cross priming of T cells specific for *Mtb*. These responses enable *Mtb*-activated DC to confer protection against challenge with viable *Mtb*, with levels of protection as good or better than those conferred by BCG.

The further understanding of the mechanisms involved in the interaction of mycobacteria with DC, and the mechanisms underlying the transfer of protective immunity, should provide important insights for the development of novel approaches to immunotherapy or for the development of new vaccines.

Acknowledgements

The process involved in producing the work on this thesis would not have been possible without the help and support of various people. Therefore, I would like to take this opportunity to express my gratitude and appreciation to them all.

First and foremost, I would like to thank the Portuguese Foundation for Science and Technology (PRAXIS XXI) for financially supporting the work carried out, and also Professora Maria de Sousa, especially during the scholarship administration stage. Subsequently, this led me to the Division of Mycobacterial Research at the National Institute for Medical Research under the watchful eye of Dr M J Colston.

On many occasions, Dr Colston has unfailingly used available resources to promote a peaceful working environment for me, especially during the write-up stage. The laboratory, I felt on many occasions has a special unique quality, where all the staff communicated within a happy and joyful atmosphere. Mainly due to two members of those staff, Dr Silvia Ragno and Dr Ricardo Tascon – they were both a great help (both physically and mentally).

Other members of staff within the Division of Mycobacterial Research whom I owe special thanks to are: Evangelos Stavropoulos and Bob Butler – they were always around and a great source for extracting useful information.

During my third working year, Dr Monica Belich from Cellular Immunology was an immense help at my first approach to molecular biology. Elizabeth Hirst from Neurobiology was responsible for the excellent electron microscopy pictures and Dr B Stockinger for promptly making the tsDC available.

Some of the excellent design skills displayed were largely due to Frank Johnson, who always worked quickly and accurately, from the Photographic Department.

Perhaps my greatest thanks would be to my valuable friends. Some of my happiest moments have been in the company of: Gili Bachrach, Susana Gonzalez-Rico, Sapna

Manglani, Krishna Gopal, Richard Speight, Colin Anderson, and Fran Johnson. Most undoubtedly, my version of "War and Peace" included Nazy Movahedzadeh and Cristiane Frota – my two adopted sisters. Cristiane – "thank you for keeping me sane and in sound mind".

Lastly, to one of the most special persons of all "through sickness and in health", Peter - I adore you!

My family I dedicate this message to you:
Aos meus queridos pais e irmao pelo apoio nos momentos de crise e nos momentos de alegria sem os quais nao completaria os meus estudos nem conseguiria estar tanto tempo em Londres (as visitas sao caras, mas foram muitas!!!!)
Aos meus queridos avos por estarem vivos e de saude, sempre prontos a pagarem as infinitas contas de telefone, que a neta teve!!!!!!

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Abbreviations

AEBSF	4- (2- Aminoethyl) benzenesulfonyl fluoride
AIM- V	Serum-free lymphocyte medium
Ag-DC	Antigen-pulsed dendritic cell
AMG	Aminoguanidine
APC	Antigen presenting cell
APS	Ammonium persulfate
BCG	Bacille Calmette Guerin
bmDC	Bone marrow- derived dendritic cells
bp	Base pairs
BSA	Bovine serum albumin
CD	Clusters of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
CMI	Cell mediated immunity
CTL	Cytotoxic T cell
Da	Daltons
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate
ddH_2O	Double distilled water
DMEM	Dulbecco`s modified eagles`s minimal essential medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide 5`- triphosphates
ECL	Electrogenerated chemiluminescence
EDTA	Ethylene diaminetetra-acetic acid
EGTA	Ethyleneglycol-bis[β- aminoethylether]-N,N,N`,N`-tetra-acetic acid
ELISA	Enzyme-linked Immunoadsorbent Assay
EM	Electron Microscopy

EMSA	Electrophoretic mobility shift assays
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluoroscein isothiocyanate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HSA	Heat Stable Antigen
hsp	Heat Shock Protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IMDM	Iscove`s modified Dulbecco`s medium
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of NF-κB
LAM	Lipoarabinomannan
L-NAME	N-nitro-L-arginine methyl ester
L-Norv	L-Norvaline
KO	Knockout
L-NMMA	N- Monomethyl-l-arginine monoacetate
LPS	Lipopolyssaccharide (endotoxin)
Mϕ	Macrophage
MHC	Major Histocompatibility Complex
mRNA	Messenger ribonucleic acid
Mtb	*Mycobacterium tuberculosis*
NF-κB	Nuclear Factor κB
NK	Natural killer cells
NO	Nitric Oxide
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
RNA	Ribonucleic acid
Rnase	Ribonuclease

RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RT-PCR	Reverse Transcriptase –Polymerase Chain Reaction
SPI	Small peptide inhibitors
TB	Tuberculosis
TBE	Tris-borate EDTA buffer
Tc	Cytotoxic T cell
Th	Helper T cell
TMB-8	8-(diethylamino) octyl-3,4,5-trimethoxybenzoate
TNF- α	Tumour necrosis factor-α
tsDC	Thermo sensitive dendritic cell line

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Chapter 1
Introduction

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1 INTRODUCTION

1.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (*Mtb*), commonly known as the "tubercle bacillus" is an Actinomycete and belongs to the group of slow growing Mycobacteria; it divides just once every 14 hours when growing optimally in culture medium. *Mtb* is Gram-positive and although normally aerobic, can adapt to microaerophilic environments. *Mtb* grows at an optimum temperature of 37°C and its morphology can vary, but usually consists of straight or slightly curved rods approximately 1μm in length. Mycobacteria have an unusual cell wall with a high lipid content, which makes them more resistant to the activity of antibacterial agents than most other bacteria. *Mtb* is a facultative intracellular bacterium, since it has the capacity to multiply and survive inside phagocytic cells such as macrophages, adapting to the biochemical and biophysical conditions of its host.

1.1.1 Aetiology and transmission

Mtb causes tuberculosis (TB), a disease that kills 2 million people each year; it has been estimated that between 2000 and 2020, nearly one billion people will be newly infected with *Mtb* (WHO, 2000). These frightening numbers are compounded by the variable efficacy of the vaccine BCG, by the spread of HIV and by the emergence of multidrug-resistant strains of *Mtb*. Situations where social infrastructure and health care systems have broken down create ideal conditions for the spread of *Mtb*, as does the increased migration of people (Lifson, 2000).

TB spreads through the air, in droplets that are inhaled and reach the alveoli of the infected individual. Once infected, most individuals do not develop clinical disease. The infection remains latent for the entire period of the infected individuals life. Occasionally, a latent infection can develop into clinical TB, perhaps as a consequence of diminished immunological function caused by ageing, concurrent infection with another agent such as HIV, or by nutritional factors (Sperber and Gornish, 1992). When this occurs, it is referred to as "reactivation" or "secondary TB". Primary TB, in which clinical symptoms develop shortly after infection, is also more likely to occur in individuals whose immune system is impaired.

Clinical TB is treated with a combination of specific antibiotics, which include rifampicin, isoniazid, ethambutol and pyrazinamide. A complete course of treatment usually lasts for 6 months. If the individual is infected with drug-sensitive *Mtb*, and the appropriate course of treatment is strictly adhered to, the treatment is invariably successful. However, poor compliance or inappropriate treatment regimes increase the risk of relapse and also favour the development of secondary drug-resistance.

Since it is estimated that one third of the world's population is infected with *Mtb* (Sudre *et al.*, 1992), and for the most part this remains as latent, subclinical infection, the development of novel strategies to eliminate latent organisms represents a major challenge for the future of TB control.

1.1.2 *Mtb* as a pathogen of man

Robert Koch identified *Mtb* as the aetiological agent of TB in 1882 (Koch, 1882). However the mechanisms involved in pathogenesis and disease progression of TB have remained unclear, hindering what is now an urgent need for the development of new drugs and vaccines.

Pulmonary tuberculosis usually begins with infection in the alveolar spaces of the lower lungs. *Mtb* is phagocytosed by resident alveolar macrophages and more phagocytes are attracted to the site of infection by inflammatory cytokines and chemokines (Robinson *et al.*, 1994). The accumulation of blood-monocyte derived macrophages at the focus of the infection generates a granuloma, the characteristic "tubercle" of *Mtb* infection (Saunders and Cooper, 2000). In primary TB the granuloma can heal or persist.

As discussed above, latent, viable *Mtb* may persist within the granuloma for many years, and may eventually result in reactivation. Thus *Mtb* is able to survive within the hostile environment of the granuloma macrophages. It is this ability to survive in the hostile environment of the macrophage which most clearly defines *Mtb* as a human pathogen. The mechanisms that contribute to the intramacrophage survival of *Mtb* are discussed in detail in section **1.2.1.1**.

1.1.3 The Mycobacterial cell envelope

The *Mtb* envelope is highly complex, with a high lipid content, which enables the mycobacteria to resist adverse conditions or chemical agents, and probably contributes to survival inside phagocytes. The cell wall skeleton contains peptidoglycan and arabinogalactan with a highly immunologically active molecule, lipoarabinomannan (LAM). The cell wall also contains a layer of long-chain fatty acids, called mycolic acids, which form a permeability barrier to polar molecules - for review see (Daffe and Draper, 1998).

The outer part of the cell envelope, or the "capsule", consists of a mixture of polysaccharides, proteins and lipids. Capsular components are suggested to be involved in the pathogenecity of the tubercle bacillus, contributing, for example to

adhesion and penetration into the host cell and protecting the mycobacteria in the host environment (Daffe and Etienne, 1999).

The mycobacterial envelope is also important as a target of antimycobacterial drugs. Several of the existing drugs target enzymes involved in cell wall synthesis; for example isoniazid targets the inhibition of mycolic acid synthesis (Winder and Collins, 1970) and ethambutol is thought to inhibit the incorporation of glucose into arabinomannan and arabinogalactan (Takayama and Kilburn, 1989). The cell envelope is also important in the transfer of molecules into and out of mycobacteria; the high lipid content creates a permeability barrier. Pore-forming molecules, or porins, are thought to create hydrophilic channels, which allow the access of polar molecules across the cell envelope (Draper, 1998).

The envelope is a dynamic structure in the growing mycobacteria, with molecules moving within and through the envelope. Even the very stable wall skeleton is continuously being reconstructed (Daffe and Draper, 1998).

1.1.3 The *Mtb* genome

The mycobacterial genome, as with most other bacteria, consists of one single, circular chromosome plus other extrachromosomal elements such as plasmids and phages. The mycobacteria belong to the high guanosine/cytosine (C+G) group of Gram-positive bacteria, with a G+C content of 62-70% with the exception of *M.leprae* which has a G+C content of 56% (Clark-Curtiss *et al.*, 1985).

The complete sequence of the genome of *Mtb* strain H37Rv, the most widely used laboratory strain of *Mtb,* has been determined (Cole *et al.*, 1998). Approximately

4,000 genes have been identified; of these approximately 40% have a clearly identifiable function, a further 40% can be identified as belonging to previously identified classes of genes, and 20% are completely unknown.

A number of broad features can be described from this huge amount of genomic information:

(i) *Mtb* has a much broader range of metabolic capabilities than previously thought. This includes the ability to adapt to anaerobic environments, in addition to growing aerobically.

(ii) A significant part of the genome encodes genes involved in lipid synthesis and lipid metabolism.

(iii) *Mtb* has a wide range of mechanisms for different levels of gene regulation. This includes 13 sigma factors, several eukaryotic-like serine/threonine protein kinases, 11 sensor histidine kinases, and more than 100 transcriptional regulator proteins.

(iv) Approximately 10% of the genome encodes a family of closely related genes (termed PGRS sequences). The function of this highly conserved family of genes/proteins is unknown, but a role in antigenic variation (Cole *et al.*, 1998) or virulence (Cole, 1999) has been suggested.

The challenge now is to exploit the genome information to understand the molecular basis of mycobacterial pathogenicity, immunogenicity and immunopathology. A range of post-genomic techniques are now available to facilitate this type of analysis.

Comparative genomics involves the detailed comparison of the genomes of closely related species or strains. In addition to the *Mtb* H37Rv genome, a variety of other mycobacterial genomes are currently being sequenced. These include a recent clinical

isolate of *Mtb*–CDC 1551 (Valway *et al.*, 1998) BCG, *M.leprae*, *M.bovis* and *M.smegmatis;* comparisons of these genomes will provide important clues as to the molecular basis of pathogenicity, host-range, etc.

Targeted gene deletion techniques or random mutagenesis approaches (using transposons, illegitimate recombination or signature-tagged mutagenesis) are now available to investigate genes of unknown function and to identify genes, which are important in pathogenesis (Cole, 1999). Techniques such as proteomics (Rosenkrands *et al.*, 2000) or microarray analysis (Talaat *et al.*, 2000) are available to investigate the global genomic response to different growth conditions; such techniques will be used over the next few years to understand genes which are important for intracellular growth and survival.

1.2 The immune response in tuberculosis

The first line of defence against *Mtb* involves phagocytes of the innate immune system; these cells play a crucial role in the initial control of the infection, and in the subsequent direction of the adaptive immune response. The initial inflammatory response to the invading mycobacteria involves neutrophils, macrophages and dendritic cells which, following attachment of the mycobacteria, engulf the bacteria and release a range of cytokines that influence the behaviour of other cells of both the innate and acquired immune system. This subject will be discussed further in section **1.2.6** .

1.2.1 Invasion of the host cell

The invasion of the host cell- most commonly the macrophage- is not initially a destructive process; *Mtb* is an intracellular pathogen and relies on the macrophage environment for nutrients. At the same time the macrophage responds by producing toxic products aimed at eliminating the infectious agent. This balance can lead to unrestricted cellular growth of *Mtb* on the one hand, or elimination of the infection, on the other. Under some circumstances, a balance is maintained whereby the bacteria can survive without multiplying in the macrophage for several years. This phenomenon is known as "latency" or "dormancy"(see section **1.3**).

1.2.1.1 Phagocytosis and the intracellular fate of *Mtb*

The adherence of *Mtb* to the macrophage is mediated by a variety of receptors, including complement C1, C3 and C4 (Schlesinger *et al.*, 1990), mannose (Schlesinger, 1993) and Fc receptors (Nibbering *et al.*, 1989). Previous work has identified mannose units at the terminal end of LAM as the mycobacterial ligand for the mannose receptor (Schlesinger *et al.*, 1994).

In order to survive the toxic environment of the macrophage following phagocytosis, *Mtb* has evolved a number of strategies. Entrance into the phagocyte by way of the mannose receptor could itself be a survival strategy, in that this has been claimed to avoid generation of toxic superoxide anions (Venkataprasad *et al.*, 1999; Yu *et al.*, 1999).

One of the best-characterized survival strategies is the ability to inhibit phagosomal fusion with lysosomes and hence avoid exposure to toxic lysosomal enzymes. Inhibition of phagosome-lysosome fusion was first described by Armstrong and Hart in 1971 (Armstrong, 1971), and similar strategies have been described for other

intracellular bacteria and parasites such as *Salmonella, Bordatella pertussis and Legionella* (Ishibashi and Arai, 1990; Steed *et al.*, 1991; Hacker *et al.*, 1991). More recently, this process of inhibition of phagosome-lysosome fusion has been shown to be associated with the arrest of phagosome maturation. This is thought to be associated with the failure to acidify the mycobacterium-containing phagosome by exclusion or inactivation of the proton pump ATPase (Sturgill-Koszycki *et al.*, 1994; reviewed by Russell *et al.*, 1997). An alternative or additional mechanism of avoiding phagosome lysosome fusion has recently been described. It has been shown that cholesterol is an essential component of the macrophage for the uptake of *Mtb* (Gatfield and Pieters, 2000). *Mtb* is taken up at cholesterol-rich domains of the macrophage plasma membrane; this ensures that the phagosome is coated with a protein called TACO (tryptophane aspartate-containing coat protein) (Ferrari *et al.*, 1999) TACO-coated phagosomes do not fuse with lysosomes.

It has also been claimed that *Mtb* can avoid exposure to lysosomal enzymes by escaping from the phagosome into the cytoplasm (McDonough *et al.*, 1993) similar to what has been described for *Listeria monocytogenes* (Drevets *et al.*, 1992). However, this has not been confirmed by others.

1.2.1.2 Intracellular killing of *Mtb* by macrophages

As discussed above, *Mtb* avoids exposure to low pH and harmful lysosomal enzymes by blocking maturation of the phagosome. In addition, a variety of other mechanisms are used by macrophages to control the growth of *Mtb*. These include the production of radical oxygen and radical nitrogen intermediates (ROI and RNI respectively), a variety of other antimicrobial molecules, and mechanisms involving apoptosis of the host macrophage. It is likely that such mechanisms act synergistically to control the

intracellular growth of *Mtb*. Oxygen independent antimicrobial mechanisms include the production of bactericidal proteins such as Cathepsin D (Converse *et al.*, 1996; Vishwanath *et al.*, 1997) and defensins (Lichtenstein, 1991), which can damage the mycobacterial membrane. Macrophages can also produce lysozymes that are able to damage the bacterial cell wall, and Lactoferrin (Bezwoda *et al.*, 1985) which complexes with iron, depriving the bacteria of an essential growth element. The production of RNI, which are generated during the conversion of arginine to citrulline is an important antimycobacterial mechanism (Figure 1.1); mice which lack inducible nitric oxide synthase (iNOS) or in which the RNI pathway is inhibited, are extremely susceptible to infection with *Mtb* (Chan *et al.*, 1992).

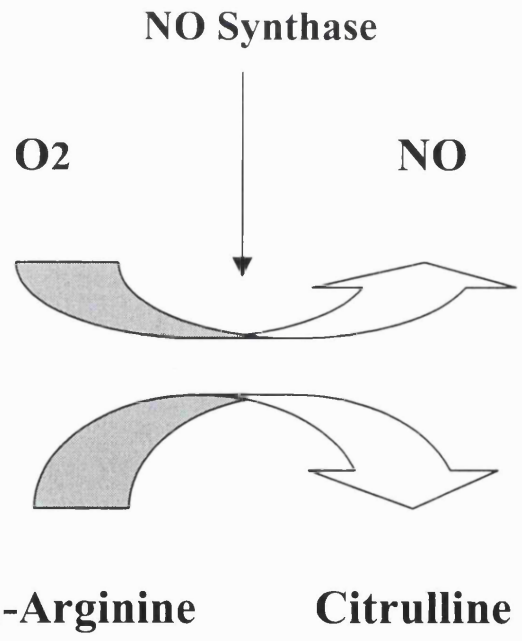

Figure 1.1 – The production of nitric oxide via the L-arginine pathway

Oxygen dependent mechanisms of killing mycobacteria involve the formation of reactive oxygen intermediates (ROI) such as H_2O_2, superoxide anions and

myeloperoxidase; these ROI can cause lipid peroxidation and damage to the cell membrane. They can also damage DNA and proteins, and in some cases alter the phagosomal pH, although it has been suggested that such mechanisms are unlikely to be significantly involved in killing *Mtb* (Chan *et al.*, 1992).

Free fatty acids (FFA) found in macrophages, such as arachidonic acid, display strong anti-*Mtb* activity *in vitro*. This activity of FFA is synergistic with the activity of RNI and with the H_2O_2-halogenation system, reinforcing the fact that many of these antibactericidal activities of macrophages act in concert and are not mutually exclusive (Akaki *et al.*, 2000). Furthermore, as mentioned above, the failure to produce ROI and RNI results in enhanced infections in mice. Mice that lack a functional subunit of the phagocyte cytochrome b are unable to produce ROI and mice which are deleted for the iNOS inducible gene fail to produce RNI, resulting in the enhanced intracellular growth of *Mtb* (Adams *et al.*, 1997).

Infection with *Mtb* can induce programmed cell death or apoptosis of macrophages by pathways not necessarily dependent on NO production (Rojas *et al.*, 1998). The role of macrophage apoptosis in controlling growth or killing of *Mtb* is unclear. Some groups have claimed that apoptosis of cells infected with intracellular pathogens may benefit the host by eliminating a supportive environment for bacterial growth (Keane *et al.*, 2000), or by preventing the spread of the pathogen by sequestration in apoptotic bodies (Fratazzi *et al.*, 1999). On the other hand, Mustafa and colleagues (Mustafa *et al.*, 1999) claim that apoptosis *via* the Fas-Fas ligand pathway constitutes a novel evasion mechanism for *Mtb* because it protects the macrophage from attack by cytotoxic T cells and the activation of bactericidal mechanisms by Th1 lymphocytes. There is also a link between apoptosis and RNI production, via the Nramp 1 gene

(Rojas *et al.*, 1997). Nramp 1 is thought to be involved in the control of NO production in response to macrophage activation, and apoptosis was found to correlate with NO-production, suggesting that apoptosis may be a critical mechanism by which Nramp 1 controls mycobacterial growth in macrophages (Rojas *et al.*, 1997). It is now clear that the mechanism for inducing apoptosis is important in determining the outcome for the mycobacteria. Lammas and colleagues (Lammas *et al.*, 1997) compared the fate of intracellular BCG when macrophages were induced to apoptose by complement-mediated lysis, Fas ligation, CD69 ligation and by addition of exogenous ATP. Only ATP was found to induce both cell death and killing of the BCG, supporting earlier findings with BCG (Molloy *et al.*, 1994) and *M.avium* (Laochumroonvorapong *et al.*, 1996). This ATP-induced killing of mycobacteria by macrophages has been shown to be mediated by the pore-forming purinergic receptor, P2X7 (Lammas *et al.*, 1997). The availability of P2X7 knockout mice will establish the importance of this mechanism in the *in vivo* infection.

Proteins released by CD8+ T cells can also cause lysis of the *Mtb* infected macrophage by the release of perforins and granzyme (Stenger *et al.*, 1997). Granulysin directly kills extracellular *Mtb*, altering the membrane integrity of the bacillus and in combination with perforin decreases the viability of intracellular *Mtb* (Stenger and Modlin, 1998); recent work illustrates that this intracellular CTL-mediated killing of *Mtb* is independent of nuclear DNA degradation (Thoma-Uszynski *et al.*, 2000).

1.2.2 The role of the macrophage in initiating immune responses

As discussed above, the macrophage acts as the host cell in which *Mtb* can survive and multiply, or be killed as a result of immune mechanisms. In addition, the macrophage

plays a key role in initiating both innate and acquired immune responses. It is clear that, immediately following infection with *Mtb*, macrophages release a wide range of inflammatory cytokines and chemokines. Among proinflammatory cytokines, IL-1β (Wilkinson *et al.*, 1999), TNF-α (Rojas *et al.*, 1999; Manca *et al.*, 1999) and IL-12 (Ladel *et al.*, 1997) have been shown to be upregulated in *Mtb*-infected macrophages.

A wide range of chemokines and proteins involved in cell migration have been reported as being produced by macrophages following infection with *Mtb*. Thus the chemokine IL-8 is produced by human alveolar macrophages (Pace *et al.*, 1999), the monocyte cell line THP1 (Friedland *et al.*, 1993) as well as bronchial epithelial cells (Wickremasinghe *et al.*, 1999) and polymorphonuclear granulocytes (Riedel and Kaufmann, 1997). Other chemokines which have been reported to be upregulated in *Mtb*-infected macrophages include osteopontin (or ETA-1; (Nau *et al.*, 1997)), MCP-1 (Lin *et al.*, 1998; Sadek *et al.*, 1998; Friedland *et al.*, 1993), MIP-1α (Sadek *et al.*, 1998; Kasahara *et al.*, 1998) and RANTES (Sadek *et al.*, 1998), Thus it would appear that one of the major roles of macrophages following *Mtb* infection is to mobilise and recruit a wide range of other immunoregulatory and effector cells to the site of infection.

In addition to the release of cytokines and chemokines which can orchestrate the cellular response, macrophages are also important as antigen presenting cells (Unanue, 1984) Although dendritic cells (DC) are the most efficient and effective antigen presenting cells (see section 1.5.1), macrophages also process and present antigen in association with MHC Class I and II molecules, to T cells. Other accessory molecules such as the costimulatory molecules B7 and the adhesion molecule ICAM-1 are expressed in stimulated macrophages (Razi-Wolf *et al.*, 1992; Damle and Aruffo, 1991; Van Seventer *et al.*, 1990).

Interestingly, a number of studies have suggested that macrophages which are infected with *Mtb* have reduced antigen presenting capabilities (Gercken *et al.*, 1994; Pancholi *et al.*, 1993) either by inhibiting expression of MHC Class II molecules (Hmama *et al.*, 1998) or other antigen presenting molecules (Stenger and Modlin, 1998), or by inhibiting antigen processing (Noss *et al.*, 2000). Thus *Mtb* may evade recognition by specific T cells by reducing the antigen presenting capability of the cell in which it resides.

1.2.3 The role of CD4+ T lymphocytes

Cell-mediated immunity plays the key role in containing mycobacterial infection. The absolute requirement for CD4+ T cells has been demonstrated in mice (North, 1973; Orme *et al.*, 1992; Colston and Hilson, 1976; Tascon *et al.*, 1998) and in man by the increased susceptibility of AIDS patients to a wide range of mycobacterial infections, eg. (Hopewell, 1992). Selective depletion of T cell subsets, using monoclonal antibodies, have shown that depletion of CD4+ T cells markedly shortened survival of *Mtb*-infected mice (Leveton *et al.*, 1989).

One of the key roles of CD4+ T cells is to respond to specific antigens by producing cytokines, which activate, and increase the microbicidial capacity of macrophages. The main cytokine in this regard is IFNγ. IFNγ has been shown to induce antimycobacterial activity in macrophages *in vitro* (Rook *et al.*, 1986; Flesch and Kaufmann, 1987), and mice with a targeted deletion in the IFNγ gene are less able to control *Mtb* infections than normal mice (Cooper *et al.*, 1993; Flynn *et al.*, 1993). The activation of human macrophages to kill *Mtb* by IFNγ has been much more difficult to demonstrate (Rook *et al.*, 1986; Douvas *et al.*, 1985). However the recent demonstration that people with genetic defects in the IFNγ pathway are more

susceptible to mycobacterial infections (Lammas *et al.*, 2000; Doffinger *et al.*, 1999; Jouanguy *et al.*, 1999) suggests that this pathway is also important in man.

Although the main role of CD4+ T cells is thought to be to provide cytokine-mediated help to macrophages, CD4+ T cells have also been shown to have cytotoxic activity towards mycobacteria-infected macrophages (Ottenhoff and Mutis, 1990; Lorgat *et al.*, 1992; Tsuyuguchi, 1990; Fazal *et al.*, 1995; Tan *et al.*, 1997). However the role of this activity *in vivo* is not understood.

1.2.4 The role of CD8+ T lymphocytes

Although CD4+ T cells are thought to play the major role in controlling mycobacterial infections, there has been increasing evidence in recent years of the importance of CD8+ T cells. The presence of mycobacteria specific CD8+ T cells in humans has been widely reported (see for example (Lalvani *et al.*, 1998; Lewinsohn *et al.*, 1998; Bothamley *et al.*, 1992b; Smith and Dockrell, 2000; Smith *et al.*, 1999). However it is work in mice which has provided compelling evidence for an important role in protective immunity. Cell depletion studies indicated the involvement of CD8+ T cells in control of *Mtb* infection in a murine model (Orme, 1987). Mice deficient in β-2-microglobulin showed increased susceptibility to tuberculosis, indicating a role for MHC Class I-restricted cells (Flynn *et al.*, 1992). This was further supported by the demonstration that mice which lack the TAP1 molecule, which is involved in transport of processed peptides from the cytosol to the endoplasmic reticulum for loading onto MHC Class I molecules, are also more susceptible to *Mtb* infection (Behar *et al.*, 1999).

CD8+ T cells could contribute to the control of *Mtb* infection in one of two ways; either through cytotoxic mechanisms, or through T-helper activity by producing

macrophage-activating cytokines. Support for the importance of the latter is provided by a number of studies. Transfer of both CD4+ and CD8+ T cells into *Mtb*-infected athymic mice, provides equivalent levels of protection; however, transfer of CD8+ T cells from mice with a targeted deletion in the IFNγ gene is unable to protect (Tascon *et al.*, 1998), suggesting that at least in this model, IFNγ production is the major role of CD8+ T cells. This is supported by the fact that mice which lack cytotoxic T cell mechanisms are little different from wild-type mice in their ability to control *Mtb* (Laochumroonvorapong *et al.*, 1997; Cooper *et al.*, 1997; Sousa *et al.*, 1999). On the other hand, although in *Mtb* infected mice both CD4+ T cells and CD8+ T cells are found in the lungs as early as one week post-infection, recent data show that the majority of IFNγ produced is by CD4+ T cells (Serbina and Flynn, 1999).

In addition to classical MHC class I-restricted CD8+ T cells, a number of other types of CD8+ T cells which are not MHC Class I-restricted, have been demonstrated. Some CD8+ T cells recognize non-peptide antigens of *Mtb* in the context of CD1 molecules (Stenger *et al.*, 1997; Gong *et al.*, 1998). An additional class of CD8+ T cells recognize antigen when presented by MHC Class Ib molecules other than CD1 (Lewinsohn *et al.*, 1998). The murine equivalent of MHC Class Ib molecules are termed H2-M3, which present bacterial N-formyl peptides (Lindahl *et al.*, 1997). H2-M3-restricted CD8+ T cells with specificity for mycobacterial N-formyl peptides have been reported (Flynn and Ernst, 2000).

Although the role of cytotoxicity in controlling *Mtb* infections is unclear, recent evidence has suggested that killing of *Mtb* infected macrophages can result in concurrent death of the bacteria. CD8+ T cells which kill *Mtb*-infected target cells do so by granule exocytosis, in contrast to $CD8^{-}CD4^{-}$ double negative (DN) cytotoxic cells which utilise a Fas-FasL pathway (Stenger *et al.*, 1997). CD8+ T cell-killing of

infected targets involves the concerted action of perforin and granulysin, and results in killing of the intracellular microbe in addition to the target cell. By contrast DN cells, using the Fas-FasL pathway kill the target cell, but do not kill the intracellular microbes (Stenger *et al.*, 1997). Thus it is possible that CD8+ T cells can contribute to protection against *Mtb* infection by the granulysin/perforin pathway.

1.2.5 The role of other T cell subsets

In addition to macrophages, CD4+ and CD8+ T cells, many other cell types contribute to the recognition of and response to *Mtb*. A range of minor T cell subsets which often recognise unusual mycobacterial products have been identified. For example T cells expressing the γδ T cell receptor, and expressing both cytotoxic and helper activities have been shown to recognise either heat shock proteins (Boom *et al.*, 1992; O'Brien *et al.*, 1989) or phosphorylated bacterial products (Constant *et al.*, 1994; Schoel *et al.*, 1994). It has been suggested that γδ T cells are involved in the early, innate arm of the immune response to infection (Skeen and Ziegler, 1993).

DN CD1 restricted T cells recognise mycolic acids (Beckman *et al.*, 1994) or LAM (Sieling *et al.*, 1995), both major constituents of the mycobacterial cell envelope. These cells are capable of producing IFNγ and expressing cytotoxic activity (Porcelli *et al.*, 1992; Beckman *et al.*, 1994), via the Fas-FasL pathway (Stenger *et al.*, 1997).

1.2.6 The importance of cells other than macrophages and T cells

In addition to macrophages and T cells, a number of other cell-types are thought to play a role in *Mtb* infection.

Natural killer (NK) cells are involved in the early response to *Mtb* infection; they have elevated lytic activity against *Mtb*-infected monocytes following IL-2 or IL-12

stimulation (Denis, 1994) and also produce IFNγ (Ye *et al.*, 1995). Antibody-mediated depletion of NK cells in mice makes them more susceptible to *M.avium* infection, confirming an important role in the control of intracellular pathogens (Harshan and Gangadharam, 1991).

Neutrophils, phagocytes usually associated with protection against extracellular parasites, also play an important role in *Mtb* infection. In rabbits neutrophils release chemotaxins, which attract monocytes to the site of inflammation contributing to granuloma formation following mycobacterial infection (Antony *et al.*, 1985). These cells were also found to directly eradicate *Mtb* by oxidative killing (H_2O_2) or by a combination of components with microbicidial activity (H_2O_2, MPO and Cl^-) (Brown *et al.*, 1987). Also, during mycobacterial infection, T cells and macrophages are primed to recruit neutrophils, which may represent a way to cope with secondary infections (Appelberg, 1992). Depletion of neutrophils completely abrogates the resistance conferred by activated macrophages induced during mycobacterial infection (Leal *et al.*, 1996), and has a direct effect on bacterial proliferation (Pedrosa *et al.*, 2000). Most important of all is the immunomodulatory activity of these cells: they are capable of producing cytokines such as: TNF-α, IL-12 and IL-1β (Petrofsky and Bermudez, 1999), and hence contributing to the global cellular response to infection.

B-lymphocytes initially were considered to have no role in the control of the *Mtb* infection. Subsequently studies indicated that B-cell deficient mice, unable to produce antibodies of any class, were more susceptible to *Mtb* infection (Vordermeier *et al.*, 1996). However mice that are deficient in the production of B lymphocytes have severe lesion formation and delayed bacterial dissemination which was found to be B-cell dependent but not antibody-dependent (Bosio *et al.*, 2000). In fact the role of the antibodies in the *Mtb* infection is still poorly understood. Recent work on

mycobacterial components have shown that the administration of LAM to mice caused an increase in the levels of IgM and could modify the course of the mycobacterial infection (Glatman-Freedman *et al.*, 2000).

1.2.7 The cytokine network

Cytokines secreted by monocytes/macrophages following initial contact with *Mtb* play a role in determining the ultimate evolution of the immune response and are responsible for many of the clinical manifestations of tuberculosis (IL-1β and TNF-α producing fever and wasting, for example). The inflammatory response and production of chemokines was discussed in detail earlier (see section **1.2.2**). It is this cytokine milieu, provided by both macrophages and dendritic cells (DC; see section **1.5.1**) which is largely responsible for the types of T cells which are recruited to the site of infection and for the phenotypic differentiation of these T cells. For example IL-15 is thought to induce CD8+ memory T cells (Lodolce *et al.*, 1998; Kennedy *et al.*, 2000) and is induced in macrophages by mycobacteria (Doherty *et al.*, 1996). Similarly IL-12 and IL-18 are produced by macrophages and are important in generating protective immunity against mycobacteria (Cooper *et al.*, 1995; Sugawara *et al.*, 1999) through their stimulation of IFNγ-production (Kohno *et al.*, 1997; Matsui *et al.*, 1997 ;Okamura *et al.*, 1995).

As discussed earlier, the major contribution to protective immunity is through helper T cells (both CD4+ and CD8+) producing cytokines, which activate macrophages, particularly IFNγ. Such T cells can be subdivided on the basis of the range of cytokines they produce; thus IFNγ is produced by Th1 cells, IL-4, IL-10 and IL-13 by Th2 cells, while Th0 cells can have an intermediate phenotype (Mosmann and Coffman, 1989; Mosmann *et al.*, 1986). However, there is considerable feedback, both

positive and negative, between Th1, Th2 and macrophage cytokine production. Thus, IL-10 and IL-4, produced by Th2 cells suppress Th1 cells, while IFNγ, produced by Th1 cells, suppresses Th2 cells. A gross oversimplification of the pathway leading to protective immunity would be: macrophages produce IL-12, IL-15 and IL-18 which stimulates IFNγ production by Th1 cells; IFNγ suppresses Th2 cells and activates macrophages to become microbicidial and to produce more IL-12, etc. TNF-α, which can be produced by macrophages, Th1 and Th2 cells, is important in granuloma formation (Kindler *et al.*, 1989) and in macrophage activation (Bermudez and Young, 1988) but is also responsible for much of the immunopathology associated with tuberculosis (Kaplan, 1994), emphasising the balance between protective immune mechanisms and immunopathology.

1.3 Reactivation of persistent infection: latent tuberculosis

Mtb is able to survive for long periods within macrophages or infected hosts; even if infected individuals control the infection they may not eliminate all viable mycobacteria, and in this case the tubercle bacillus is said to be in a state of dormancy. The ability to survive in the host in a latent state, only to revive years later is an important component of pathogenicity and has been the subject of investigation for many years, using model systems.

One such system involves the hypothesis that dormant bacilli shift from a O_2 –rich environment to an environment where O_2 is limited.; it is thought that this involves a first step from rapid to slow replication of the mycobacteria and a second step with complete shutdown of replication, but not death (Wayne, 1994). An explanation for this phenomenon is that nitrate reduction actually supports the hypoxic shiftdown of *Mtb*; this response may help the bacilli to survive in oxygen-depleted regions of

inflammatory or necrotic tissues, where nitrate can occur as a degradation product of nitric oxide (Wayne and Hayes, 1998). The genome sequencing project has revealed that *Mtb* has the genetic potential to survive in anaerobic conditions (Cole *et al.*, 1998).

It has also recently been shown that the pattern of cytokines produced by T cells from mice with latent TB and reactivated TB is different: reactivation of latent TB was associated with a shift in cytokines from a type 1 (Th1) to a type 2 (Th2) cytokine response; control of mycobacterial growth resulted in a return to the type 1 cytokine pattern found during latent disease (Howard and Zwilling, 1999).

Reactivation of clinically latent infection is responsible for a large proportion of active tuberculosis cases: one cause of reactivation is the development of immunossupression resulting from HIV infection (Murray, 1991) suggesting a role of the CD4+ T cells in dormancy maintenance (Di Perri *et al.*, 1993). In mice, antibody–mediated depletion of CD4+ T cells results in rapid reactivation of a persistent infection (Scanga *et al.*, 2000). It has also been found that corticosteroid therapy can reactivate latent *Mtb* infection (Sperber and Gornish, 1992).

1.4 BCG vaccination

The BCG vaccine, an attenuated form of the bovine tubercle bacillus, is widely used but has extremely variable efficacy in protecting against disease, in different parts of the world (reviewed by (Fine, 1995)). The reason for this variation (from 78% efficacy in the UK, to 0% in Malawi, for example (Fine, 1995) is unknown, but probably involves multiple factors. It has been suggested for example that exposure to environmental mycobacteria provides a level of background protection against *Mtb* which masks any protective effect conferred by BCG (Fine *et al.*, 1994). Alternatively

exposure to some particular environmental mycobacteria might subvert the protective immune response conferred by *Mtb* (Stanford and Rook, 1983). In most trials BCG has been found to be effective in childhood, extrapulmonary forms of the disease, but in many cases it is ineffective over the long periods required for a disease which can remain dormant for many years. Thus, although BCG is inexpensive, safe and widely used, there is an urgent need for the development of new vaccines that eradicate already established infection and protect against primary infection (Hess and Kaufmann, 1999).

1.4.1 New approaches for the development of alternative vaccines

-Improvement of BCG efficacy by manipulating the host response at the time of vaccination and promoting optimal protective responses.

BCG has been genetically engineered to secrete cytokines that can influence the generation of a Th1 response. Such cytokines include IFNγ, GM-CSF, TNF-α, IL-12 and IL-2 (Marshall *et al.*, 1997; Murray *et al.*, 1996; Kong and Kunimoto, 1995). More recently BCG vaccination of mice has been enhanced by the simultaneous administration of IL-12, and immunostimulatory DNA CpG motifs (Freidag *et al.*, 2000).

-Development of new attenuated forms of *Mtb*

Recent advances in mycobacterial molecular genetics have allowed a more targeted approach to the development of attenuated *Mtb*. Thus, for example several groups have produced auxotrophic mutants of *Mtb* which are unable to infect mice; some show protective responses if used to vaccinated mice which are then challenged with *Mtb* (Jackson *et al.*, 1999; Hondalus *et al.*, 2000). Alternative strategies involve the

use of random mutagenesis and signature target transposon mutagenesis (Camacho *et al.*, 1999) which allows the avirulent mutant to be selected for in the mouse.

-Development of subunit vaccines

The use of purified proteins, either recombinant or native proteins, was investigated as a potential approach to developing new vaccines (Table 1.1). Proteins which appear early in the filtrate of *Mtb* cultured in broth medium have been shown to elicit strong immune responses in BCG-vaccinated mice (Boesen *et al.*, 1995), and to protect guinea pigs (Pal and Horwitz, 1992) and mice (Andersen, 1994). Some of the specific proteins found in these culture filtrates have been identified as having protective effects, most notably the 85 antigen (Horwitz *et al.*, 1995). The success of the subunit proteins as vaccines is dependent on the use of appropriate adjuvants, some of which can particularly promote Th1 responses. It is not yet clear whether a subunit vaccine of this type is capable of generating the long-lasting immunity required for protection against *Mtb*.

-Development of DNA vaccines

Following the initial observations that inoculation of a DNA plasmid into a host, with subsequent expression of the encoded protein *in vitro* results in immune responses to that protein (Tang *et al.*, 1992); many studies have demonstrated this to be a promising approach to vaccination against viral (Ulmer *et al.*, 1993; Xiang and Ertl, 1994; Boyer *et al.*, 1997; Webster *et al.*, 1994), parasitic (Xu *et al.*, 1995) and bacterial, including mycobacterial, infections (Huygen *et al.*, 1996; Tascon *et al.*, 1996). Intramuscular injection of DNA plasmids results in expression of the encoded protein in myocytes (Wolff *et al.*, 1990) and keratinocytes (Akbari *et al.*, 1999) from where it is picked up and transported to lymphoid tissue by antigen presenting cells (Akbari *et al.*, 1999). DNA vaccination has been shown to be an excellent method for generating both helper

Name	kDa	Localization	Functions	References
DnaK	70	Somatic/ extra cellular	hsp	(Garsia *et al.*, 1989)
GroEl	65	Somatic	hsp	(Shinnick, 1987)
Urease	62	Somatic	Nitrogen metabolism	(Clemens *et al.*, 1995)
Glutamine Synthetase	58	Somatic/extra cellular	Cell wall synthesis	(Harth *et al.*, 1994)
Proline Rich Complex (APA)	45-47	Extracellular	Target for DTH and antibody responses after immunization	(Laqueyrerie *et al.*, 1995)
Phosphate Binding protein	38	Outer cell wall	Phosphate Metabolism Target for T-cell responses	(Andersen *et al.*, 1990)
Antigen 85 complex	30-32	Extracellular	Fibronectin binding/T cell target	(Abou-Zeid *et al.*, 1988)
HBHA (Heparin Binding Hemaglutinin)	28	Outer cell wall	Adherence to cell surfaces	(Abou-Zeid *et al.*, 1988; Menozzi *et al.*, 1996)
MPT 64	26	Extracellular	Deleted in some strains of BCG/potent inducer of DTH	(Wilcke *et al.*, 1996; Johnson *et al.*, 1999)
Superoxide dismutase	28	Somatic/extracell ular	Cleaves toxic superoxide radicals	(Thangaraj *et al.*, 1990)
19 kDa Lipoprotein	19	Outer cell wall	Phosphate uptake?????	(Bothamley *et al.*, 1992a)
α- crystallin	16	Somatic/Extracell ular	hsp	(Wilkinson *et al.*, 1998)
GroES	12	Somatic/Extracell ular	hsp	(Baird *et al.*, 1988)
ESAT-6	6	Extracellular	Deleted in all strains of BCG/ target for memory cells	(Sorensen *et al.*, 1995)

Table 1.1 - Major protein antigens of *M.tuberculosis*

and cytotoxic immune responses, which probably accounts for its success in experimental studies against a wide range of infectious agents. Whether this success in experimental studies can be converted into clinical use, and issues involving the long-term safety of DNA vaccination can be overcome, remain to be seen.

1.5 Antigen presentation and dendritic cells (DC)

Antigen presenting cells express both MHC Class I and II molecules and are hence capable of presenting antigen to both CD8+ and CD4+ T cells. The APC convert, or process antigens into short peptides. These short peptides are bound to proteins of the MHC class I and II complex and presented at the cell surface of the APC where, together with costimulatory molecules, they initiate the immune response through interactions with T cells. The APC vary in the mechanisms of antigen uptake, expression of MHC class II molecules, costimulatory molecules and localisation in the body. The major types of APC are dendritic cells (DC), macrophages and B cells.

DC are the most potent APC, with the ability to induce primary immune responses (Steinman, 1991; Hart, 1997). In addition to processing and presenting antigen to T cells, DC play important roles in the sensing and capture of antigen in peripheral tissue, in the transport of captured antigen to T cell-rich lymphoid tissue and in providing a cytokine milieu which influences the differentiation of activated T cells. In the following section these roles of DC will be considered in more detail.

1.5.1 DC subsets

DC can be derived from lymphoid (Vremec and Shortman, 1997 ; Leenen *et al.*, 1998 ; Hart, 1997; Saunders *et al.*, 1996) or myeloid tissue (Inaba *et al.*, 1992). These two lineages of DC can be differentiated by the expression of a number of cell surface markers. Murine DC of lymphoid origin express CD8α, DEC-205, with low level expression of CD11b. Murine myeloid DC do not express CD8α or DEC-205, but express high levels of CD11b (reviewed by (Reid *et al.*, 2000)) Human DC can also be

generated from lymphoid or myeloid tissue (Grouard *et al.*, 1997; Rissoan *et al.*, 1999b; Caux *et al.*, 1996; Romani *et al.*, 1994).

DC can also be distinguished on the basis of their tissue localisation. These include skin epidermal DC (Langerhans cells), thymic, liver and peripheral blood DC, germinal centre DC, T-zone interdigiting DC and splenic marginal DC. Although DC from different tissues can, to some extent, be differentiated phenotypically, differences in ontogeny, function and maturation are not clear (Table 1.2).

DC type	Function
Peripheral blood DC	Migration
Spleen DC	Antigen presentation to primed T cells
Langerhan DC	Antigen uptake and transport to Lymph nodes
Interdigiting DC	Antigen presentation
Thymic DC	Induction of in vivo proliferation of mature thymocytes
Dermal dendrocytes	????????
Follicular DC	Antigen presentation in B cells and memory maintenance

Table 1.2- Main categories of DC and functions

In addition to lineage and tissue localisation DC subsets can also be differentiated on the basis of function. DC provide the environment in which T cells differentiate into for example Th1 and Th2 cells. These different environments are largely dependent on

IL-12 secretion (Macatonia *et al.*, 1995). CD11c+ DC can produce IL-12 (Rissoan *et al.*, 1999b) and drive both CD4+ (Rissoan *et al.*, 1999b) and CD8+ and NK T cells towards a Th1 phenotype (Rissoan *et al.*, 1999a). In mice, the response towards Th1 or Th2 may be differentiated by expression of CD8α, with CD8α+ DC reflecting lymphoid origin, production of IL-12 and promotion of Th1 differentiation (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999; Pulendran *et al.*, 1998), and CD8α- DC, reflecting myeloid origin and promotion of Th2 differentiation (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999), possibly by selectively expressing IL-13 (McKenzie *et al.*, 1998) and IL-6 (Rincon *et al.*, 1997). Although distinct DC subsets may be responsible for promoting Th1 or Th2 responses, it is also clear that the cytokine milieu of DC can also influence their ability to promote polarisation of the T cell response (Buelens *et al.*, 1995; Caux *et al.*, 1994; Liu *et al.*, 1998). The tissue localisation of DC can also influence their ability to promote Th1 or Th2 responses, with splenic DC tending to induce Th1 differentiation and DC from Payers Patches inducing Th2 differentiation (Iwasaki and Kelsall, 1999).

1.5.2 Differentiation of DC *in vitro*

The ability to grow DC *in vitro*, and to control the differentiation of different lineages has greatly assisted the analysis of DC function in recent years. Human DC can be grown from peripheral blood or bone marrow by culturing with IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994; Romani *et al.*, 1994). Such *in vitro* grown DC exhibit characteristic morphological features and different immunophenotypes (Egner and Hart, 1995; reviewed by Gluckman *et al.*, 1997). DC generated from adherent peripheral blood-derived monocytes in the presence of cytokines such as IL-4, display a similar morphology, phenotype and immunostimulatory activity to bone-marrow

derived DC (Schreurs *et al.*, 1999). DC differentiated from monocytes, in this way, and further maturing them with superantigens can give rise to two functionally diametrically opposite types of DC, one stimulatory and the other inhibitory (Chakraborty *et al.*, 2000). The shift from monocyte to DC during culture is associated with changes in expression of surface markers; the first 24 hours is marked by the rapid upregulation of markers of monocyte activation (CD13, CD14, CD98 and HLA-DR), followed by CD14 down-regulation and CD1 and CD86 up-regulation between days 2 and 3. Ultimately there is a gradual increase of DC markers such as CD18 and CD43 (Woodhead *et al.*, 1998).

1.5.3 DC migration and maturation

In vivo, DC exist at different functional and morphological stages of maturation; as immature cells distributed throughout the tissues in non-lymphoid organs, where they exert a sentinel function, and as mature cells when they acquire and process antigen, migrating to the T cell-dependent areas of secondary lymphoid organs and interacting physically with T lymphocytes (Cumberbatch *et al.*, 1991). Immature DC in the peripheral tissue arise by migration from the bone marrow to become resident cells. These immature DC are specialised as " antigen capture" cells. They can acquire antigen by a variety of pathways, including macropinocytosis (Sallusto *et al.*, 1995; Steinman and Swanson, 1995), receptor-mediated endocytosis via for example the mannose receptor DEC-205 (Engering *et al.*, 1997; Jiang *et al.*, 1995; Sallusto *et al.*, 1995; Tan *et al.*, 1997) and phagocytosis. Phagocytosis by immature peripheral DC is clearly important for immunity to pathogens. Latex beads (Matsuno *et al.*, 1996), bacteria (Inaba *et al.*, 1993; Rescigno *et al.*, 1999) and higher parasites such as Leishmania (Moll, 1993; Moll *et al.*, 1995), have all been shown to be internalised by

immature DC. Immature DC can also phagocytose apoptosing and necrosing cells, and this is thought to be important in "cross-priming" of T cells (Albert *et al.*, 1998a; Albert *et al.*, 1998b; Rubartelli *et al.*, 1997; Inaba *et al.*, 1993); the cross-priming of T cells is discussed in more detail below. In addition to exposure to antigens, maturation of immature peripheral DC can also be triggered by a wide range of molecules, including molecular products of pathogens such as LPS (Roake *et al.*, 1995; Rescigno *et al.*, 1999), CpG DNA motifs (Hacker *et al.*, 1998; Hartmann *et al.*, 1999) and double stranded RNA (Cella *et al.*, 1999) (Figure 1.2).

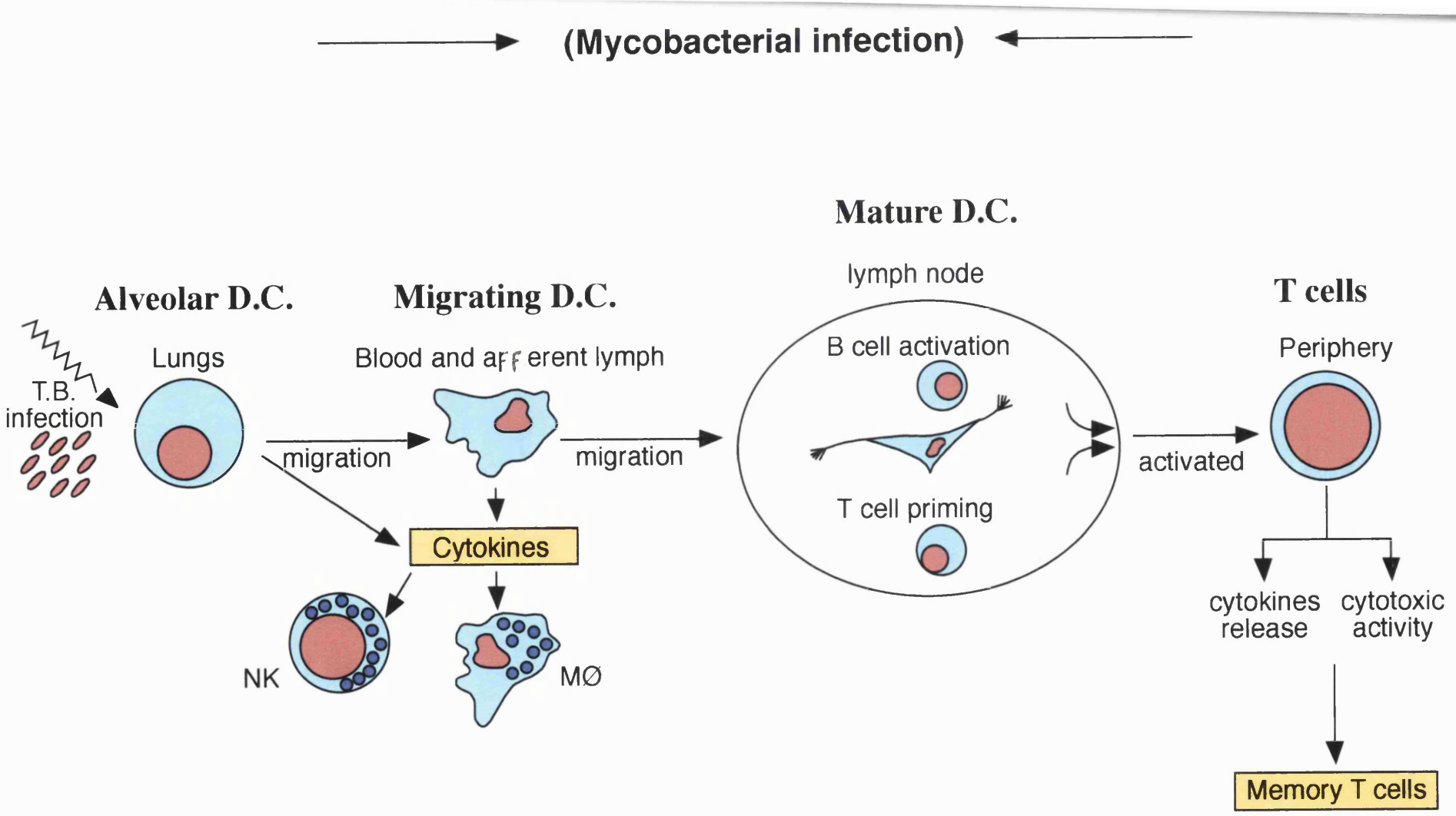

Figure 1.2 Model of *Mtb* / DC interactions and T cell immune response

The cytokine milieu of the immediate environment can also stimulate maturation of peripheral DC. Maturation is associated with marked phenotypic changes in the DC. Morphologically, DC undergo cytoskeleton remodelling to become larger, with an increase in the number of dendritic projections (Ross *et al.*, 1998). Maturing DC down-regulate receptors involved in antigen capture and upregulate costimulatory molecules such as CD86, CD80, CD58 and CD40 (Watts, 1997). The high levels of intracellular MHC Class II molecules which are present in immature DC are now expressed at the surface. Immature DC contain Class II-rich organelles caled MIICs (Nijman *et al.*,1995; Kleijmeer *et al.*, 1997). Antigen is directed to MIICs (Lutz *et al.*, 1997) for binding to MHC Class II molecules and translocation to the cell surface. Thus antigen or cytokine mediated maturation results in further differentiation of DC from an immature, antigen-capturing cell towards a mature, antigen-presenting cell (Figure 1.3).

This maturation procedure is also associated with a change in chemokine receptor expression (Sallusto *et al.*, 1998; Dieu *et al.*, 1998), with down- regulation of the MIP 3α receptor and upregulation of the MIP 3β receptor CCR7, thereby promoting the migration of mature DC into the draining lymph nodes via the afferent lymph. The expression of MIP 3β and 6Ckine results in the amplified attraction of both mature DC and naïve T cells, thereby assuring that the conditions for antigen presentation are optimal. Mice deficient for 6C kine or CCR7 exhibit aberrant migration of T cell and DC homing into lymph nodes (Gunn *et al.*, 1999; Nakano *et al.*, 1997; Nakano, 1998).

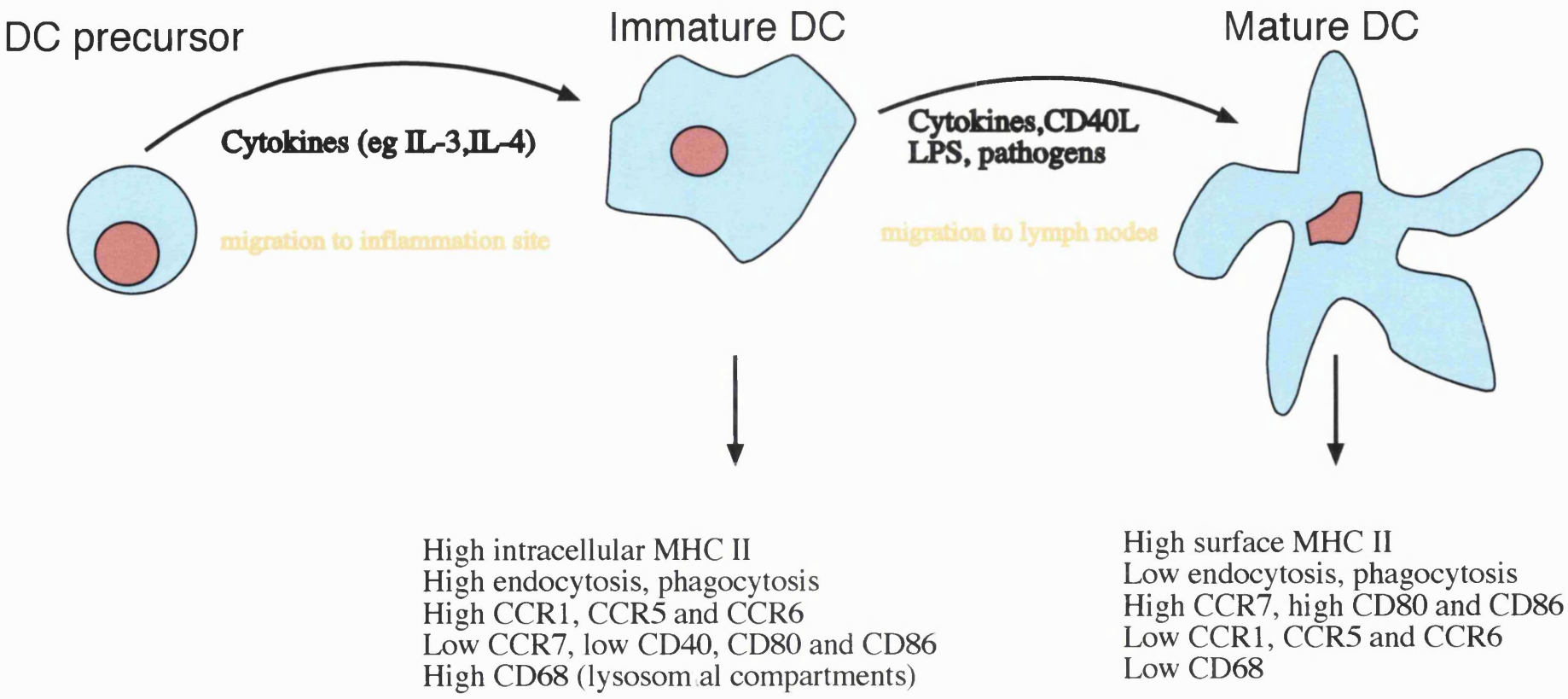

Figure 1.3 - Major property changes occurring during DC maturation

1.5.4 Interactions with T cells

DC present antigen to naïve CD4+ T cells in association with MHC Class II molecules, and to naïve CD8+ T cells in association with MHC Class I molecules. This unique ability to prime naïve T cells is probably associated with the very high concentration of MHC-peptide complexes expressed on the cell-surface (Inaba *et al.*, 1997), and with the increased expression of adhesion molecules (see above). Recently a novel protein, referred to as DC-SIGN, has been shown to interact with ICAM-3, thereby establishing the contact between DC and naïve T cells (Geijtenbeek *et al.*, 2000). DC-SIGN is specific to DC (DC-Specific ICAM-3 Grabbing Nonintegrin) and hence may be the key molecule in defining the unique ability of DC to stimulate naïve T cells. CD86 expressed on DC is crucial for amplification of T cell responses (Caux *et al.*, 199; Inaba *et al.*, 1994), and cytokine production following T cell activation both amplifies and directs the nature of the T cell response (discussed previously in sections **1.2.7** and **1.5.1**). The key event in activating DC to regulate the CD86 and cytokine production following interaction with T cells, is mutual signalling through CD40 (on DC) and CD40-ligand on T cells (Bennett *et al.*, 1998; Schoenberger *et al.*, 1998).

1.5.5 Cross-priming of CD8+ T cells

DC can prime naïve CD8+ T cells by two pathways: the endogenous pathway and the exogenous pathway. The endogenous pathway is the classical pathway by which cytosolic proteins are degraded and the resultant peptides loaded onto MHC Class I molecules within the endoplasmic reticulum. The exogenous pathway is thought to occur, at least in part, by the capture of exogenous antigens by DC and their presentation to T cells (Nouri-Shirazi *et al.*, 2000; Albert *et al.*, 1998a). Processing

via this alternative pathway can be TAP-independent (Schoenberger *et al.*, 1998) or TAP-dependent (Kovacsovics-Bankowski and Rock, 1995).

This process by which peptides derived from extracellular antigens (e.g. apoptotic bodies) are transferred to host cells for presentation by host MHC Class I molecules is termed "cross-priming" and is associated with inflammation or autoimmunity, and with normal cell death, tissue damage or cell deletion (Rovere *et al.*, 1999) (Figure 1.4). It is also thought to have an important role in self-tolerance, tumour immunity and vaccine development (Nelson *et al.*, 2000).

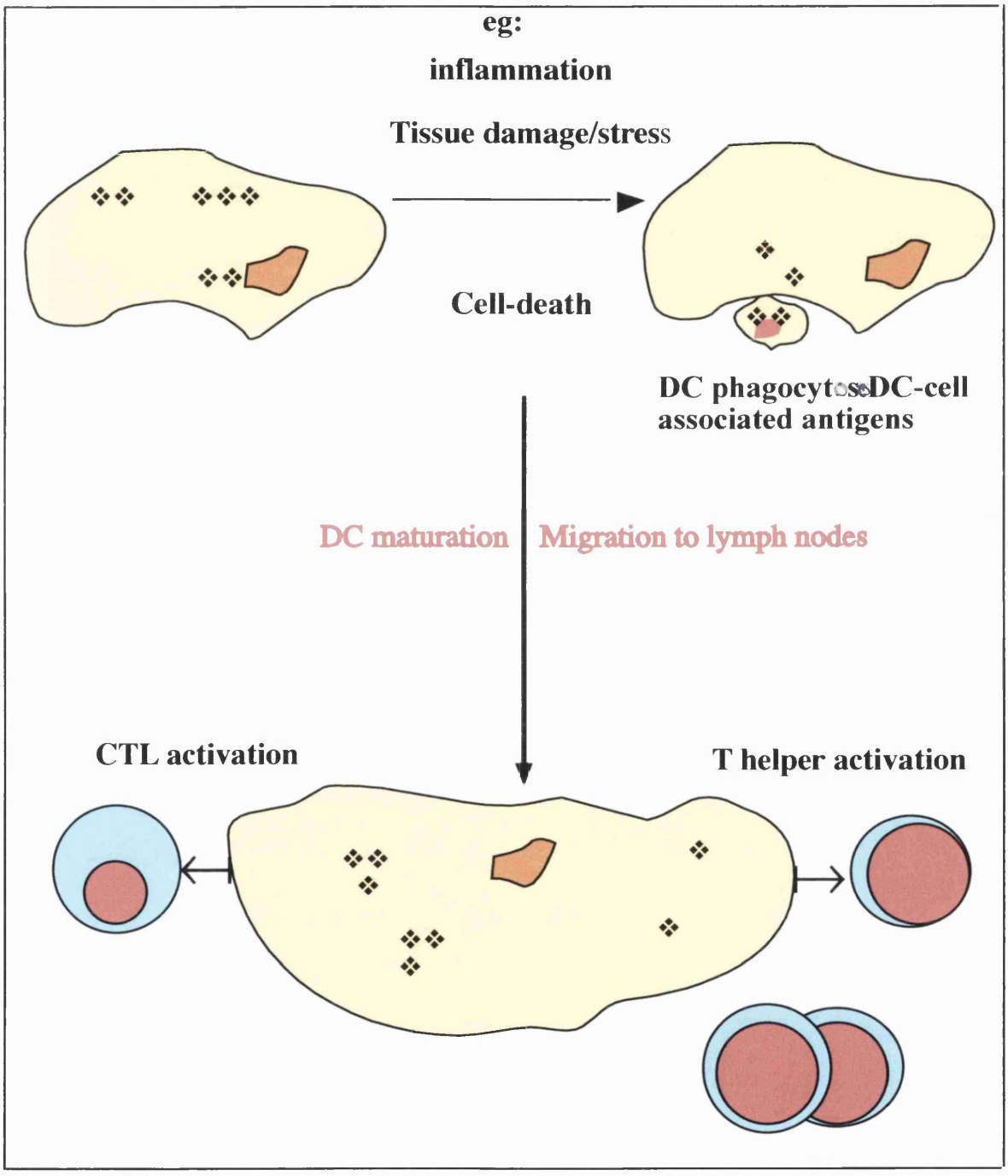

Figure 1.4- Cross-presentation: mechanism for immunity

1.5.6 Intracellular signalling

Toll-like receptors (TLR; see section **5.1**) have been demonstrated to be important in the recognition of pathogens by DC, and in the subsequent activation of the innate immune response (Muzio *et al.*, 2000). Stimulation of DC by lipoproteins or LPS results in the increased expression of TLR2 (Thoma-Uszynski *et al.*, 2000). Similar results have been obtained when BCG is used to stimulate DC (Tsuji *et al.*, 2000). The kinase cascade leading to full activation of the DC is poorly understood but involves mitogen-activated protein kinases (MAPK), ERK kinases and phosphorylation cascades (Saklatvala *et al.*, 1999). Downstream of the signalling cascade, NFκB is the main mediator of the cellular response to pathogens (Figure 1.5).

Dendritic Cells (NFκB activation)

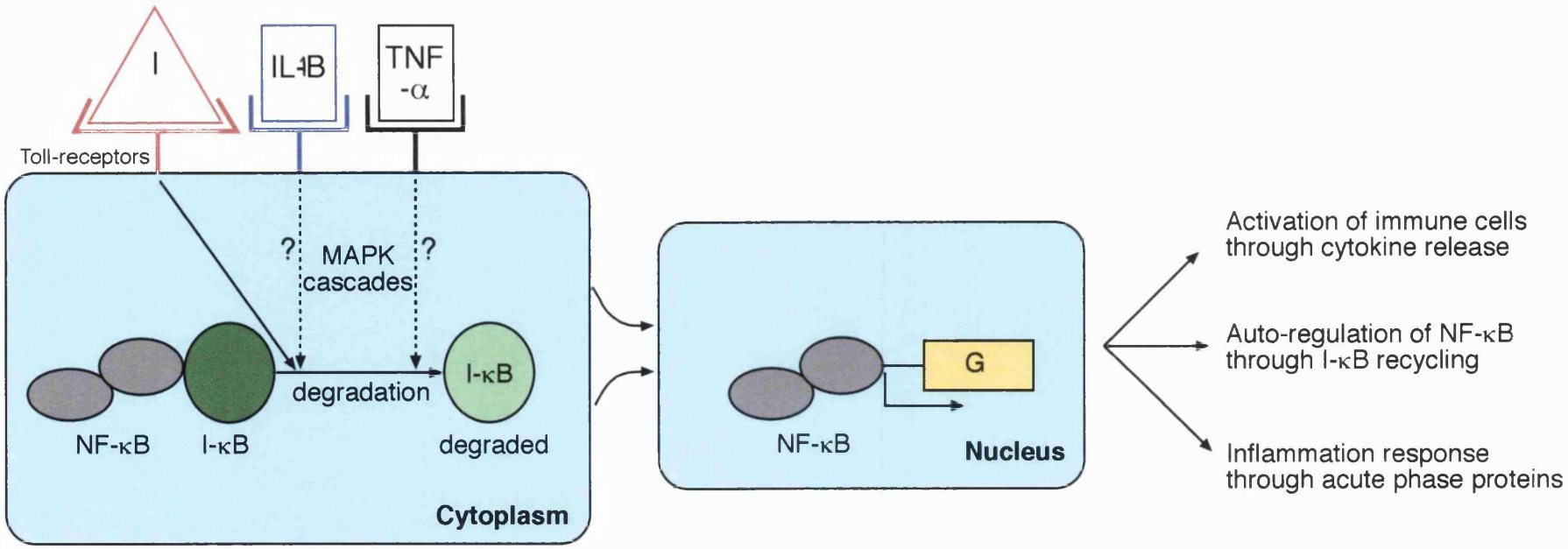

Fig. 1.5- Proposed pathway of NFκB activation in dendritic cells.Stimuli like **I** (bacterial infection, stress or LPS) and cytokines IL1-B and/or TNF-α induce this factor to be released from Iκ-B and translocate to the nucleus, where it upregulates transcription of specific genes (G)

NFκB is a major inducible transcription factor; in the inactivated state NFκB is complexed with an inhibitory subunit, IκB. Activation of the cell results in phosphorylation and degradation of IκB, resulting in the dissociation of the IκB-NFκB complex and translocation of NFκB from the cytoplasm to the nucleus where it binds DNA and activates transcription (Alkalay *et al.*, 1995).

The NFκB family of transcription factors includes the proteins p50, p52, p65, c-Rel and Rel B that can form homo or heterodimers giving rise to different protein complexes (Jensen and Whitehead, 1998). The Rel/NFκB transcription factors are not only key regulators of the immune, inflammatory and acute phase responses, they are also implicated in the control of cell proliferation and apoptosis (Rayet and Gelinas, 1999). The deletion of Rel genes in mice disrupts DC development and maturation (Wu *et al.*, 1998; Gerondakis *et al.*, 1999). Other studies have demonstrate that the Rel/NFκB family directly activates expression of the apoptosis inhibitor Bcl-xL, and that the Bcl-X promoter is directly controlled by c-Rel (Chen *et al.*, 2000). Thus, activation of DC is also accompanied by increased expression of the anti-apoptotic protein Bcl-xL in DC, suggesting a possible mechanism in protecting DC from apoptotic death (Pirtskhalaishvili *et al.*, 2000).

Thus, while little is known about the recognition of *Mtb* by DC, and the resultant kinase cascade, it seems likely that NFκB would be the main down-stream mediator of the cellular response, and could also play an important role in regulating DC maturation and death.

1.5.7 DC as clinical immunotherapeutic or prophylactic agents

Because of their pivotal role as APC in inducing T cell responses, DC are being widely studied for their possible use as immunotherapeutic or immunoprophylactic

agents. Several logistic issues have to be considered when developing DC for clinical use: they have to be generated in sufficient numbers, they should display morphological, phenotypic and functional properties of DC and they should be able to present antigens (Citterio *et al.*, 1999). Although the numbers of DC in circulation are low, recent advances have made it possible to generate DC in culture, from various sources including bone marrow, and peripheral blood; however, for clinical applications, DC must be generated in serum-free medium and cryopreserved for future use (Hajek and Butch, 2000). Although the requirement for the DC to present antigen is obvious, the fact that pathogenic organisms might have evolved mechanisms for inhibiting DC function (Urban *et al.*, 1999), has to be considered.

DC efficiently present antigens to cytotoxic T lymphocytes (CTL); tumour peptide antigens which can be presented in the context of MHC Class I, have been pulsed to DC and can induce protective immunity against lethal challenge by a tumour transfected with the gene encoding the antigen (Celluzzi *et al.*, 1996; Young and Inaba, 1996). In the same model, DC pulsed with RNA was also an effective way of inducing CTL responses and tumour immunity (Boczkowski *et al.*, 1996). Therapeutic immunity could also be conferred by transfer of DC that are presenting tumour antigens into tumour-bearing mice (Schuler and Steinman, 1997).

Experimental DC-based vaccines have also been studied against a range of infectious diseases, including Chlamydial and Toxoplasma infections (Ojcius *et al.*, 1998; Bourguin *et al.*, 1998). BCG-pulsed DC transferred into the lungs of mice, which are then challenged with *Mtb* have also been shown to confer protection (Demangel *et al.*, 1999).

In addition to using DC themselves as vaccines, other approaches to stimulating DC activity *in vivo* are being studied. In particular the activation of DC by ligation of the

CD40 signalling molecule has been shown to enhance immunity against experimental lymphomas (French *et al.*, 1999) or colon carcinomas (Kikuchi *et al.*, 2000; Sun *et al.*, 2000) or infectious agents such as *Leishmania major* (Campbell *et al.*, 1996; Ferlin *et al.*, 1998).

Yet another approach has followed from the identification of antigen presenting vesicles, or exos omes, which are secreted by DC, and that expressed MHC Class I and II and T cell-costimulatory molecules (Zitvogel *et al.*, 1998). Exos omes prepared from DC pulsed with tumour peptides can substitute for intact DC in priming CTL *in vivo*, and suppressing the growth of established murine tumours (Zitvogel *et al.*, 1998). Currently the use of DC-based vaccines is being actively persued, particularly for the treatment of tumours in man (Dhodapkar and Bhardwaj, 2000).

1.5.8 Immortalised DC lines

The development of immortalised DC lines provides a useful model for studying the process of maturation, migration and T cell activation. The murine DC line CB1 was generated using a retroviral vector for a fusion gene. This cell line displayed most of the morphologic, phenotypic and functional attributes of mature DC, with constitutive expression of MHC Class II molecules, costimulatory molecules, heat-stable antigen and intracellular adhesion molecule ICAM–1.The CB1 line is capable of efficient antigen presentation to T cells (Paglia *et al.*, 1993).

DC lines exhibiting an immature phenotype have also been produced (Granucci *et al.*, 1999). Murine splenic DC and Langerhans cells which could phagocytose bacteria, and respond by producing TNF-α and IL-6 (Riva *et al.*, 1996), and which displayed DC morphology and specific DC surface markers (Ohnishi *et al.*, 1995) have been reported.

The DC line used in many of the experiments reported in this study was derived from bone marrow cells of mice transgenic for the thermolabile mutant of the SV40 large T antigen. The resulting cell line, referred to as tsDC matures following contact with T cells, T cell-derived cytokines or with a shift in temperature to 39°C (Volkmann *et al.*, 1996) The tsDC cell line exhibits the following characteristics:

- Grows indefinitely as immature DC in the absence of GM-CSF
- High endocytic activity
- Constitutive expression of MHC Class II molecules
- Maturation at 39°C involves cessation of growth and upregulation of surface markers such as B7-1, CD40 and ICAM-1

1.6 Aims of this study

The aim of this study was to investigate the interactions between DC and *Mtb* at the cellular and molecular levels. Most of the experiments were performed with the tsDC immortalised cell line, although many of the findings were also confirmed in primary DC. The study is divided into four parts:

(i) A morphological study of the fate of *Mtb* following phagocytosis by DC.

(ii) An investigation of maturation of DC following exposure to *Mtb*. The expression of cytokines, accessory molecules and MHC Class II molecules was investigated.

(iii) A study of activation of *Mtb*-infected DC at the molecular level, looking specifically at the role of the nuclear transcription factor NFκB.

(iv) An investigation of the ability of *Mtb*-infected DC to prime protective immune responses and to cross-prime immune responses *in vivo*.

Chapter 2
Materials and methods

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial Strains and cultures

Mycobacterium tuberculosis H37Rv was grown in Middlebrook medium and stock cultures of mid-log phase bacilli were stored in aliquots containing 20% of heat-inactivated fetal calf serum (FCS-GlobePharm) in liquid nitrogen. BCG was obtained from Glaxo (Glaxo,UK).

2.2 Cell culture and conditions

2.2.1 Immortalised DC line (tsDC)

The thermosensitive immortalised dendritic cell line kindly provided by Dr. B. Stockinger (Volkmann *et al.*, 1996) was used as a continuous source of DC (tsDC). The cells were cultured in Iscove's Modified Dulbeccos Medium (IMDM;Gibco-Life Technologies) supplemented with FCS 5% and L-Glutamine (2mM-Sigma).The medium was filtered through a 0.22 μm filter (Millipore) and the cells were grown at 34-35°C in a humidified incubator in an atmosphere of 5% CO_2.

2.2.2 Primary bone-marrow DC

Bone marrow cells were collected from 6-8 week old female BALB/c or C57BL/6 mice, and washed in IMDM (Gibco-Life Technologies). The mice were killed by cervical dislocation, and the femurs surgically removed using sterile forceps and scissors. The cells were extracted from the femurs by flushing out the bone marrow cells using a 5ml syringe (Sherwood) and a 0.5x16mm needle (Kendall) with IMDM.

The cells were washed and ressuspended in 10ml of IMDM medium containing 5% filtered FCS, L-glutamine (2mM) and 0.1M mercaptoethanol (ME; Sigma) in 9cm petri dishes (Nunc). After adding 1ml [25U] of granulocyte-monocyte colony stimulating factor (GM-CSF), the cells were kept at 37°C and 5% CO_2. On the third day the medium was changed to fresh IMDM and 1ml of GM-CSF was again added. Four days later, the plates were washed with IMDM and the semi-adherent DC transferred to new plates. Purity of the DC was assayed by CD11c FACS staining. The DC were used after a total of 12 days in culture (Figure 2.1).

2.2.3 Peritoneal macrophages

Peritoneal macrophages were flushed from the peritoneal cavities of Balb/c mice using Dulbeccos Modified Eagles Medium (DMEM). The cells were washed twice in DMEM and cultured at $1x10^6$ per well in 6x25mm tissue culture wells (Nunc) using DMEM supplemented with 20% FCS and 2-ME (0.1ml stock).

2.3 Infection of cells with *Mtb*

Cells (DC or macrophages) were aliquotted to 25mm culture wells (Nunc) at a concentration of 10^6 cells *per* well. After one day the cells were infected with live *Mtb* (strain H37Rv) at a ratio of 5:1 (bacteria: DC) by adding the appropriate volume from the stock bacterial suspension. One well of cells was fixed with a solution of 2% paraformaldehyde (BDH) adjusted to pH 7.2 with hydrochloric acid (HCl) and 24 hours later Ziehl-Neelsen staining was carried out to confirm that the *Mtb* had been phagocytosed.

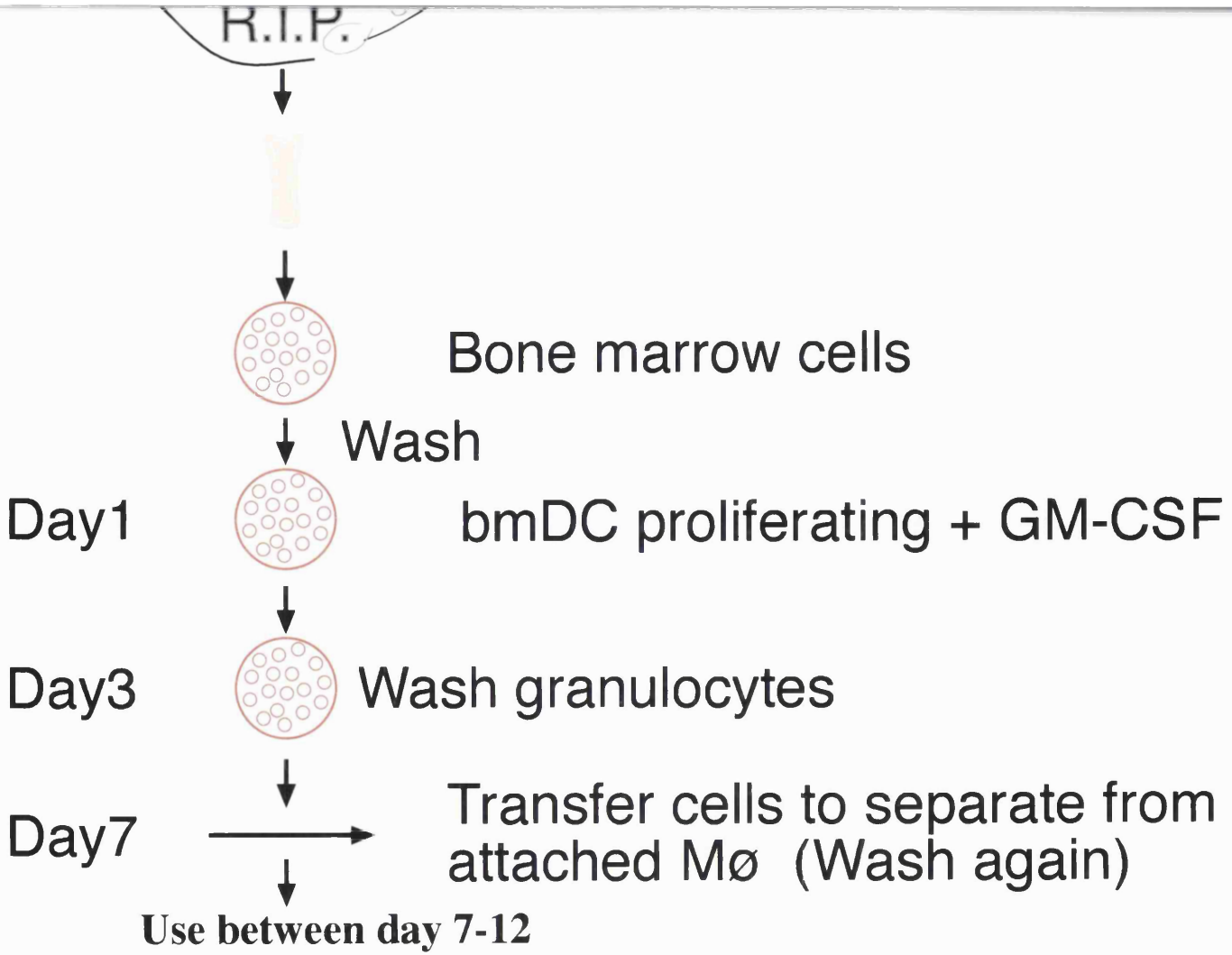

Figure 2.1 Extraction and purification of murine bone marrow-derived dendritic cells

2.3.1 Determination of the number of tsDC which had phagocytosed *Mtb*

To determine the percentage of tsDC, which had phagocytosed *Mtb*, the cells were fixed 12 hours after infection, as described above, and Ziehl-Neelsen staining was performed. In each well one hundred randomly selected cells were observed by microscopy, the number of cells that had phagocytosed *Mtb* was counted and the percentage of infected cells determined.

2.3.2 Treatment of infected tsDC with colchicine

Phagocytosis was inhibited using the tubulin polymerisation inhibitor colchicine (Sigma; (Bermudez and Goodman, 1996)). For the determination of the percentage of phagocytosis of *Mtb* by tsDC treated with colchicine, 10μM of colchicine (Sigma) was added to the medium 30 minutes prior to infection; to another group of cells, saline was added (controls) and a third group was maintained with no additions to the medium (control). After 12 hours, the cells were washed. The number of cells that had phagocytosed *Mtb* was counted and the percentage of infected cells determined, as described above.

2.3.3 Ziehl-Neelsen staining

The slide or plate was flooded with Carbol Fuchsin KF (Difco) and left for four minutes and then washed gently with tap water to remove excess stain. The TB decolorizer was added (TB decolorizer;Difco) for one minute, until the colour no longer came out. The slide or plate was washed with tap water and air-dried.

2.4 Growth of *Mtb* in DC and macrophages

Cells (DC or macrophages) were cultured and after two days in culture infected with *Mtb* as described above. The infected cells were incubated at 37°C in 5% CO_2 for various time intervals. The cells were washed and lysed using 2% saponin for one hour. Viable counts of *Mtb* released following lysis of the cells were determined by performing 10-fold serial dilutions in saline and plating onto 7H11 Middlebrook (Difco Laboratories). Plates were incubated at 37°C for three weeks. The number of colonies was counted and colony-forming units (CFU) in the original cell culture calculated.

2.5 Electron microscopy

A minimum of 10^6 tsDC (uninfected or *Mtb* infected) in 25ml of IMDM in a 80 cm^2 tissue culture flask (Nunc) was used. The cells were washed with PBS and fixed for 1 hour, with a mixture of 2.5% gluteraldehyde/2% paraformaldehyde (made up freshly) and 0.1M sodium cacodylate (Na $(CH3)_2$ As $O_2.3H_2O$) buffer, pH 7.2 adjusted with HCl. The cells were then centrifuged at 6500 rpm for 10-15 minutes and the pellet fixed again for another hour. The cell pellet was post-fixed with 1% osmium tetroxide/1% aqueous uranyl acetate, dehydrated with ethyl alcohol and embedded in Epon (Agar Scientific) allowing polymerisation at 70 °C. Sections of 55nm were cut and mounted on 200 mesh carbon coated grids and stained with ethanolic uranyl acetate/Reynolds lead citrate. Observations were made with a JEOL Cx 100 Transmission Electron Microscope and analysis of the micrographs was performed (NB. the sections were cut and mounted by Ms Liz Hurst, NIMR).

2.6 Nitric oxide production

To measure the release of nitric oxide (NO), supernatants from $6x10^6$ tsDC were collected at different time points and filtered (0.22μm - Millipore). The levels of total NO released were determined using the Total Nitric Oxide Assay (R&D Systems), in which a coloured dye product of the Griess Reaction, which absorbs visible light at 540nm, is measured. In this experiment LPS (Sigma) was used at a concentration of 10μg/ml and recombinant IFNγ (Sigma) at a concentration of 500U/ml, as a positive control. Both reagents were added 12 hours prior to infection. The iNOS inhibitor aminoguanidine hydrochloride (Calbiochem; (Griffiths *et al.*, 1993)) at a concentration of 0.5M was added 18 hours prior to infection, and again after the cells had been washed.

2.7 Treatment of cells with RNI and ROI inhibitors

The growth of *Mtb* in cells exposed to different RNI and ROI inhibitors was compared. iNOS was inhibited using the competitive inhibitor L-nitro-L-arginine methyl ester (L-NAME;Calbiochem) at a concentration of 1mM (McDonald *et al.*, 1997). Arginase activity was inhibited using L-Norvaline (Calbiochem) at a concentration of 10mM (Chang *et al.*, 1998). The H_2O_2 inhibitor TMB-8 (Calbiochem) was used at a concentration of 0.1mM (Schmidt *et al.*, 1995).

To test the effect of RNI and ROI inhibitors on the intracellular growth of *Mtb,* in each case inhibitor was added at the time of *Mtb* infection; the cells were incubated overnight, washed three times to remove extracellular *Mtb*, and fresh medium containing the inhibitor was added.

2.8 Detection of cytokine gene expression by reverse transcriptase PCR (RT-PCR)

2.8.1 RNA extraction

Total RNA was extracted from DC ($3x10^6$ cells/well) by lysing them with RNAzol (Biotechnology). Cells were homogenised by adding 0.2ml of RNAzol per 10^6 cells. The cell lysate was passed several times through a pipette and incubated for 5 minutes at room temperature to dissociate nucleoprotein complexes. 2ml of chloroform was added per 1ml of RNAzol, the tubes were shaken vigorously for 15 seconds and incubated on dry ice for 5 minutes. The suspension was centrifuged at 12000g for 15 minutes at 4°C and the aqueous phase, containing the RNA, was collected. The RNA was precipitated by adding 0.5ml of isopropanol per 1ml of RNAzol used in the initial homogenisation. The samples were then incubated for 15 minutes, in dry ice, and centrifuged for another 15 minutes at 12000g (4°C). The RNA pellet was washed once with 75% ethanol, adding 1ml of ethanol per 1ml of RNAzol. The pellet was vortexed, centrifuged for 8 minutes at 7500g at 4°C, air-dried, and redissolved in RNase-free water.

2.8.2 DNase treatment

To minimise the risk of genomic DNA contamination a DNase digestion step was included. The dissolved RNA was digested with 10U of DNase I (Boehringer Mannheim) for 15 minutes at 37°C, along with 1μg of RNase inhibitor (Promega), 1M NaAC and 0.1 MgSO4. This mixture was kept for 15 minutes at 37°C. A phenol/chloroform saturated solution was added to the tubes and after shaking well they were centrifuged for 5 minutes at 13000g at 4°C. The supernatant was then

removed and reprecipitated in 2 volumes of 100% ethanol and 0.5 volumes of 3M NaAC, washing the final pellet, twice, with 70% ethanol. The RNA concentration was assessed by absorbance: OD_{260} = 1 equivalent to approximately 40μg RNA/ml.

2.8.3 Reverse transcription

The reverse transcription reaction was carried out using a solution consisting of reverse transcriptase buffer (Gibco), 10mM DTT (Life Technologies), 0.5mM dNTP (Pharmacia Biotech), 28U of RNase inhibitor and 200U of the Reverse Transcriptase Enzyme (Gibco). The reaction had 2 steps: 60 minutes at 37° C and 5 minutes at 95° C.

2.8.4 PCR amplification and product visualisation

PCR amplification was carried out using specific primers for murine TNF-α (giving a 750 bp product), IL-6 (giving a 638 bp product), IL-1β (giving a 587 bp product), IL-12p40 (giving a 394 bp product), IFN-γ (giving a 365 bp product) and IL-10 (giving a 455bp product) (Clontech, Palo Alto). The PCR mixture contained 5 μl of 10x PCR buffer, 5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP, 0.5 μl of 20 μM primers, 0.5 μl of Taq polymerase (5U/μl) and 5 μl of cDNA. The β-actin primers used as controls for the PCR reactions were; sense: 5`- ATG GAT GAC GAT ATC GCT-3`; antisense: 5`- ATG AGG TAG TCT GTC AGG T-3` giving a 540 bp product. The PCR was carried out in a thermocycler Gene Amp 9700 (PE Applied Biosystems), using the following cycle programme: 5minutes at 95°C, then 25 cycles of 20 seconds at 98°C, 20 seconds at 56°C, 30seconds at 72°C, followed by 7 minutes at 72°C.

The molecular weights of the PCR products were determined using broad range PAGE standards (Biorad). PCR products were visualised by electrophoresis through a 1.2% agarose gel containing ethidium bromide.

2.9 Cytokine protein assays

A quantitative "sandwich" enzyme-immunoassay technique was used. Supernatants from uninfected and *Mtb*-infected cultures were collected at 12, 24 and 48 hours, filter sterilised (0.22 μm; Millipore) and the following cytokines measured using standard commercially available Elisa kits: IL-6, TNF-α, IL-1 β, IFN-γ (Amersham Life Science), IL-12 (Genzyme Diagnostics, USA) and IL-10 (R&D Systems), according to the manufacturers instructions. The assays were carried out in triplicate and the results analysed by the Students t-test; p values of <0.01 were considered significant.

2.10 Flow cytometry

Uninfected and *Mtb* infected cells were washed twice with PBS and, after blocking Fc receptors using anti-mouse CD16/CD32 (Pharmigen) for 15 minutes, the cells were stained for 20 minutes on ice with directly conjugated antibodies: Phycoerythrin (PE)-conjugated anti-B7-1 clone 1G10, PE-conjugated anti-B7-2 clone GL-1, PE-conjugated anti-ICAM1 clone YNI/1.7.4, PE-conjugated anti- Heat Stable Antigen clone MI/69, FITC-conjugated streptavidin (all from Pharmigen) and biotin-conjugated anti-MHC class II I-E$\alpha_{k,d}$ clone 14.4.4 ATCC HB32 (kindly supplied by Dr. B. Stockinger, NIMR).

After staining, the cells were fixed using 4% paraformaldehyde for 40 minutes at room temperature and then washed three times with PBS. The cells were ressuspended in 1 ml of FACS reading buffer (PBS + 1% BSA+ 0.1% Azide) and acquisition was

performed on a FACScan (Benton Dickinson, Mountain View, CA) using forward and side–scatter characteristics to exclude dead cells. Data were analysed using WinMDI (The Scripps Research Institute, CA).

2.11 NFκB protein assays

2.11.1 Protein extraction

Cells pellets were centrifuged and ressuspended in 1 ml of RIPA lysis buffer, plus protease inhibitors:

RIPA buffer

1xPBS

1% Igepal CA630 (Sigma)

0.5% Sodium Deoxycholate (BDH)

0.1% SDS

Protease inhibitors

AEBSF-10μl/ml (Sigma)

Aprotinin-30μl/ml (Sigma)

Sodium orthovanadate-10μl/ml

Leupeptin- 5μg/ml (Sigma)

The mixture was incubated on ice for 30 minutes and then centrifuged for 15 minutes at 4°C. The supernatant was collected (lysate) and the protein levels were quantified using the BCA protein assay Kit (Pierce) according to the manufacturers instructions.

2.11.2 SDS polyacrylamide protein gel electrophoresis

A 10% resolving gel was prepared as below and polymerisation initiated by addition of fresh 10% APS and TEMED (Biorad). The gel was allowed to set for 1 hour, then the 4% stacking gel, containing APS and TEMED was poured. Equal amounts of protein were loaded per lane, per gel. 10 μg to 40μg of protein was loaded depending on the size of the combs and protein gel apparatus used. The gels were run at 20-25mA and a maximum of 150V for between 1and 2 hours, until the dye reached the bottom of the gel. The running buffer used is described below.

10% Resolving Gel (25 ml)

8 ml 30% Protogel (30% acrylamyde/0.8% bisacrylamyde)

6.25 ml 0.5M Tris-HCl pH 8.8

10.75 ml ddH_2O

0.12 ml 10% APS

0.025 ml TEMED

4% Stacking Gel (10 ml)

1.33 ml 30% Protogel (30% acrylamyde/0.8% bisacrylamyde)

2.5 ml 1.5M Tris-HCl pH 6.8

6.17 ml ddH_2O

0.05 ml 10% APS

0.1 ml TEMED

5x Page Electrophoresis Buffer (1L)

15.1g Tris

94g Glycine

25ml SDS 20%

ddH_2O- up to 1L

(pH 8.3)

2.11.3 Immunoblotting

The transfer apparatus (Fastblot B33; Biometra) was assembled containing blotting buffer in the following order and on the top of each other: three sheets of 3MM paper, Immobilon-Transfer membrane (Millipore), pre-treated according to the manufacturer's instructions, protein mini-gel without the stacking part, and another three sheets of 3MM paper. Each of the sheets was previously soaked in transfer buffer and bubbles were removed by rolling with a Pasteur pipette. The gel sandwich, in the transfer apparatus, was transferred for 1 hour at 60mA at room temperature.

The membrane was removed from the sandwich, air-dried at room temperature and placed into MeOH for 10 seconds. It was then transferred to ddH_2O, immersed, rinsed and incubated overnight with gentle rocking at room temperature in 10-20 mls of blocking solution, prepared as follows: 10g non-fat/dry milk, 100 mls of TTBS and 400 µl 0.5 M EDTA. The appropriate dilution of the antibody was prepared in a solution containing: 0.6g non-fat/dry milk, 20mls of TTBS and 80 µl 0.5 M EDTA. The membrane was vacuum-sealed in plastic with the solution, and incubated for 1 hour at room temperature with gentle rocking. The membrane was then washed vigorously with TTBS for 5 minutes for three times, to achieve low background staining. A 1:1000 to 1:2000 dilution of the second layer antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse; DAKO) was prepared in 20 ml of a solution prepared as above. The membrane was again vacuum-sealed in plastic with

the solution and incubated for 1 hour at room temperature, with gentle rocking. Again the membrane was washed in TTBS for 5 minutes three times, washed twice for another 5 minutes in TBS and finally washed once with 1xPBS for five minutes.

The antibodies used were as follows: IκB-α (Santa Cruz), Phospho IκB-α (Pharmigen), c-Rel (Santa Cruz), Bcl-x (L) (Pharmigen), Bcl-2 (Pharmigen), β-actin (Sigma), M1 and HeLa cell lysates (Pharmigen).

TBS 5x (1L)

Tris - 12.1g 100mM pH 7.5

NaCl- 146.2g 2.5M

ddH_2O- up to 1L

(pH 7.5 adjusted with HCl)

TTBS (1L)

TBS- 1L

Tween 20 – 1ml

Transfer Buffer (1L)

Glycine- 2.9g

Tris- 5.8g

SDS 20%- 1.85ml

MeOH - 200ml

ddH_20 – 800 ml

(pH 8.3)

The washed membrane was transferred to Saran plastic wrap with the protein side up. ECL (Western Blotting detection system) was performed using the Amersham Detection Kit by mixing 3ml of each of the solutions provided, and poured immediately onto the membrane. After 1 minute the membrane was dried using paper tissues, sealed in a plastic bag and attached to an X-ray cassette. A range of exposure times, from 10 seconds to 10 minutes, was used to obtain the ideal exposure to the film-Hyperfilm ECL (Amersham).

2.12 NFκ B Electrophoretic Mobility Shift Assays (EMSA)

2.12.1 Labelling of oligonucleotide with γ 32 P[ATP]

Using 5 pmol of the NFκB primer (2.8 μl), T4 polynucleotide Kinase and 10 x T4 polynucleotide kinase buffer with γ 32 P[ATP], a standard labelling procedure was carried out (Sambrook and Gething, 1989) with incubation for 10 minutes at 37° C.

The NFκB primer (Promega) was as follows: **5` AGA GGG GAC TTT CCG AGA GGC-3 ‘** (consensus sequence)

2.12.2 Binding reaction

Samples were prepared at room temperature and each sample contained:

EMSA 2x binding buffer- 5μl

1ug poly dIdC poly dIdC (Roche) -1μl

p32 g ATP labelled probe- 1μl

Protein extract (4μg max.) – 3 μl

The protein extract, the binding buffer and the polydIdC were mixed first and the labelled probe added last. The mixture was incubated on ice for 30 minutes.

EMSA 2x Binding buffer (1ml)

HEPES - 20µl 20mM, pH 7.9

KCl- 120 µl 120 mM

DTT- 0.8µl 0.8mM

Glycerol 20 % - 400µl

BSA - 8µl 0.4µg/µl

ddH2O – 451.2 µl

2.12.3 Nuclear extraction

Nuclear extracts were prepared by a variation of the method of Schreiber (Schreiber *et al.*, 1989). 1 x 10^7 cells were washed twice in cold-PBS (calcium/magnesium free) and pelleted by centrifugation at 12000 rpm in a microcentrifuge (Sigma) at 4° C for 5 minutes. PBS was removed by aspiration and the cell pellet was ressuspended in 500µl of cold Cytoplasmic Buffer. The cells were allowed to swell on ice for 15 minutes, after which 30 µl of a 0.6% solution of Nonidet NP-40 (BDH) was added and the tube was vortexed for 10 seconds. After 5 minutes on ice, the cells were centrifuged for 30 seconds and the supernatant containing cytoplasm and RNA was transferred to another tube. All traces of Cytoplasmic Lysis buffer were removed from the pellet. 50 to 60µl of Nuclear Lysis buffer was added to the pellet (if the pellet was too big, 1:1 volume "pellet: lysis buffer"was added). The nuclear pellet was freeze-thawed 3 times to

extract proteins, by transferring from an ethanol/dry ice bath to a 37° C water bath. The nuclear extract was then centrifuged for 10 minutes at 4°C and the supernatant transferred to a new tube. This supernatant was further microcentrifuged in an ultra-free eppendorf tube filter unit-0.22μm (Millipore) for 5 minutes at 4°C.

The protein concentration was measured by the BCA method for EMSA.

Cytoplasmic Lysis Buffer

10mM HEPES pH 7.6

1mM EDTA

0.1mM EGTA

10mM KCl

1mM DTT

20mM NaF

1mM Na pyrophosphate

1mg/ml SPI (freshly added)

1mM Sodium orthovanadate (freshly added)

Nuclear Lysis Buffer

20mM HEPES pH 7.6

0.2mM EDTA

0.1mM EGTA

25% Glycerol

0.42M NaCl

1mM DTT

20mM NaF

1mM Na pyrophosphate

1mg/ml SPI (freshly added)

1mM Sodium orthovanadate (freshly added)

Protease inhibitors (SPI)

5μg/ml Chymostatin (Sigma)

5μg/ml Antipain (Sigma)

5μg/ml Pepstatin (Sigma)

30μg/ml Leupeptin (Sigma)

2.12.3.1 NFκ B modulation by TNF-α

The anti-TNF-α antibody (Sigma) was added to the cell cultures one hour before *Mtb* infection at a blocking concentration of 1.2μg/ml. Nuclear extracts were prepared as described above and stored at -70° until the gel-shift reaction was carried out.

2.12.4 Polyacrylamide Gel Shift

A 7% polyacrylamide gel was mixed and poured in the gel-shift apparatus and allowed to polymerise at room temperature for 1 to 2 hours. The gel was then pre-run in EMSA running buffer (0.5xTBE 1% glycerol) for 1.5 hours at 130 V, at 4 °C. The buffer was then changed and the gel left to soak for 2-3 hours. The samples were loaded and the gel run for 3-4 hours, at 130V (until the bromophenol marker was 2/3 to 3/4 down the gel). The plates were carefully separated leaving the gel on one plate. The gel was removed and overlaid with Whatmann 3mm paper covered with Saran wrap and dried.

After 1 hour, the paper was removed and exposed overnight to X-ray film in a Phosphorimager cassette at –70 °C. The exposure time varied according to the experiment.

7% Gel for EMSA

30% Acrylam. /Bysacrylam (29:1)	10 ml
10xTBE	1.5ml
Glycerol 1%	0.6ml
ddH_2O	21 ml
Temed	30µl
APS 10%	300µl

2.13 Super-shift assays for NFκB

For super-shift assays, the antibodies were added to the binding mixture and incubated on ice for 30 minutes before the probe was added (Bours *et al.* 1992). The antibodies used were c-Rel and Rel-B (Santa Cruz). A 7% polyacrylamide gel, prepared as described previously was used. After the samples were loaded onto the gel, electrophoresis was carried out for 4 hours at 100-110V, at 4°C. The gel was dried as described before.

2.14 *in vivo* immunisation procedures

2.14.1 Mice

Six to eight week old mice were obtained from breeding colonies maintained under specific pathogen free conditions in the Division of Biological Services, NIMR.

2.14.2 Infection of DC and immunisation procedures

DC were grown and infected with *Mtb* as described above (Sections **2.2** and **2.3**); when irradiated *Mtb* was used, the bacterial suspensions were irradiated at 2.5 megaRads. For the immunisations the DC were incubated overnight at 37°C and irradiated at 2.5 megaRads. Between 1 $x10^6$ and 4 x 10^6 cells were injected intraperitoneally in 0.5ml of IMDM.

2.14.3 Spleen cell and T cell preparations

Spleens were removed and placed in 15 ml of IMDM medium (Gibco-Life Technologies) in a 9 cm petri dishes (Nunc). The spleens were then carefully crushed through a nylon mesh using the tip of a sterile syringe plunger. The spleen cells suspensions were centrifuged at 7500rpm for 5 minutes, ressuspended in fresh medium and recentrifuged to wash the cells. After discarding the medium, 4 ml of Red Blood Cells Lysis buffer (Sigma) was added to the cells for 4 minutes. The pellet was then washed again and ressuspended in fresh medium. Irradiation of spleen cells was carried out by exposure to 3000 rads.

T cells were purified using T cell separation columns (R&D Systems) following the manufacturer's instructions. Pooled T cells were then cultured in duplicate in 24 well-plates (Nunc) at a concentration of $3x10^5$ cells/ml per well in AIM V serum free medium (Gibco). These cells were cultured with irradiated spleen cells ($3x10^4$ cells/ml) from male or female mice at 37° C. After 48 hours, the supernatants were collected and assayed for the presence of IFN-γ and IL-2 by ELISA, as described in section **2.9**.

2.14.4 *Mtb* challenge assay

Immunised mice were infected intraperitoneally with approximately 10^6 viable *Mtb*. Six weeks later, lungs were removed and homogenised in 1ml of saline by shaking in a Bead Beater (Cambridge Scientific & Industrial Products) with 2.5mm glass beads for 20 seconds. Serial ten-fold dilutions of the tissue homogenate were spotted onto 7H11 Middlebrook (Difco Laboratories). Plates were incubated at 37°C for three weeks. The numbers of colonies were counted and colony-forming units (CFU) in the original cell tissue calculated.

2.14.5 Intracellular FACS staining

Cells were washed twice with PBS and, after blocking Fc receptors using anti-mouse CD16/CD32 (Pharmigen) for 15 minutes, stained for 20 minutes on ice with directly-conjugated surface antibodies: Phycoerythrin (PE)-conjugated anti-CD4+(clone GK 1.5) and FITC-conjugated streptavidin (Pharmigen). The procedure used for sorting cells for flow cytometry was as described in section **2.10**. Before permeabilization the cells were fixed as described before. Permeabilization of the cells to stain for intracellular cytokine was carried out by incubating the cells with permeabilization buffer for 20 minutes at 4°C, in the dark. After permeabilization the cells were washed twice and the Biotin-conjugated anti-IFNγ (clone GR 20) antibody was added to the samples and incubated for another 30 minutes. The cells were washed again, ressuspended in 1ml of FACS reading buffer and acquisition was performed on a FACScan (Benton Dickinson, Mountain View, CA) using forward and side–scatter characteristics to exclude dead cells. Data were analysed using WinMDI (The Scripps Research Institute, CA).

Permeabilization Buffer

- PBS (Ca and Mg free)
- FCS 1% heat-inactivated
- Sodium Azide 0.1% (w/v)
- Saponin 0.1% (w/v)

(Buffer adjusted to pH 7.4-7.6 and filtered)

The protocol used to test DC activation after *Mtb* infection is illustrated in Fig. 2.2: Primary bone marrow-derived DC were generated from male C57/BL6 mice. The bmDC were activated after infection with *Mtb in vitro* (uninfected DC and LPS activated DC were used as controls). $5x10^6$ DC were transferred intra-peritoneally into naïve female MHC Class II knockout mice. The DC immunisation was repeated after an interval of 4 weeks and 4 weeks later T cells ($3x10^5$) extracted from the spleens of the recipient mice were incubated with $3\text{-}5x10^4$ irradiated spleen cells from male or female mice with the same background as the recipients-C57/BL6. Supernatants were removed after 48 hours and assayed for IFNγ and IL-2.

The protocol used to test the ability of *Mtb* infected DC to cross-prime T cells (Fig. 2.3) can be briefly described: immunologically normal C57/BL6 female mice were immunised intraperitoneally with DC from MHC Class II knockout female C57/BL6 mice which had been infected *in vitro* with *Mtb* and subsequently irradiated, and with DC from immunologically normal C57/BL6 mice which had been infected and irradiated. The recipient mice received cells 3 times at 3-4 weeks intervals. T cells from these recipient mice were incubated with *Mtb*-infected or uninfected bmDC obtained from C57/BL6 mice; after 48 hours, supernatants were assayed for IFNγ. In addition CD4+ T cells were analysed for intracellular IFNγ by FACS, following 6 days of *in vitro* stimulation.

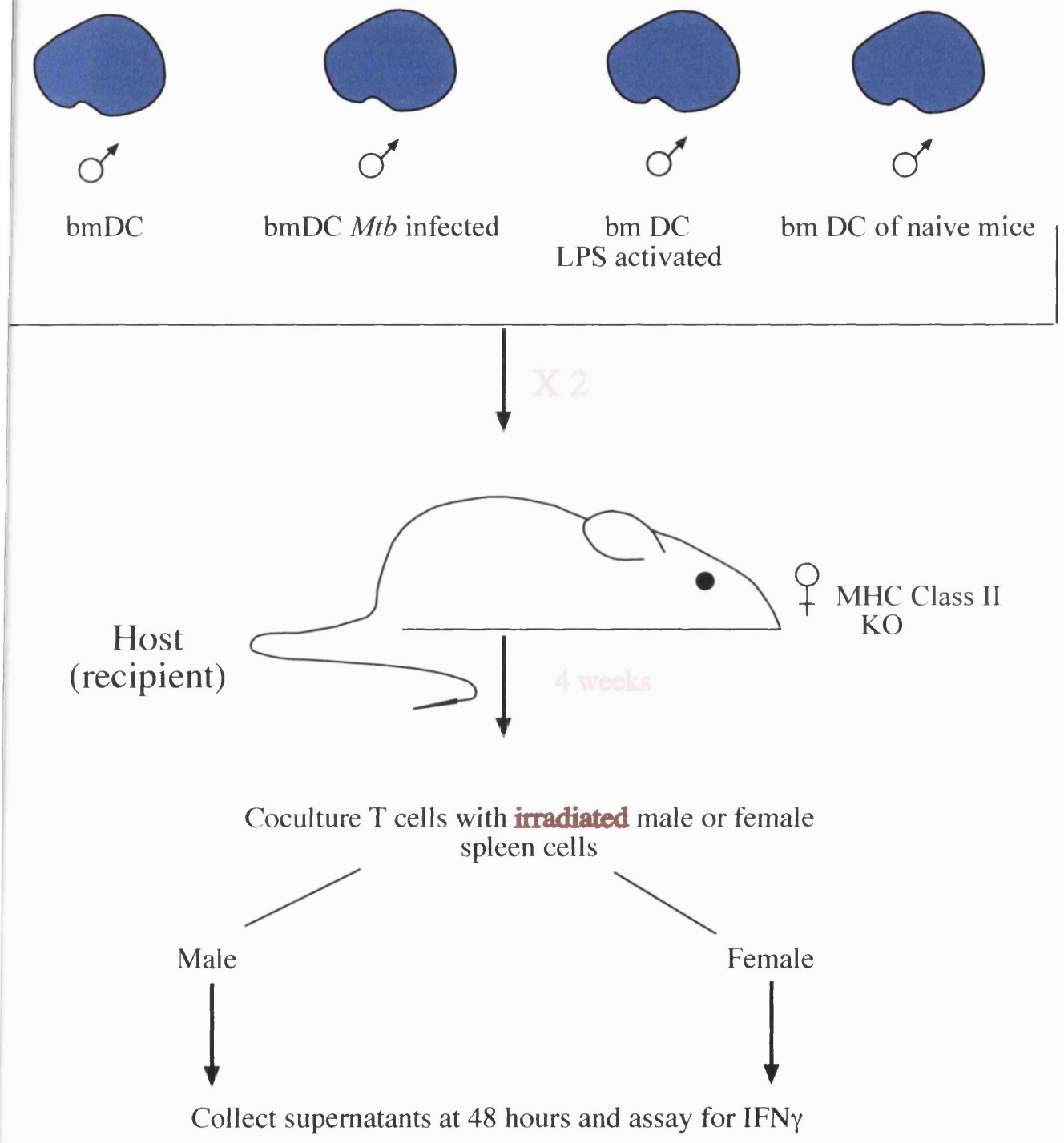

Figure 2.2 Illustration of the experimental murine model protocol used to test dendritic cell activation after *Mtb* infection.

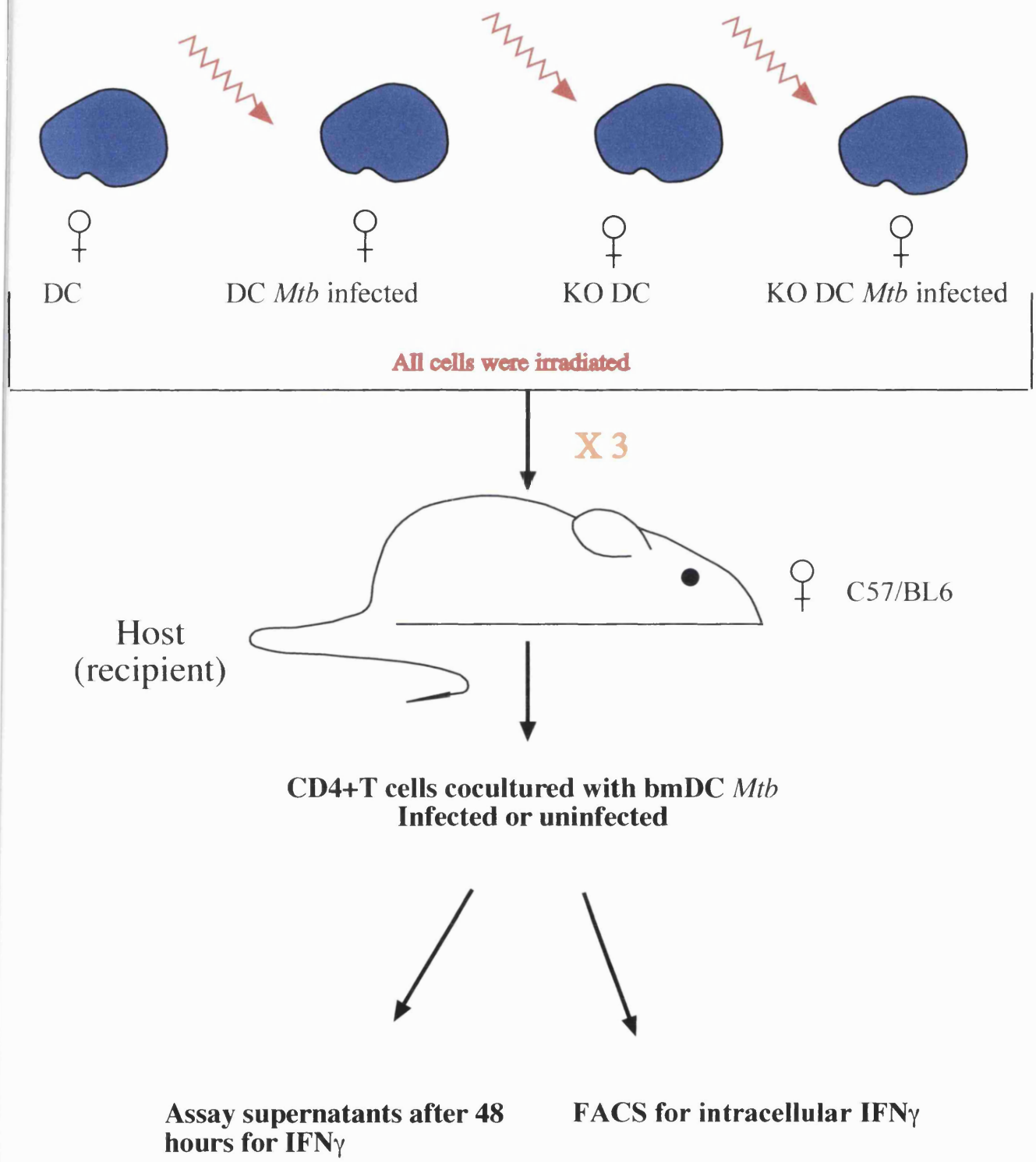

Figure 2.3 Illustration of the experimental murine model protocol used to test the ability of infected dendritic cells to cross-prime T cells specific for *Mtb*

Chapter 3
Results

Chapter 3

Uptake of *Mtb* by DC

3.1 Introduction

The primary target cell for *Mtb* following entry into the lung is the alveolar macrophage. However, the interactions between DC and infectious agents are known to be of importance in priming the acquired immune response. In recent years DC have been shown to be capable of internalising a wide range of bacteria and parasites, including *Borrelia burgdorferi* (Filgueira *et al.*, 1996), *Bordetella bronchiseptica* (Guzman *et al.*, 1994), *Chlamydia* (Igietseme *et al.*, 1998; Ojcius *et al.*, 1998), *Salmonella* (Marriott *et al.*, 1999) and *Leishmania* (Moll, 1993; Moll *et al.*, 1995). Both *Mtb* (Henderson *et al.*, 1997) and *M.bovis* BCG (Inaba *et al.*, 1993) have been shown to be taken up by DC. Thus DC, in addition to macrophages are now established as having a major role in phagocytosis of intracellular pathogens.

The fate of the infectious agent following phagocytosis by DC could be a key determinant in the presentation of antigen by DC to naïve T cells. However, there have been few studies describing the fate of bacteria or parasites inside DC.

Murine splenic DC were shown to take up *Salmonella gordonii* by phagocytosis, with the bacteria subsequently being found in a partially degraded form, within phagosomes (Rescigno *et al.*, 1998). Peripheral blood, monocyte-derived DC were found to contain phagosome-bound *Mtb* 48 hours after infection (Henderson *et al.*, 1997).

Murine DC isolated from bone marrow were shown to be capable of internalization of *Chlamydia* and the bacteria were observed to be present in vacuoles rapidly fusing with the cell lysosomes; furthermore the bacteria were killed by the DC by 24 hours

after infection (Ojcius *et al.*, 1998). *Listeria monocytogenes* was found to invade DC but then escape from DC phagosomes into the cytoplasm (Guzman *et al.*, 1995).

In this study we used the immortalised tsDC cell line to investigate the fate of *Mtb* within dendritic cells. Firstly the ultrastructural changes and intracellular localization of *Mtb* were studied using electron microscopy. Secondly the effect on the viability of *Mtb* of phagocytosis by tsDC was investigated.

3.2 Ultrastructural changes in tsDC following uptake of *Mtb*

Approximately 10^6 tsDC were infected with *Mtb* at a ratio of 5 bacteria per DC. Ziehl-Neelsen staining confirmed that, 12 hours after infection, most of the DC had taken up *Mtb*. The fate of the phagocytosed *Mtb* and the ultrastructural changes occurring in the infected DC were monitored by electron microscopy.

By 12 hours the *Mtb* had been extensively internalised. The tsDC exhibited the typical DC morphology, with many cytoplasmic processes extending into the surrounding medium (Figure 3.1).

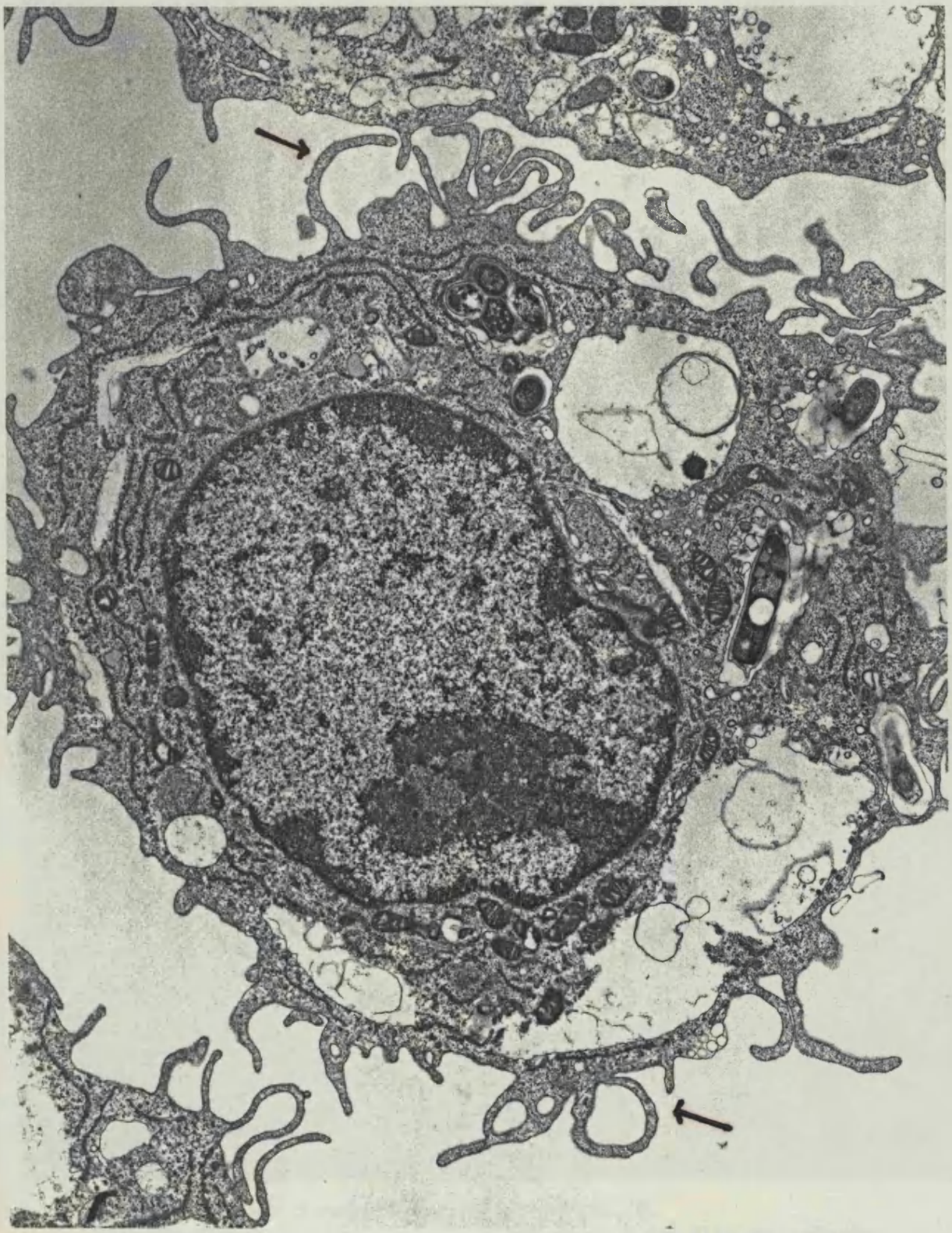

Figure 3.1 Electron micrograph showing *Mtb*-infected DC
X 12 500 magnification showing cell cytoplasmatic processes, 12 hr after infection.

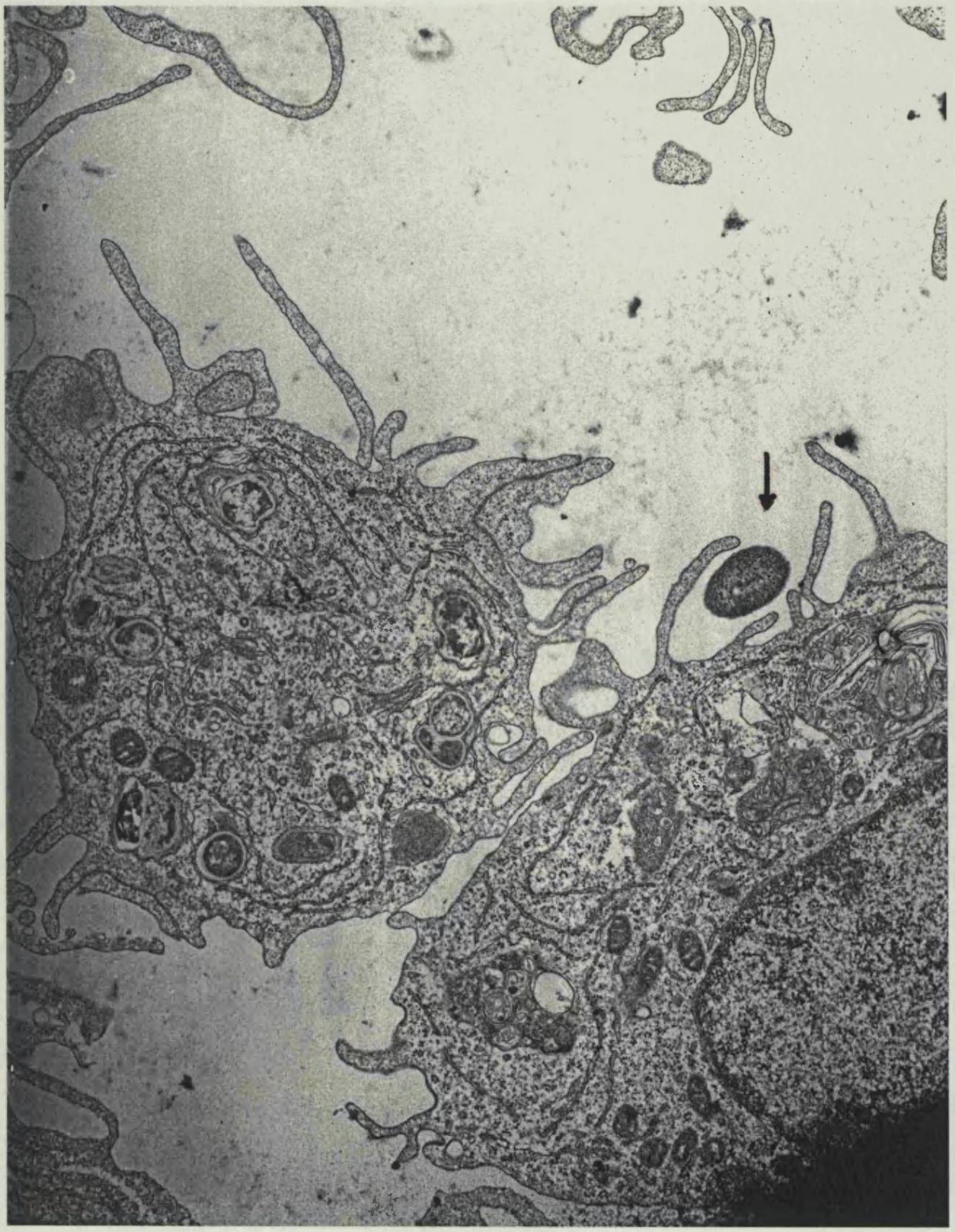

Figure 3.2 Electron micrograph showing *Mtb*-infected DC
X 16 500 magnification illustrating single *Mtb* phagocytosis, 12 hr after infection.

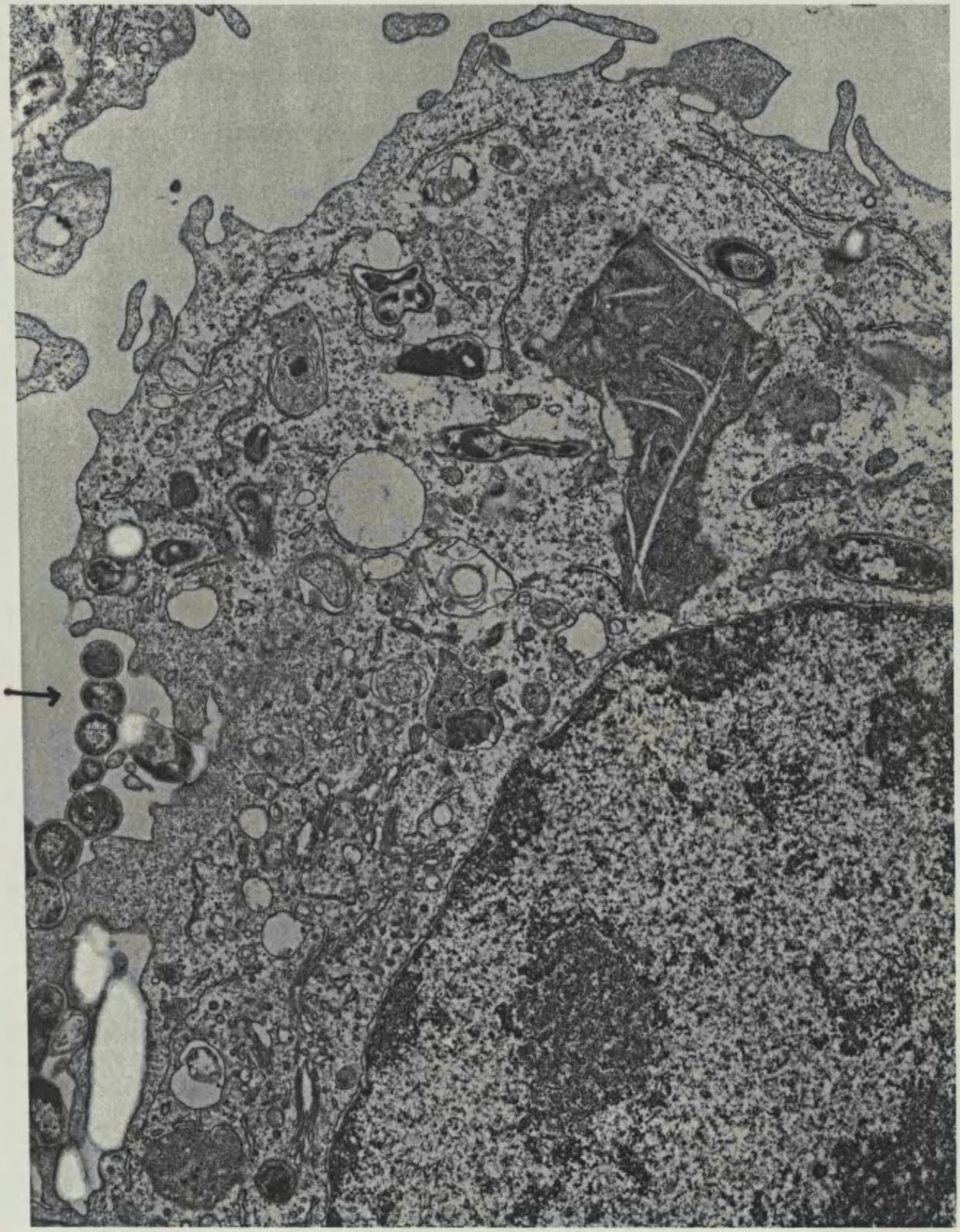

Figure 3.3 Electron micrograph showing *Mtb*-infected DC
X 13 200 magnification of *Mtb* phagocytosis in small clumps, 12 hr after infection.

It was possible to identify bacteria which were being phagocytosed, either singly (Figure 3.2) or in small clumps (Figure 3.3). Either individual or small groups of bacteria which had already been phagocytosed were evident at this stage. In some cases it was possible to see an intact phagosomal membrane, whereas in others this membrane was not visible and may have been degraded (Figure 3.4). At higher magnification (Figure 3.5) it was possible to see that some of these bacteria-containing vesicles contained extensive membrane-like material and were associated with abundantly active endoplasmic reticulum.

Similar structures were apparent at 24 hours (Figures 3.6-3.8), with some of the bacteria undergoing ultrastructural changes (Figure 3.7). The DC showed large nuclei and an abundance of ribosomes and mitochondria, indicating active protein synthesis. We also started to see the appearance of a number of pale irregular-shaped bodies with small electron-dense granules inside (Figures 3.8 and 3.9). At higher magnification, these bodies were surrounded by a layer of more dense granular material within an electron-transparent zone (Figure 3.11).

It was still possible to see bacterial phagocytosis occurring, even at 48 hours (Figures 3.9 and 3.10), with an increasing accumulation of the pale bodies (Figure 3.11), and "empty" membrane-bound vesicles associated with the endoplasmic reticulum (Figure 3.12). By 72 to 96 hours there was no evidence of necrosis of dendritic cells or any evidence of apoptosis, eg condensed nucleus, cell shrinking. Uninfected DC did not show any of these characteristics (Figure 3.13).

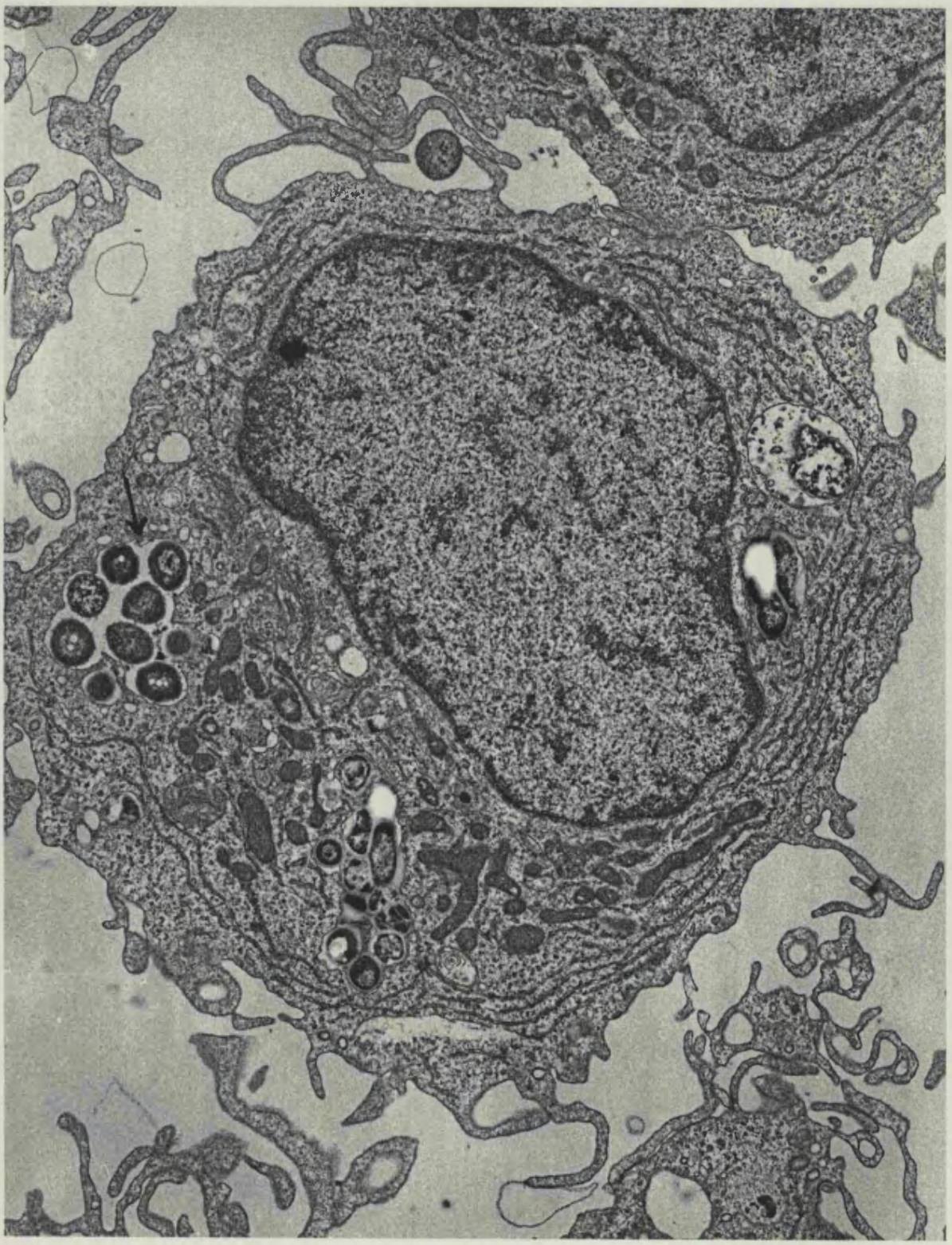

Figure 3.4 Electron micrograph showing *Mtb*-infected DC
X 12 500 magnification showing membrane-damaged bacilli in multivesicular phagocytic vacuoles, 12 hr after infection.

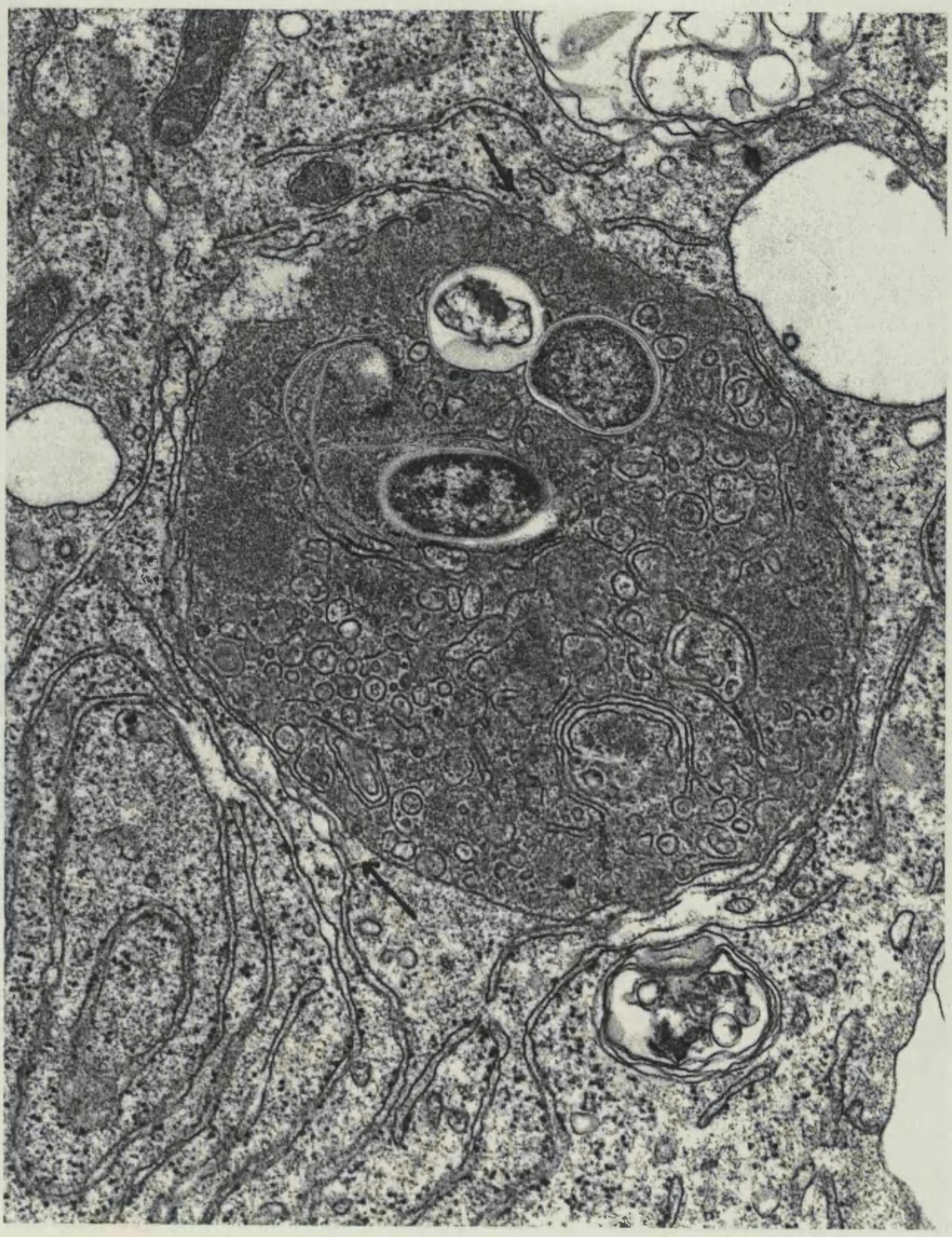

Figure 3.5 Electron micrograph showing *Mtb*-infected DC
X 40 000 magnification showing multivesicular vacuoles associated with endoplasmatic reticulum, 12 hr after infection.

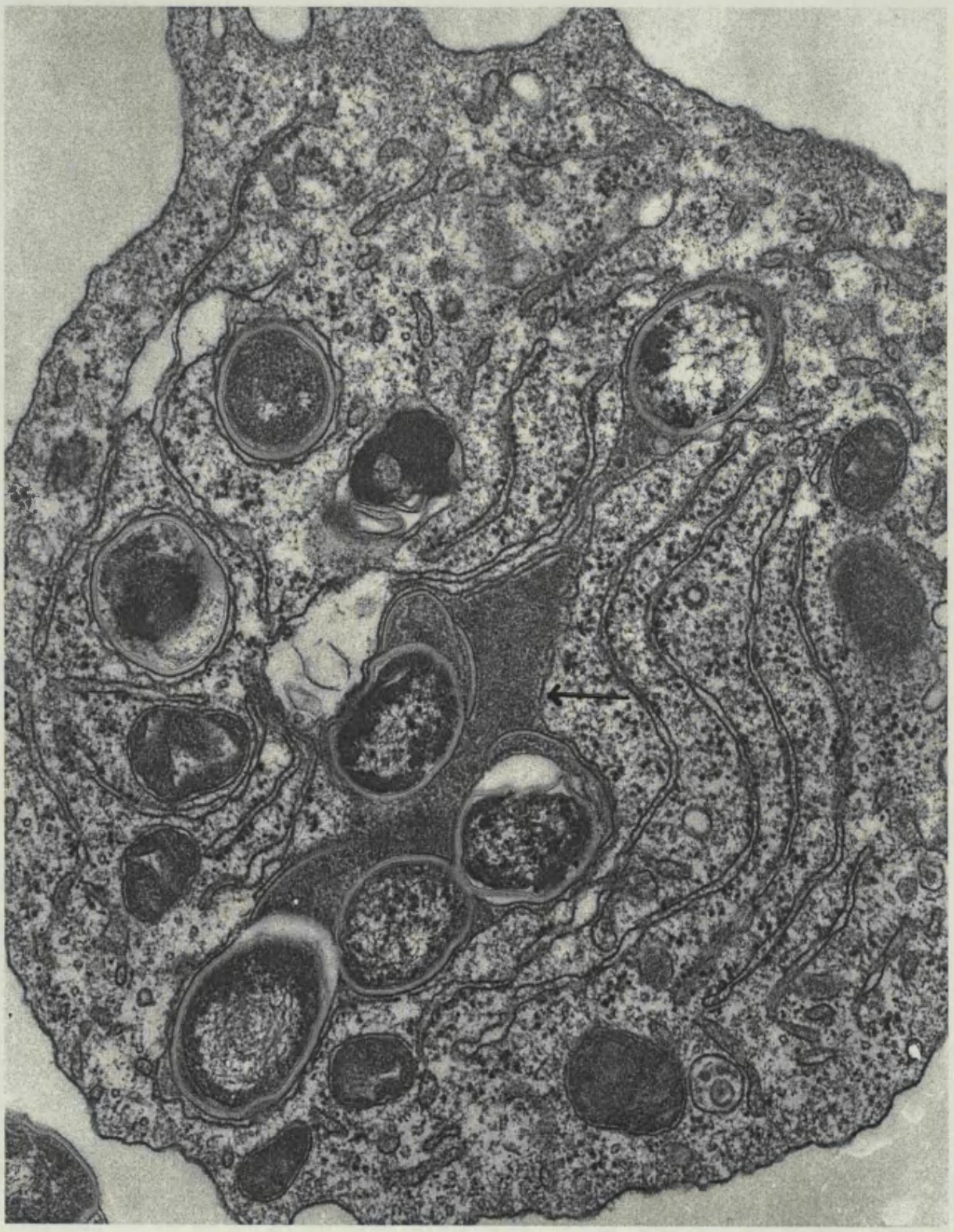

Figure 3.6 Electron micrograph showing *Mtb*-infected DC
X 50 000 magnification of membrane-bound phagosome containing *Mtb*, surrounded by endoplasmatic reticulum, 24 hr after infection.

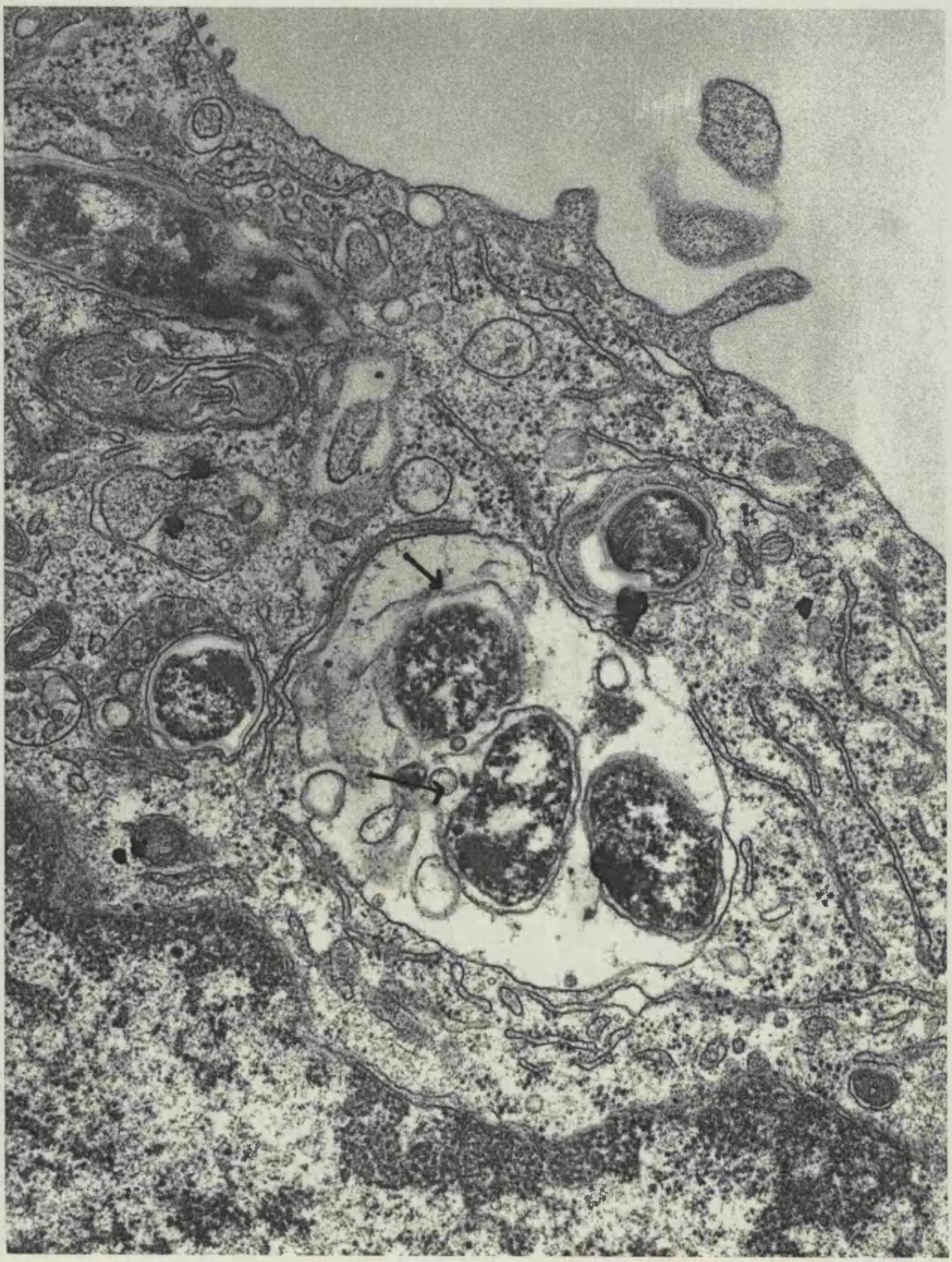

Figure 3.7 Electron micrograph showing *Mtb*-infected DC
X 40 000 magnification of *Mtb* inside the phagosome illustrating bacterial-membrane changes, 24 hr after infection.

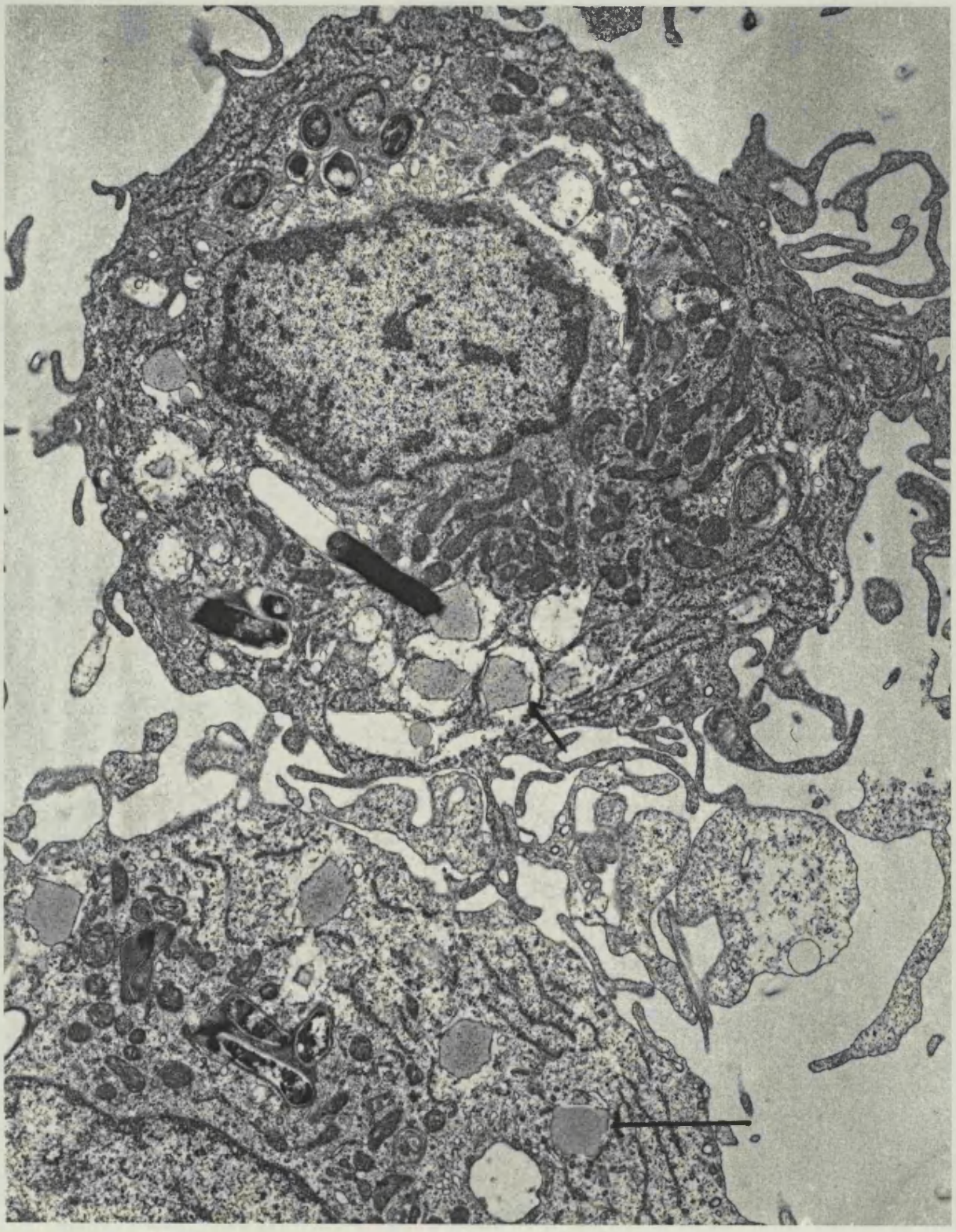

Figure 3.8 Electron micrograph showing *Mtb*-infected DC
X 12 500 magnification of DC showing the appearance of pale irregular-shaped bodies with small granules inside, 24 hr after infection.

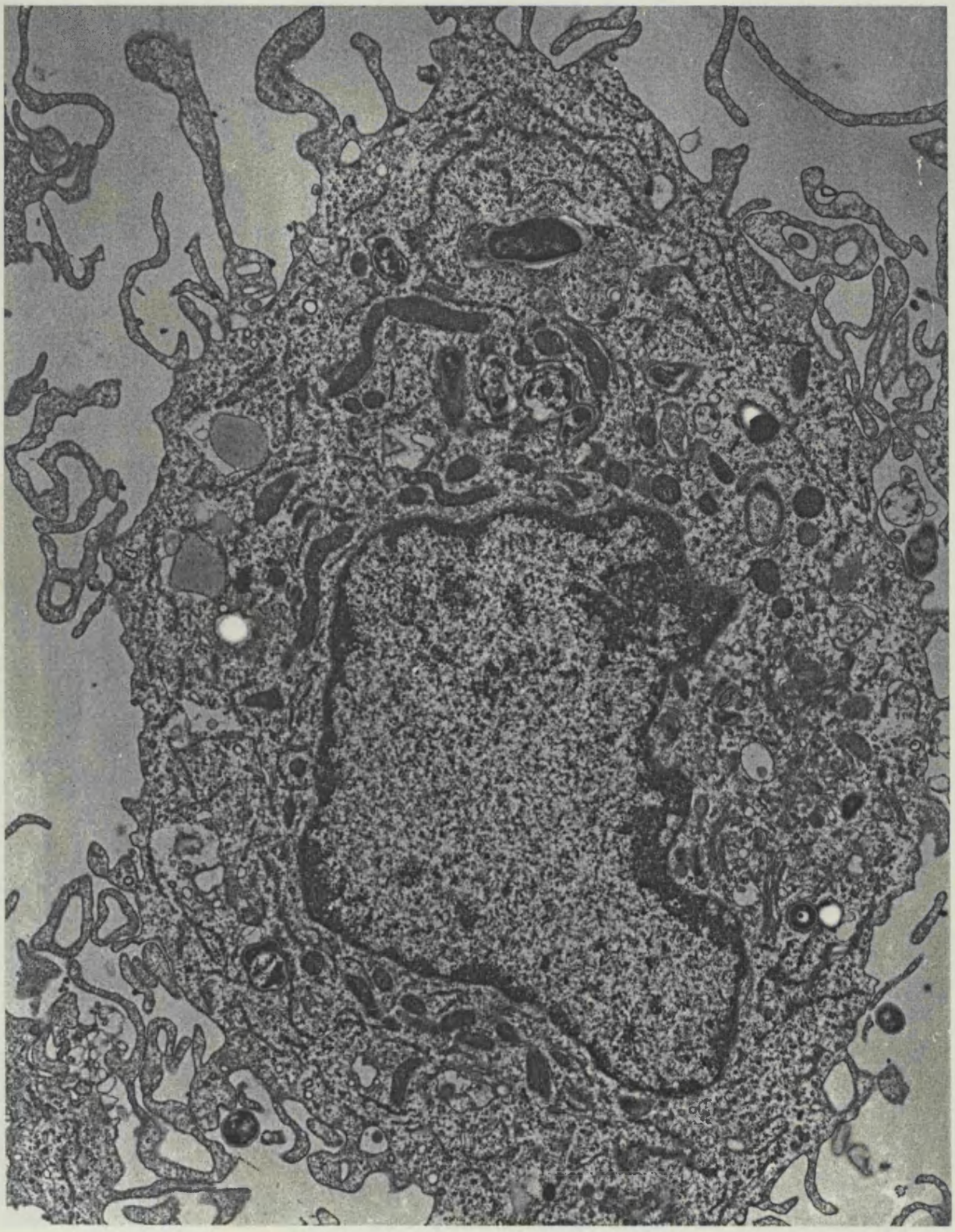

Figure 3.9 Electron micrograph showing *Mtb*-infected DC
X 12 500 magnification of DC illustrating the pale bodies, the large nuclei and the abundance of ribosomes and mitochondria, 24 hr after infection.

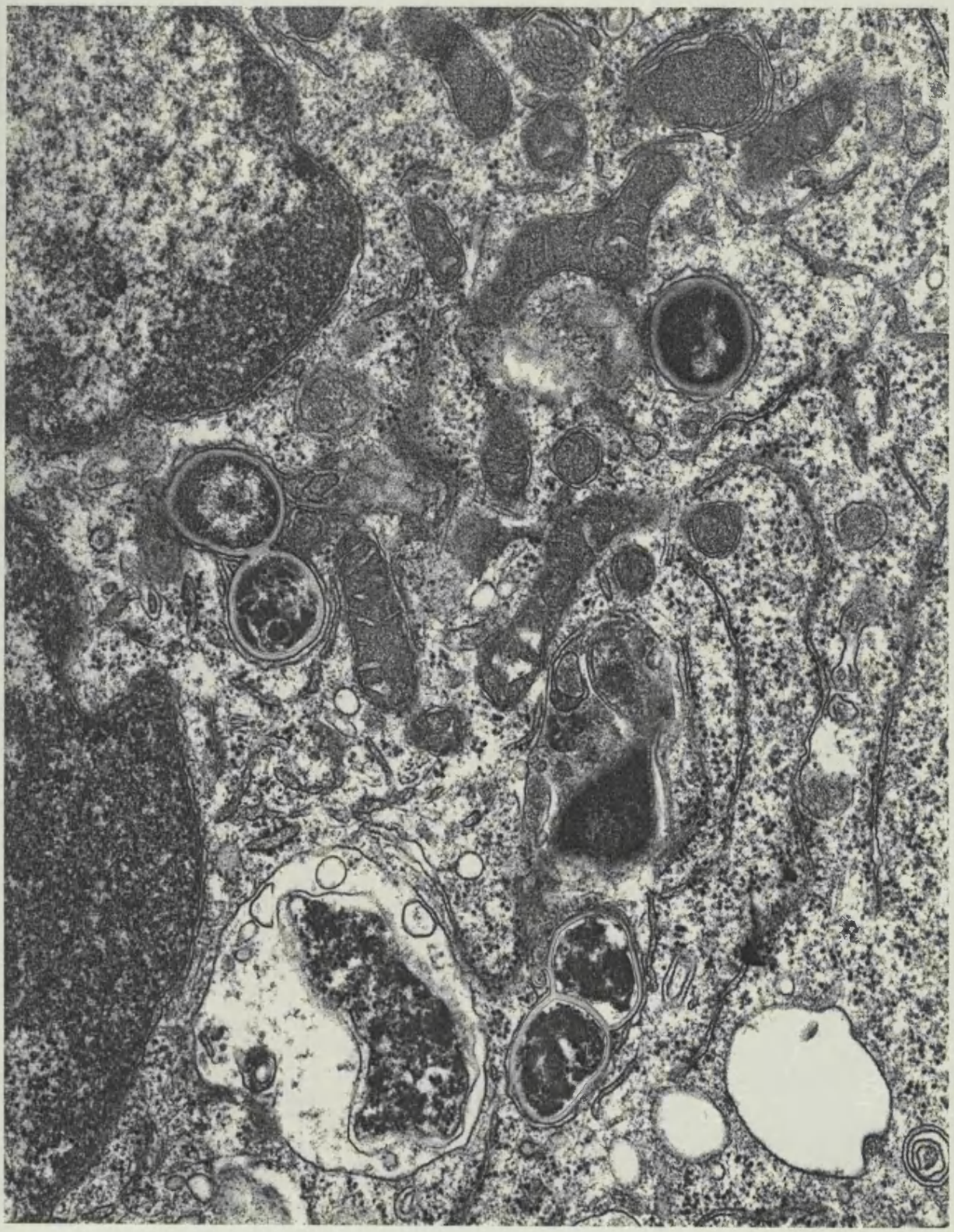

Figure 3.10 Electron micrograph showing *Mtb*-infected DC
X 40 000 magnification showing *Mtb* phagocytosed, 48 hr after infection.

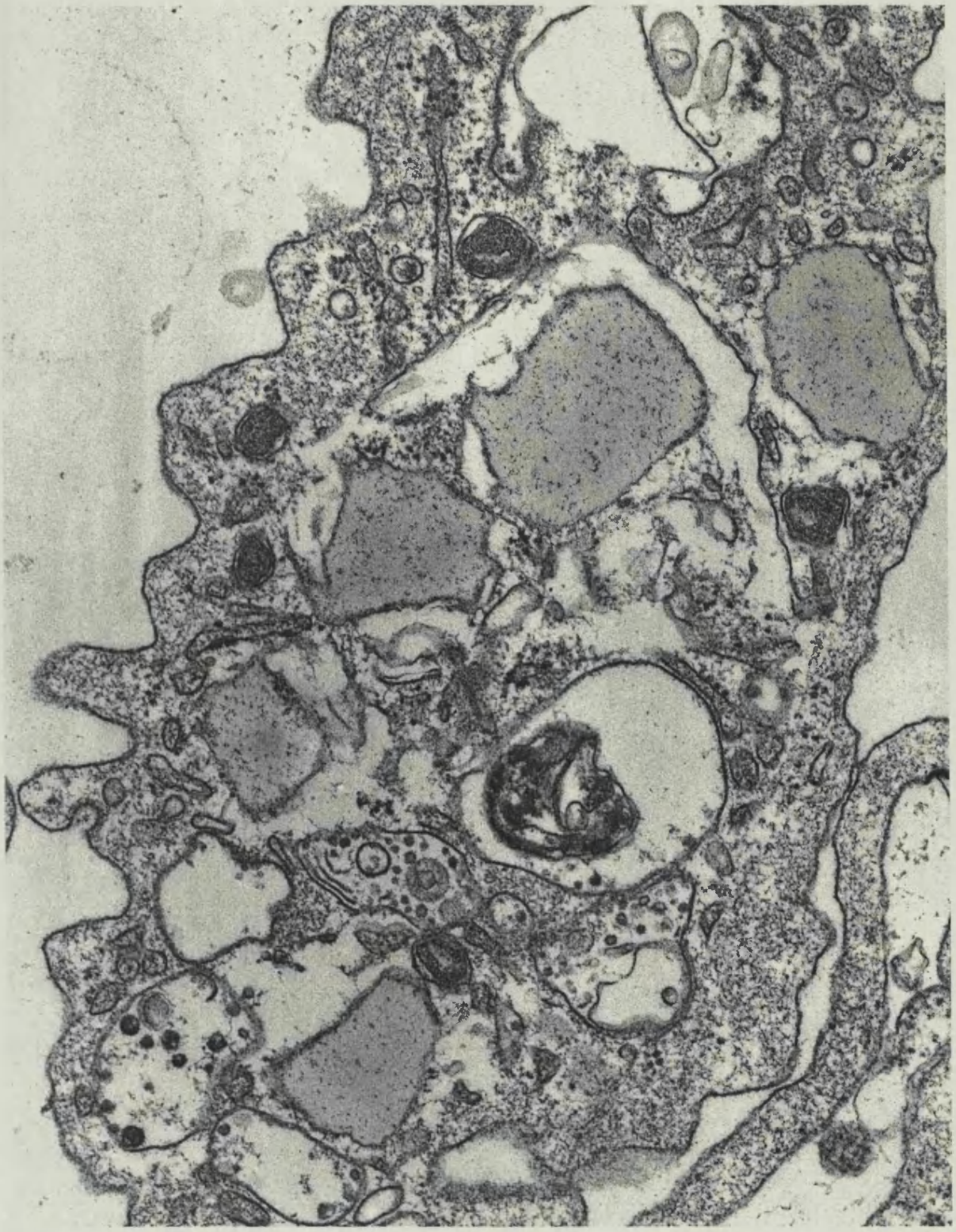

Figure 3.11 Electron micrograph showing *Mtb*-infected DC
X 50 000 magnification showing increasing accumulation of pale bodies surrounded by a layer of dense granular material within an electron-transparent zone, 48 hr after infection.

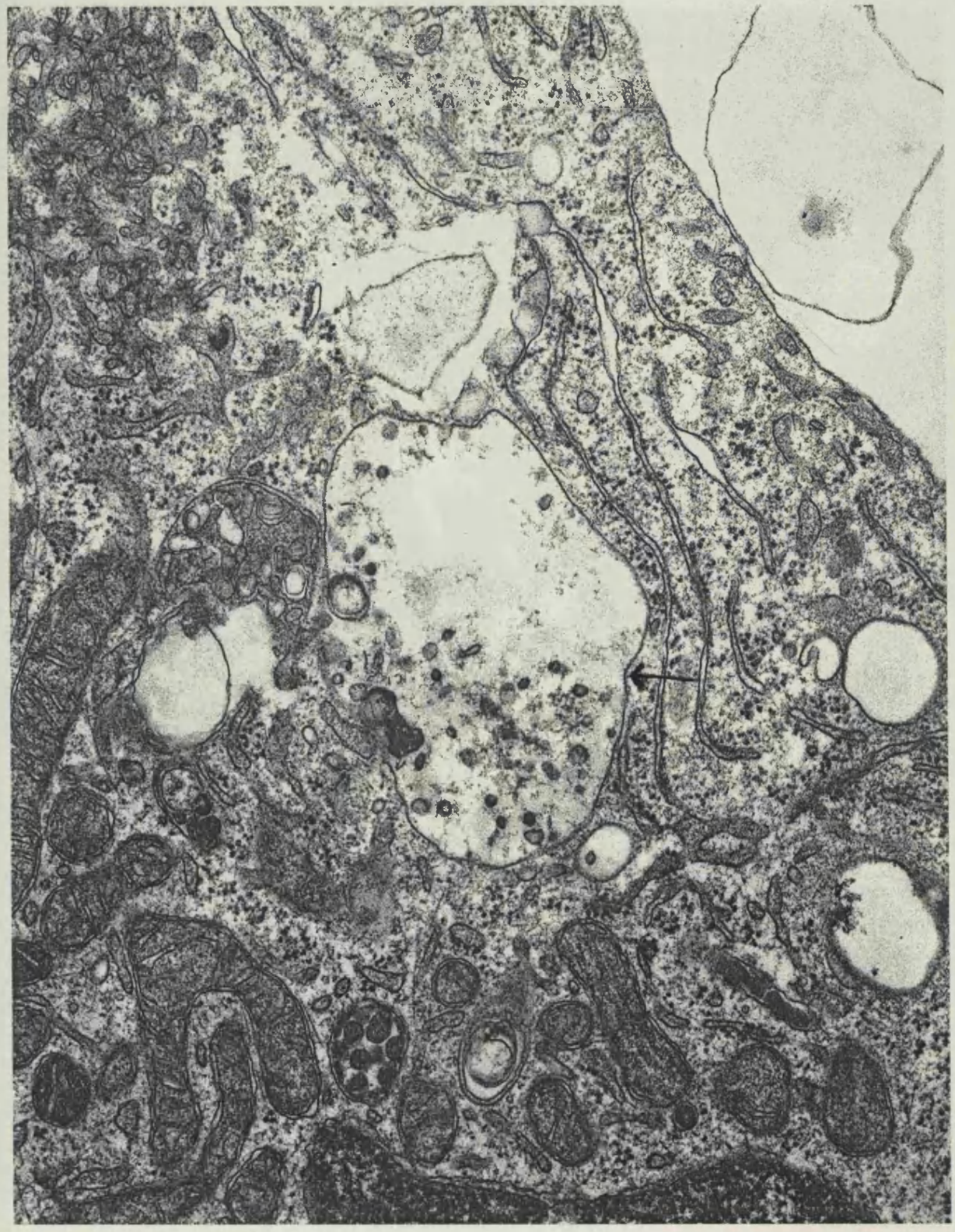

Figure 3.12 Electron micrograph showing *Mtb*-infected DC
X 40 000 magnification showing an empty membrane-bound vesicle associated with the endoplasmatic reticulum, 48 hr after infection.

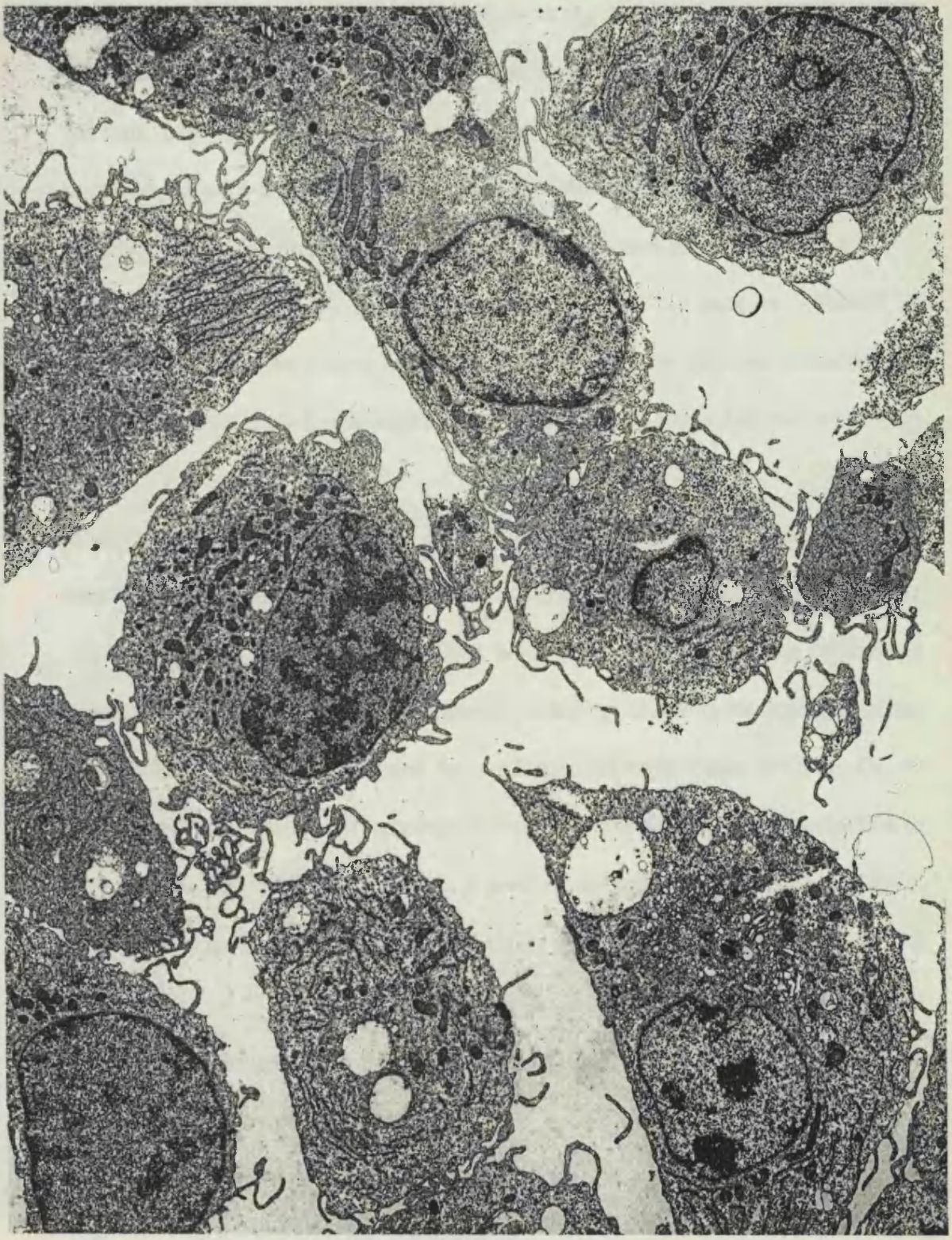

Figure 3.13 Electron micrograph showing uninfected DC
X 5 000 magnification of uninfected DC after 96 hours.

3.3 Changes in *Mtb* viability following phagocytosis by DC

The electron microscopy studies described above suggested that *Mtb* might be killed or damaged by the DC. In order to investigate this, the viability of *Mtb* at time intervals up to 5 days after infection was investigated. For comparison, peritoneal macrophages were simultaneously infected. The infected cells (macrophages and DC) were lysed at 24, 48, 72, 96 and 120 hours, and viable counts of *Mtb* carried out.

The results (Figure 3.14) confirm that the DC, were at least as effective as macrophages in phagocytosing *Mtb*. However, whereas *Mtb* appeared to multiply in the macrophages, there was a small decline in numbers of viable *Mtb* recovered from the DC.

Both radical nitrogen intermediates (RNI) and radical oxygen intermediates (ROI) have been implicated in the intracellular control of *Mtb* infections (Forrest *et al.*, 1988), (Hibbs *et al.*, 1988). Since RNI have been shown to have a particularly profound effect on the suppression of growth, or killing, of *Mtb* in macrophages (Chan *et al.*, 1992), we first investigated the production of nitric oxide (NO) by DC in response to *Mtb* infection. NO production was measured using the Griess reaction in *Mtb* infected and uninfected tsDC. As a positive control tsDC were also stimulated with rIFNγ plus LPS. Aminoguanidine (AMG) was included as an inhibitor of RNI production.

Thus, NO was determined in the following conditions:

(i) tsDC (negative control)

(ii) tsDC, *Mtb* infected

(iii) tsDC, *Mtb* infected, AMG-treated

(iv) tsDC, plus LPS/rIFNγ

(v) tsDC, plus LPS/rIFNγ, AMG-treated.

The results are shown in Figure 3.15.

By 7 hours, there was a significant increase in NO production in the tsDC treated with rIFNγ/LPS; this was maintained throughout the 72 hours observation, and was completely inhibited by AMG. In the *Mtb*-infected cells, there was a transient increase in NO production at 48 hours; however, by 72 hours, NO levels had returned to baseline levels. All the values are the mean of triplicate determinations (± SD).

In order to investigate more directly the role of RNI in controlling *Mtb* growth in tsDC the effect of inhibiting RNI production on the viability of *Mtb* within tsDC was investigated. In the first experiment the iNOS inhibitor L-NAME and the arginase inhibitor L-Norvaline were included in the medium. The results (Figure 3.16) indicate that RNI play at least some role in controlling *Mtb* infection in tsDC in that the presence of the inhibitors resulted in an increase in the numbers of viable *Mtb* recovered five days after infection.

In a second experiment, the effect of the iNOS inhibitor L-NAME was compared with that of the ROI inhibitor TMB-8 (Figure 3.17). Again, L-NAME had a significant effect on the recovery of viable *Mtb*. However TMB-8 also had a similar effect, suggesting that both ROI and RNI play a role in controlling *Mtb* infection in tsDC.

(In both experiments, determinations were triplicates ± SD, Students t-test was applied to analyse the results, and values of $p<0.01$ were considered significant- represented in the Figures by *)

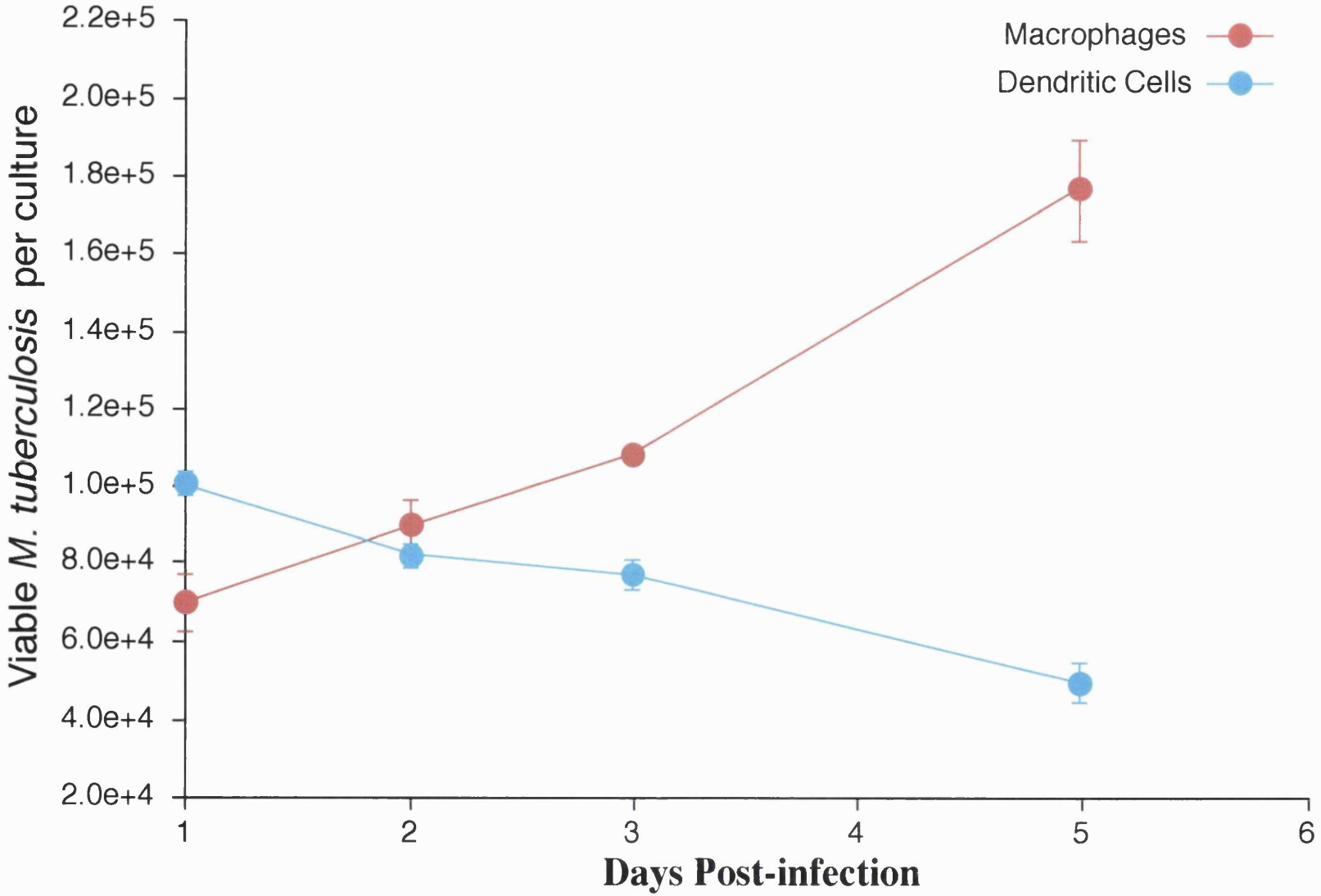

Figure 3.14 Growth of *Mtb* in tsDC and peritoneal macrophages measured by the colony-forming unit assay at different times post-infection. Data represent the mean counts from three separate well monolayers.

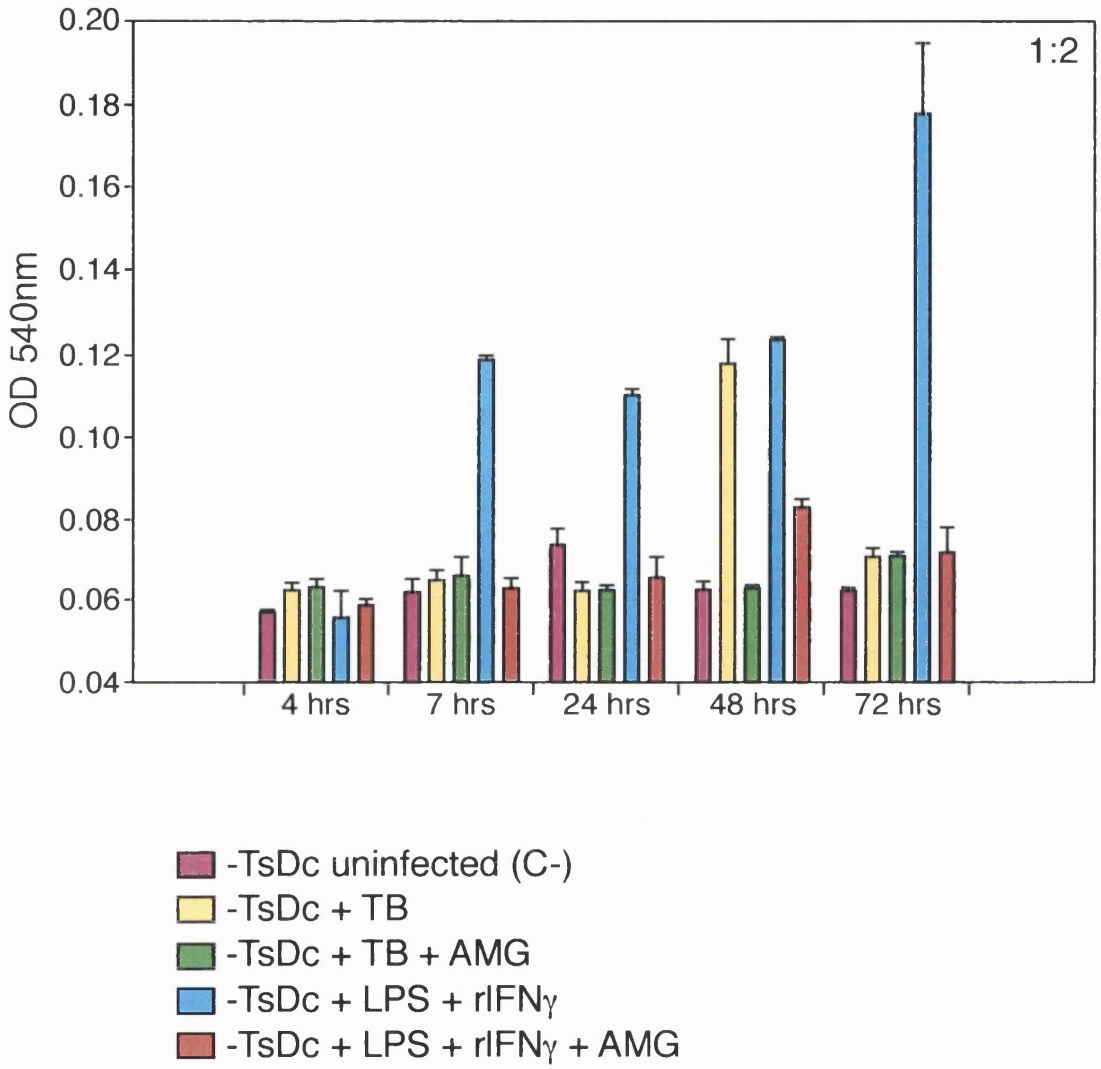

Figure 3.15 Nitric oxide production in *Mtb* infected and uninfected tsDC. As a positive control tsDC were stimulated with rIFNγ plus LPS. Aminoguanidine was included as an inhibitor of RNI production. Supernatants were collected between 4-72 hrs and anaysed by ELISA (1:2 dilution). All the values are the mean of triplicate determinations.

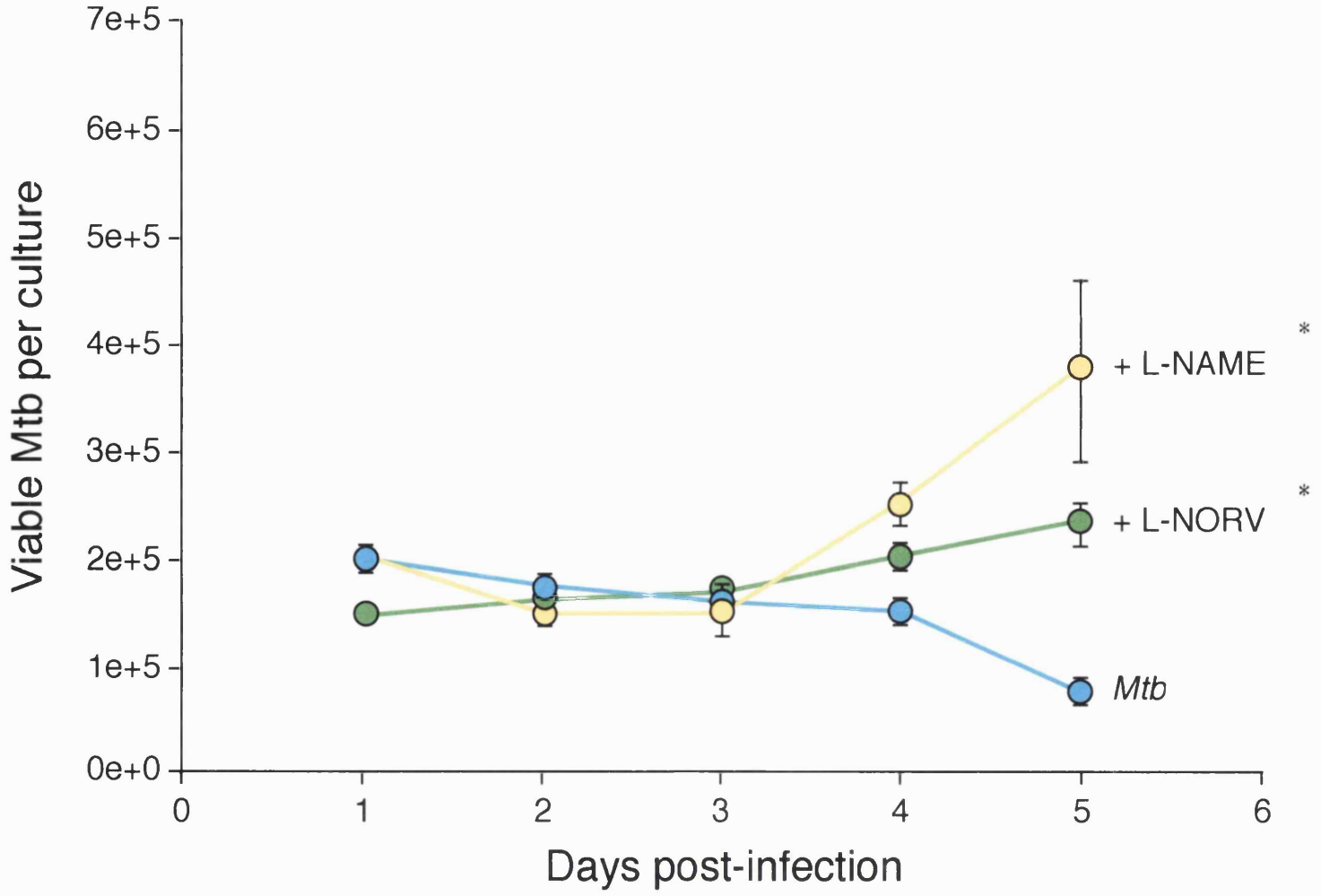

Figure 3.16 Growth of *Mtb* measured by the colony-forming unit assay at different times post-infection, in tsDC alone (blue) and in tsDC *Mtb*- infected with added L-NAME (yellow) or L-Norvaline (green). Data represent the mean counts from four separate well monolayers. * - $p<0.01$ (significative difference from tsDC cultured with *Mtb* alone).

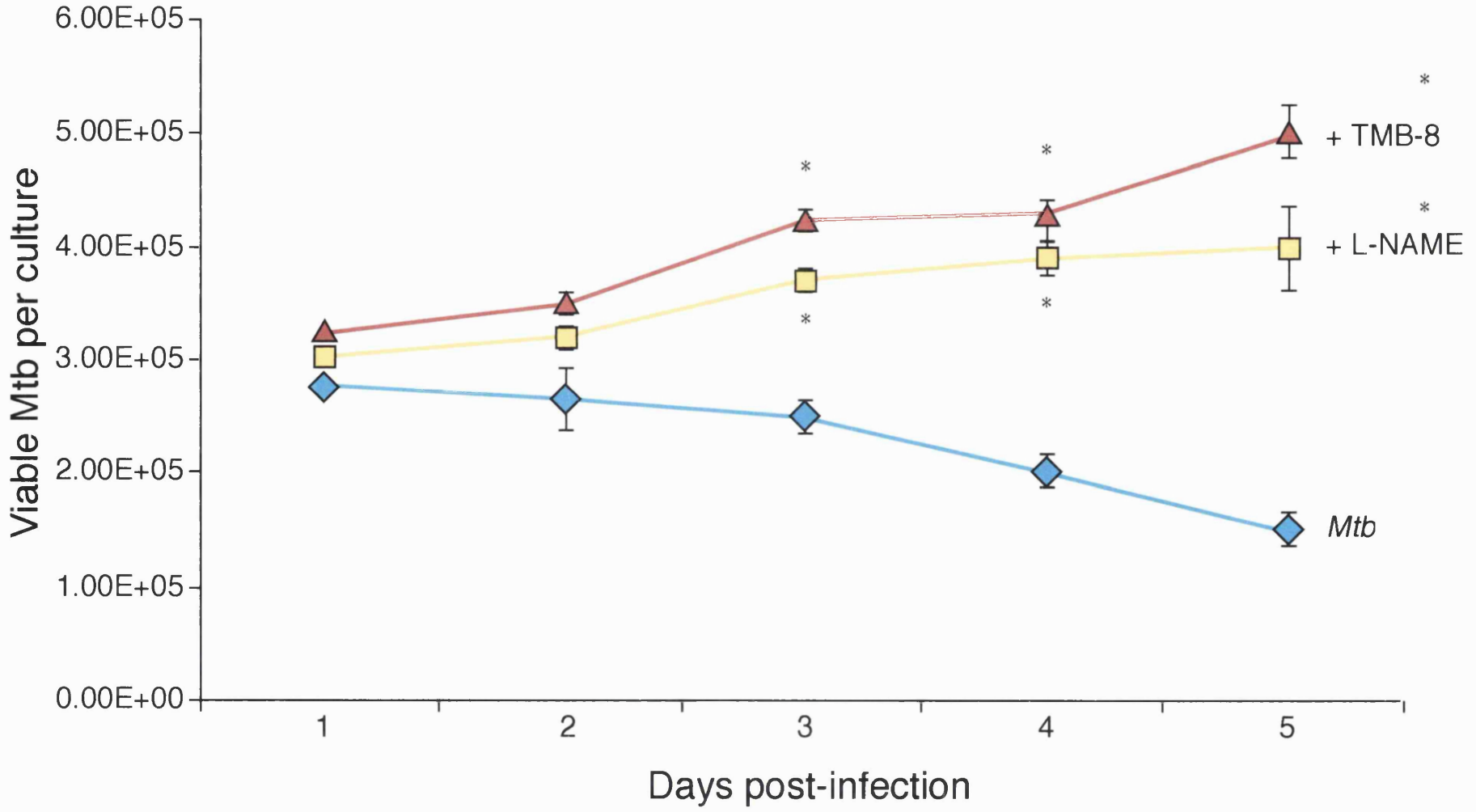

Figure 3.17 Growth of *Mtb* measured by the colony-forming unit assay at different times post-infection, in tsDC alone (blue) and in tsDC-*Mtb* infected with added L-NAME (yellow) or TMB-8 (red). Data represent the mean counts of four separate well monolayers.*-$p<0.01$ (significative difference from tsDC cultured with *Mtb* alone)

3.4 Discussion

The main objective of these experiments was to carry out a preliminary investigation of the interaction between *Mtb* and DC, in order to establish the phagocytic capacity of the tsDC cell line, and to investigate how the cells handled the *Mtb* infection.

The tsDC were found to effectively phagocytose *Mtb* as assessed by electron microscopy, by conventional Ziehl-Neelsen staining and by lysing the cells and recovering viable *Mtb*. Although the intracellular localisation of the phagocytosed *Mtb* was not studied in depth, it was possible to draw a number of broad conclusions.

Following phagocytosis, the *Mtb*-containing phagosomes appear to fuse to form large vesicles which usually contain several mycobacteria; these resemble the previously reported MIIC vesicles (Nijman *et al.*, 1995), which are part of the late endocytic pathway and are rich in MHC class II molecules (Nijman *et al.*, 1995). Time constraints meant that it was not possible to use fluorescence microscopy or immunoelectron microscopy to investigate the nature of these vesicles further. Similarly the nature of the pale bodies which accumulated in the cells during the course of the infection was not investigated. It is possible that these contained mycobacterial lipids, since *Mtb*-infected macrophages are known to contain large amounts of extrabacillary lipids (Berman *et al.*, 1996). Alternatively they could be similar to the previously reported exosomes (Zitvogel *et al.*, 1998) which express MHC molecules and are effective antigen-presenting vesicles.

Experiments aimed at investigating the ability of *Mtb* to replicate in tsDC indicated that, unlike peritoneal macrophages, tsDC were able to inhibit growth of the bacteria. However, the extent of any mycobacterial killing by the tsDC was very small, suggesting that, DC which phagocytose *Mtb* in tissue such as lung are likely to traffic

to the lymph nodes while carrying live *Mtb*, hence enabling *Mtb* to gain access to the lymph node.

Activated murine macrophages have been shown to inhibit the growth of *Mtb* by the production of RNI (Chan *et al.*, 1992; Flesch and Kaufmann, 1991). Our preliminary experiments indicate that both RNI and ROI are involved in the inhibition of *Mtb* growth in tsDC. There are few studies investigating the ability of other bacteria and parasites to survive and /or grow within DC. However, while *Salmonella* (Marriott *et al.*, 1999) and *Leishmania* (Moll, 1993; Moll *et al.*, 1995) appear to survive within DC, *Chlamydia* are killed by a phagosome-lysosome fusion pathway (Ojcius *et al.*, 1998).

Time did not permit further investigation of the interaction between DC and *Mtb*. Future work of particular interest would be to investigate the nature of the *Mtb*-containing vesicles, and the pale, membrane bodies using immunofluorescence or immuno-electron microscopy, to try and identify the cellular origins of these organelles.

Chapter 4
Results

Chapter 4

Cytokine and surface molecule expression by *Mtb* infected DC

4.1 Introduction

In the previous chapter it was apparent that DC were capable of phagocytosing *Mtb*. In peripheral tissue DC are present in an immature state, unable to stimulate T cells and specialised in antigen-sensing or antigen-uptake. However, after an interaction with antigen or inflammatory products, DC develop a more mature phenotype in which they express higher surface levels of MHC Class II molecules and costimulatory molecules, and secrete increased levels of cytokines (Banchereau and Steinman, 1998; Guery *et al.*, 1996). It is in this mature, differentiated state that DC migrate to lymphoid tissue and there are able to prime specific T cells (Larsen *et al.*, 1990).

In addition to being efficient antigen presentation cells, DC are also involved in modulating the immune response, by controlling the development of TH1 or TH2-type responses following exposure to infectious agents (Sousa *et al.*, 1999). The production of IL-12 by DC as a rapid response to an intracellular parasite is thought to be a major determinant in establishing a TH1 response (Sousa *et al.*, 1997), and thus providing a link between the innate and the acquired immune response (Trinchieri and Gerosa, 1996; Trinchieri, 1998). This is likely to be particularly important in mycobacterial infections where TH1-type responses are important in protection against disease, although it is important to realise that other factors such as the type and amount of antigenic stimulation and the expression of costimulatory molecules are likely to be involved.

Previous studies (Henderson *et al.*, 1997) have shown that human dendritic cells phagocytose *Mtb* and differentiate towards a mature phenotype. This involves the

secretion of both TH1 (IL-12, IL-1, TNF-α) and TH2 (IL-10, IL-6) cytokines, and up-regulated surface expression of MHC Class I and II, B7-1, CD40 and ICAM-1. Similar results have been reported for BCG-infected, primary murine DC (Demangel *et al.*, 1999). This ability of DC to produce both TH1 and TH2 cytokines in response to mycobacterial infection emphasises that the factors which influence the polarisation of the TH response may be complex.

The experiments described in this Chapter are aimed at investigating the maturation of the cell line tsDC following infection with *Mtb*, and comparing this to the responses seen in primary murine DC.

4.2 Cytokine production

4.2.1 The production of cytokines by tsDC following phagocytosis of *Mtb*

The expression of TNF-α, IL-1β, IL-6, IL-10, IL-18 and IFN-γ was monitored by RT-PCR using RNA isolated from tsDC 24 hours after infection with *Mtb*. The production of IFN-γ, TNF-α, IL-6, IL-12 and IL-1β, was also monitored by ELISA at 12, 24 and 48 hours after infection.

As shown in Figure 4.1, expression of IL-6, IL-12, TNF-α and IL-1β mRNA was upregulated following *Mtb* infection, whereas IL-18 (Figure 4.1), IL-10 and IFN-γ (Figure 4.2) mRNA could not be detected either pre or post-infection. To confirm these results supernatants from *Mtb* infected tsDC were compared to supernatants from uninfected tsDC for the presence of cytokines. High levels of TNF-α and IL-6, and low levels of IL-1β and IL-12 were detectable in the infected tsDC supernatants, significantly higher than levels seen in uninfected controls (Figure 4.3). There was no significant production of IFN-γ (Figure 4.4).

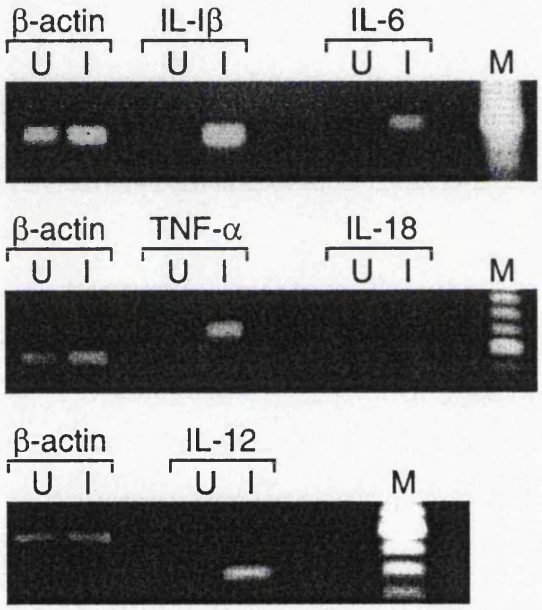

Figure 4.1 Infection of tsDC with *Mtb* and cytokine expression.
RT-PCR analysis for the detection of IL-1β, IL-6, TNF-α, IL-18, IL-12 and β-actin specific mRNAs after *Mtb* infection. RNA obtained from non-infected or from 24 hr infected tsDC was reverse transcribed and amplified with specific primers for these cytokines or β-actin as positive control.

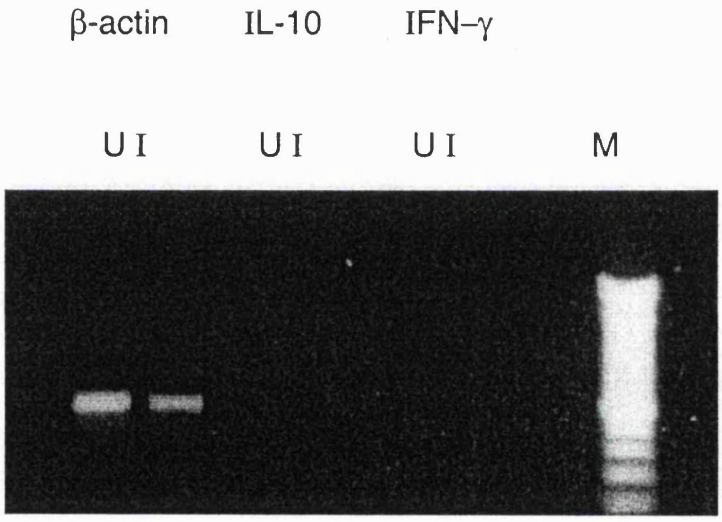

Figure 4.2 Infection of tsDC with *Mtb* and cytokine expression.
RT-PCR analysis for the detection of IL-10, IFNγ and β-actin specific mRNAs after *Mtb* infection. RNA obtained from non-infected or from 24 hr infected tsDC was reverse transcribed and amplified with specific primers for these cytokines or β-actin as positive control.

The cytokine concentrations of each group were expressed as geometric means of pg/ml ± SD. Student's t-test was carried out to analyse statistical significance of the results; *p*-values lower than 0.01 were considered to be significant – represented in the Figures by *)

4.2.2 Production of cytokines by primary, bone marrow-derived DC following phagocytosis of *Mtb*

In order to compare the tsDC response to *Mtb* infection with that of primary DC, the cytokine production by bone marrow-derived DC following infection with *Mtb* was investigated, using protocols identical to those used for tsDC. In general the results obtained with the primary DC were similar to those obtained with the tsDC, except that the levels of cytokine production tended to be higher (Figure 4.5). Once more there was no evidence of IFN-γ secretion (Figure 4.6), although there was significant production of IL-10 by the *Mtb* infected DC (Figure 4.7).

The cytokine concentrations of the groups, as previously, were expressed as geometric means of pg/ml ± SD. Student's t-test was carried out to analyse statistical significance of the results; *p*-values lower than 0.01 were considered to be significant-represented in the Figures by *.

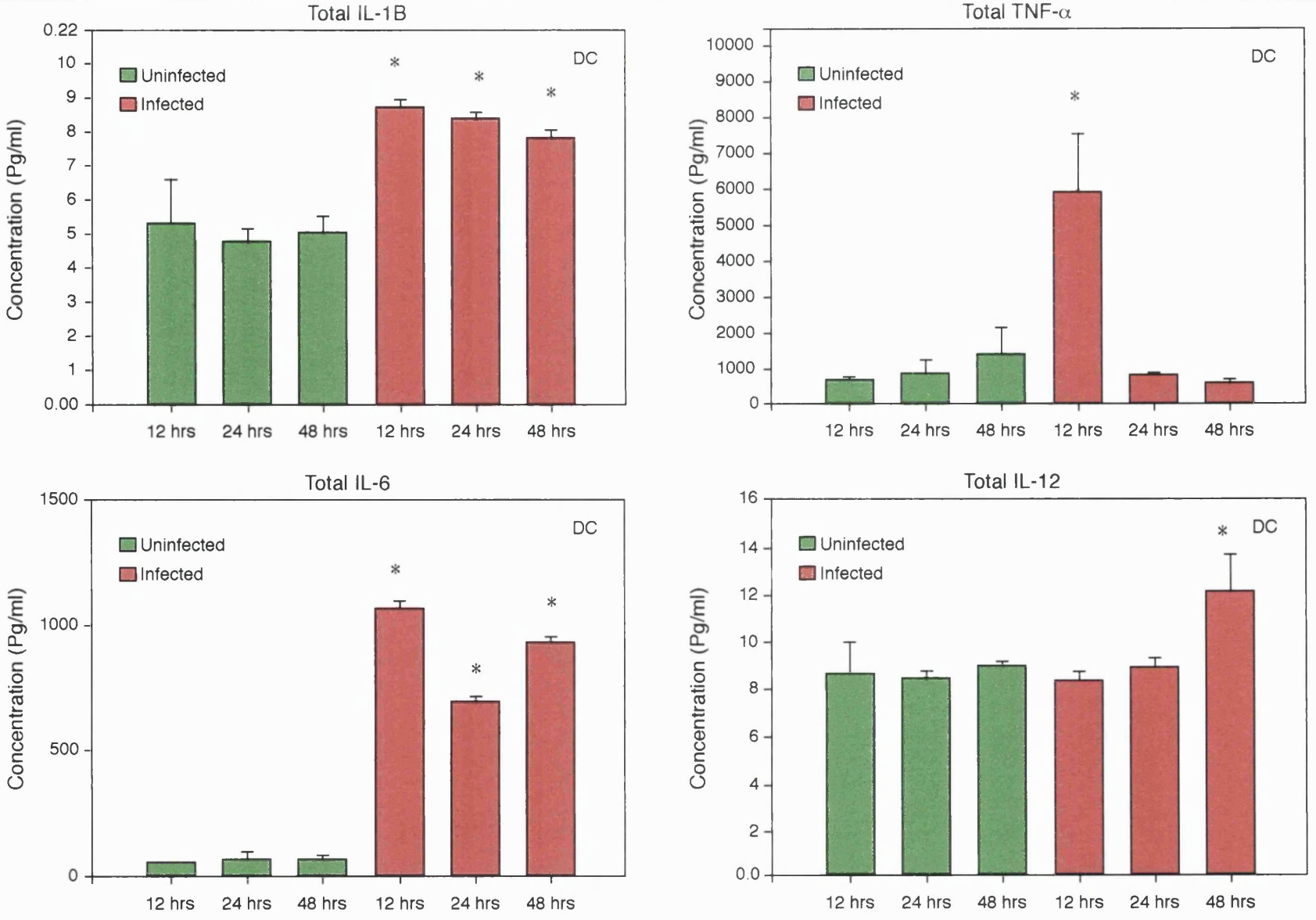

Figure 4.3 Infection of tsDC with *Mtb* and cytokine expression. IL-1B, IL-6, TNF-α and IL-12 cytokine secretion by tsDC or *Mtb* infected tsDC. Supernatants were collected 12 hr, 24 hr and 48 hr after infection and analysed by ELISA. All the values are the means of triplicate determinations (±SD). If $p<0.01$ there is a statistic difference from *Mtb*-infected to the uninfected cells-*

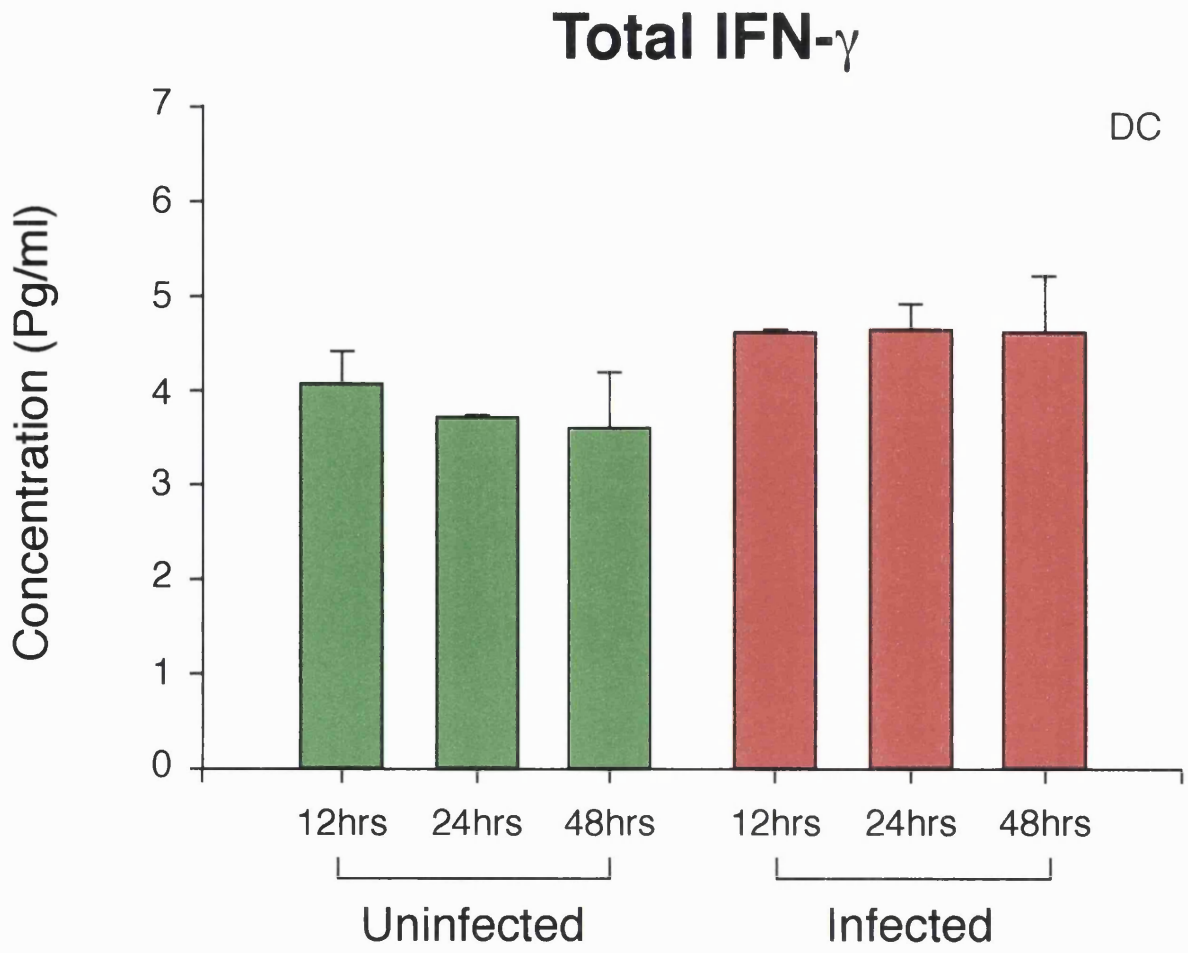

Figure 4.4 Infection of tsDC with *Mtb* and cytokine expression. IFN-γ secretion by tsDC or *Mtb* infected tsDC. Supernatants were collected 12hr, 24 hr and 48 hr after infection and analysed by ELISA. All the values are the means of triplicate determinations (±SD).

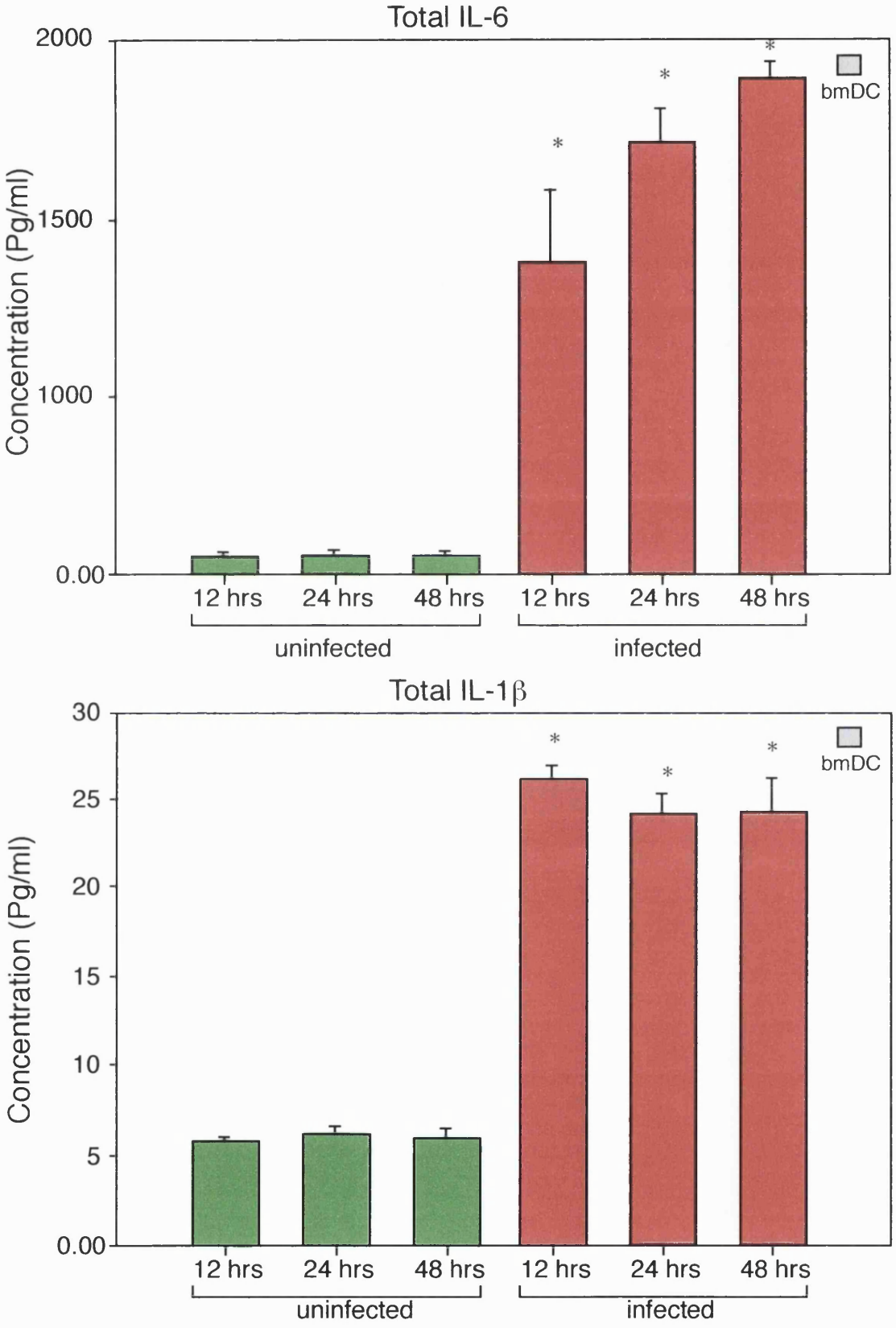

Figure 4.5 Infection of bmDC with *Mtb* and cytokine expression. IL-6 and IL-1B secretion by bmDC or *Mtb* infected bmDC. Supernatants were collected 12 hr, 24hr and 48 hr after infection and analysed by ELISA. All the values are the means of triplicate determinations (±SD).If $p<0.01$ there is a statistic difference from *Mtb*-infected to the uninfected cells.

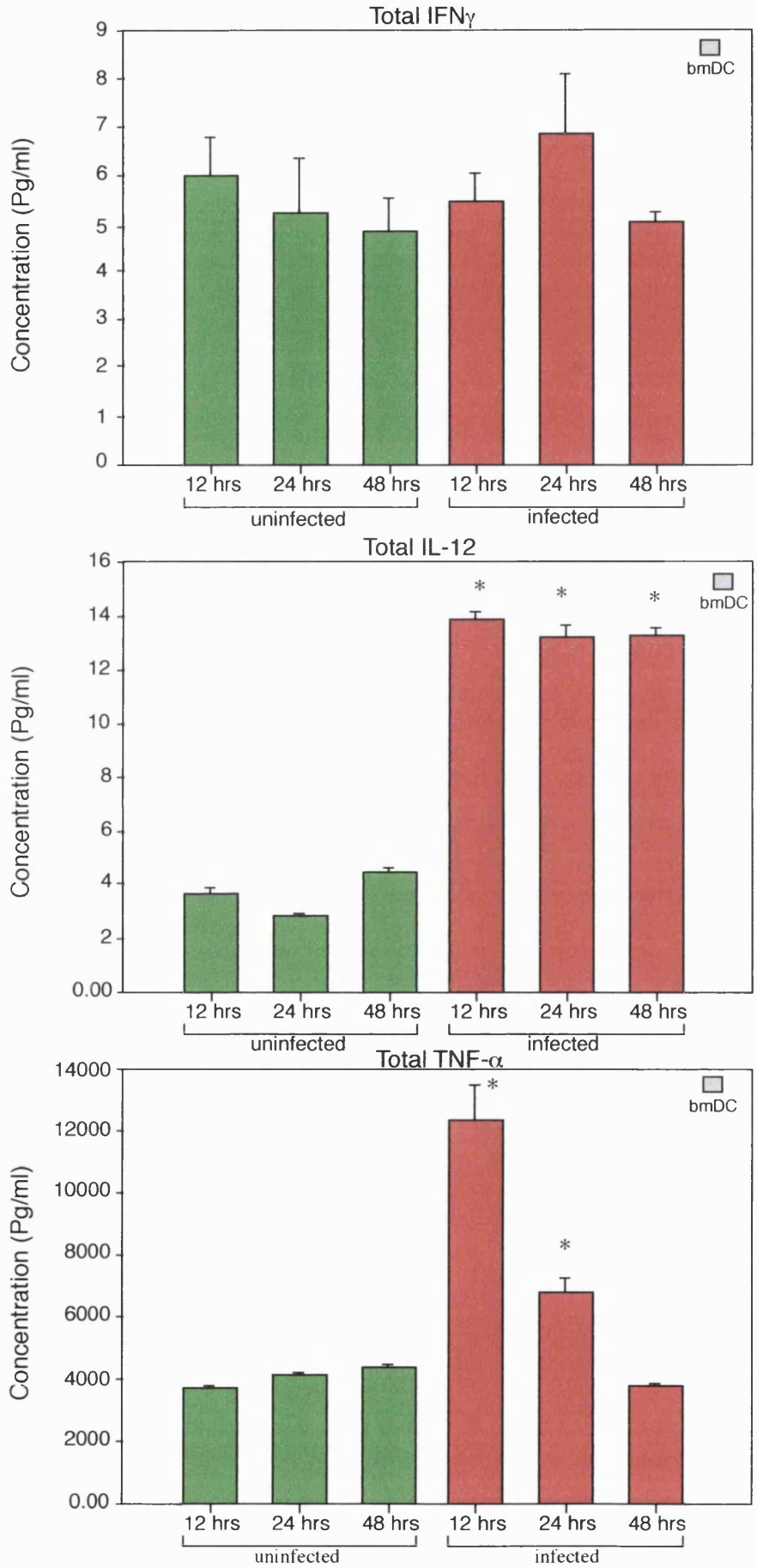

Figure 4.6 Infection of bmDC with *Mtb* and cytokine expression. IFNγ , IL-12 and TNF-α secretion by bmDC or *Mtb* infected bmDC. Supernatants were collected 12 hr, 24hr and 48 hr after infection and analysed by ELISA. All the vallues are the means of triplicate determinations (±SD).If $p<0.01$ there is a statistic difference from *Mtb*-infected to the uninfected cells.

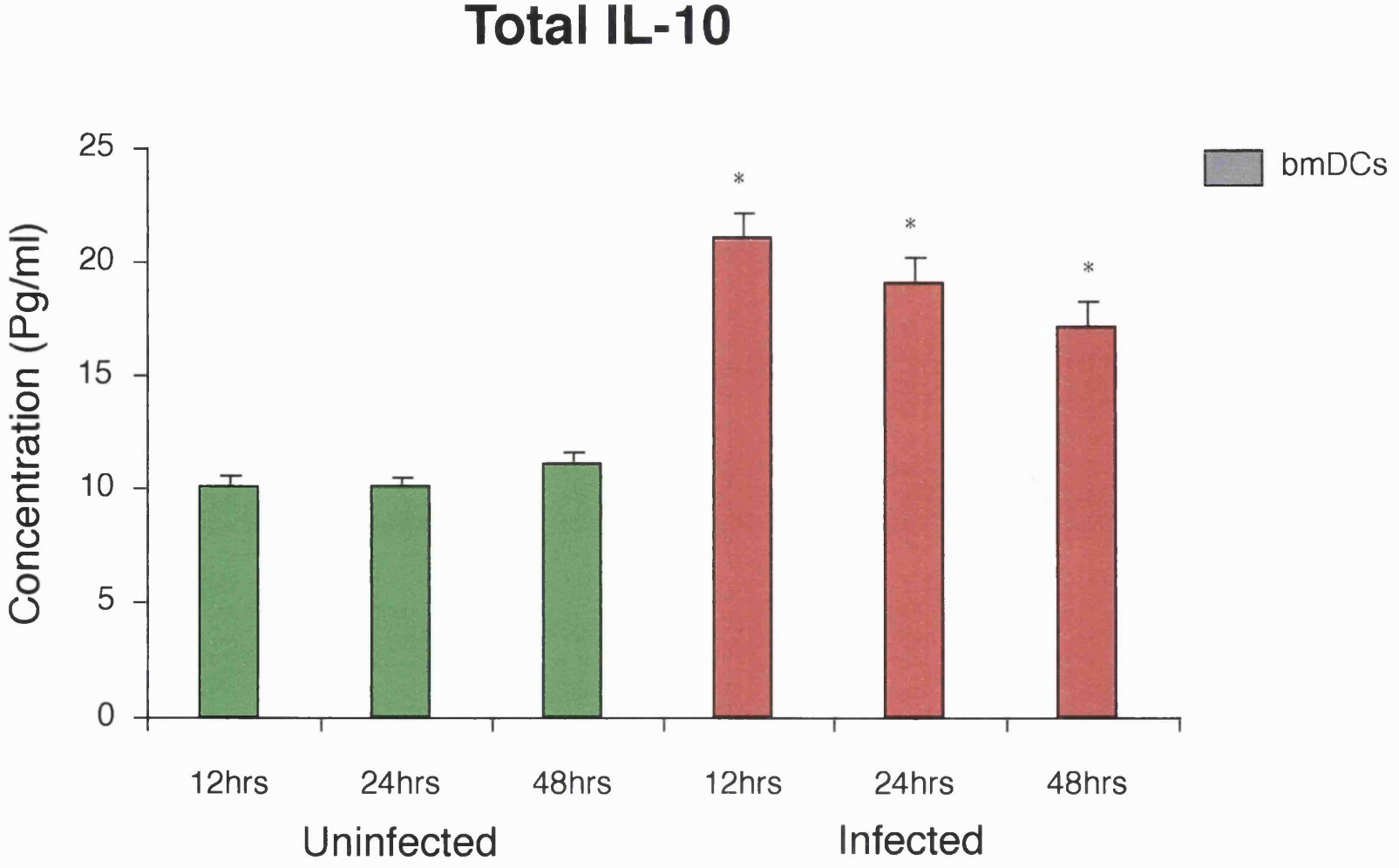

Figure 4.7 Infection of bmDC with *Mtb* and cytokine expression. IL-10 secretion by bmDC or *Mtb* infected bmDC. Supernatants were collected 12 hr, 24 hr and 48 hr after infection and analysed by ELISA. All the values are the means of triplicate determinations (±SD). If $p<0.01$ there is a statistic difference from *Mtb*-infected to the uninfected cells.

4.3 Changes in cell surface phenotype of tsDC infected with *Mtb*

Maturation of DC following exposure to antigen is accompanied with the increased expression of costimulatory molecules on the cell-surface. The surface expression of B7-1, B7-2, ICAM-1, heat stable antigen (HSA) and MHC Class II molecules was analysed by FACS on tsDC before and 48 hours after infection with *Mtb*. The results (Figure 4.8) show that MHC Class II, B7-1 and B7-2 were significantly up regulated following *Mtb* infection; there was a small upregulation of ICAM-1 but no increased expression of HSA.

4.4 Discussion

Immature DC are capable of sensing and taking up antigen. Following this they mature into antigen presenting cells, capable of migrating to T cell rich-areas of lymphoid tissue, presenting antigen to specific T cells and producing cytokines which can influence the differentiation of the T cells. In this Chapter we have investigated some of these changes using the tsDC cell line. The results on cytokine production are summarised in Table 4.1.

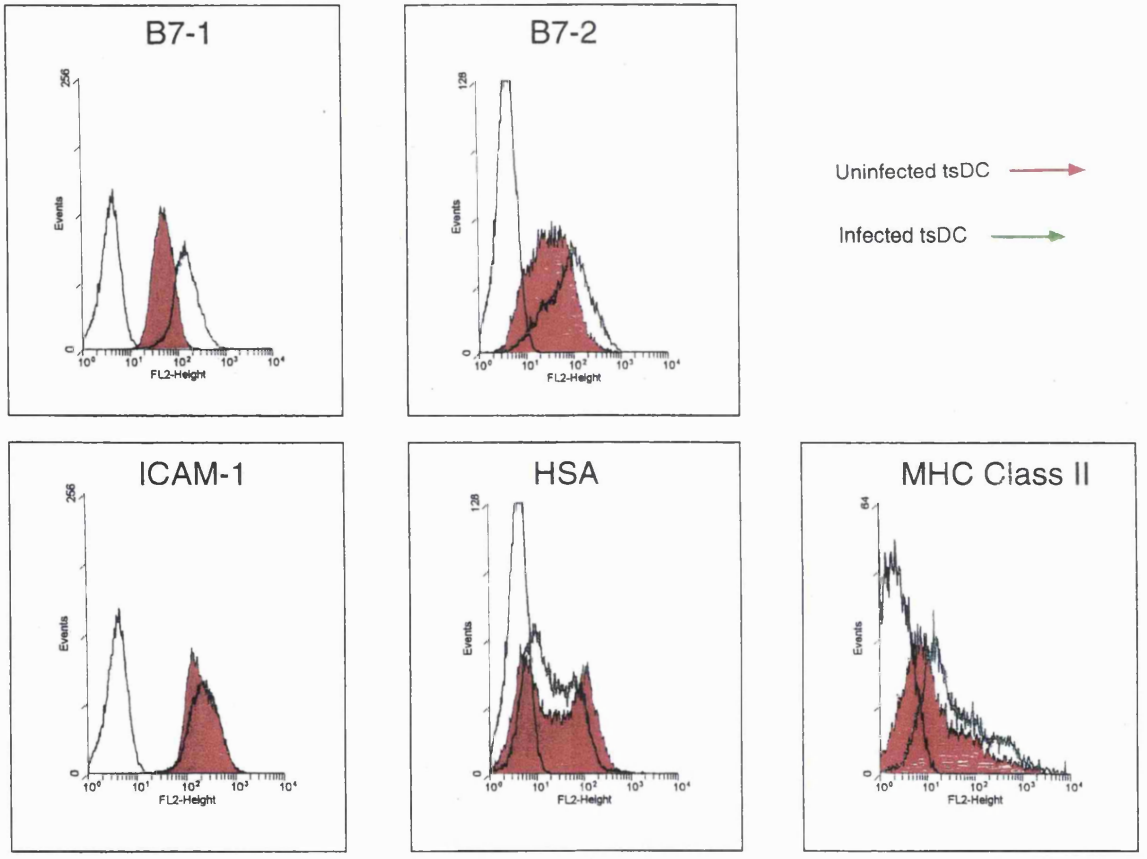

Figure 4.8 FACS analysis of tsDC or *Mtb* infected tsDC at 48 hr, for surface expression of the indicated antigens. Staining with isotype-matched control antibodies as negative controls is shown in black open histograms, non-infected tsDC in red histograms and *Mtb* infected tsDC in green histograms.

	Effect (protein)		Effect (mRNA)	
Cytokine	**tsDC**	**primary DC**	**tsDC**	**primary DC**
IL-12	↑	↑↑	↑↑	ND
IL-10	ND	↑↑	-	ND
IL-6	↑↑↑	↑↑↑	↑↑	ND
IL-18	ND	ND	-	ND
TNF-α	↑↑↑	↑↑↑	↑↑	ND
IFN-γ	-	-	-	ND
IL-1β	↑	↑↑↑	↑↑↑	ND

Table 4.1 The production of cytokines (protein or mRNA) by tsDC or primary DC following infection with *Mtb*. ↑,↑↑, ↑↑↑ indicates low, moderate and strong upregulation, respectively. -Indicates no effect, ND indicates not done.

The results confirm that phagocytosis of *Mtb* rapidly produces an induction of proinflammatory cytokines, consistent with a more mature phenotype.The results with the cell line tsDC were generally consistent with those of the primary DC, although the latter cells generally gave higher levels of cytokines and also produced IL-10. The latter could be due to contamination of the bone marrow–derived DC with macrophages and/or NK cells. However both human and murine DC have previously been shown to produce IL-10 in response to mycobacteria (Henderson *et al.*, 1997; Demangel *et al.*, 1999), suggesting that the cytokine milieu in which DC priming of T cells occurs may not result in a purely polar TH1 response.

The maturation of tsDC following phagocytosis of *Mtb* is further illustrated by the changes in cell surface phenotype. The upregulation of surface expression of MHC Class II molecules, along with the costimulatory molecules B7-1 and B7-2 is consistent with an increase in antigen presenting capacity. The very small increase in ICAM-1 expression is less than that reported for *Mtb*- stimulated primary human DC (Henderson *et al.*, 1997). Integrin and adhesion molecules such as ICAM-1 are involved in migration of cells and their interaction with vessel walls (Van de Stolpe and Van der Saag, 1996); it may be that regulation of such molecules is lost during prolonged *in vitro* culture, as has occurred with the tsDC cell line.

HSA showed no variance in its surface levels after *Mtb* infection; earlier recognised as a DC surface marker, HSA is known to participate in T lymphocyte stimulation (Williams *et al.*, 1996). It belongs to a sequestered pool of proteins that are exposed co-ordinately at the cell surface via a protein kinase signalling mechanism that detects phagocytic events (De Bruijn *et al.*, 1996). The invariability of HSA at the surface of *Mtb* infected tsDC, could also be explained by the prolonged *in vitro* culture of the

cells, but the mechanisms underlying the action of this molecule are still poorly understood.

Overall however, the phenotypic changes seen in these experiments confirm that exposure to *Mtb* results in maturation of DC from an antigen detecting to an antigen presenting cell phenotype.

Chapter 5
Results

Chapter 5

NFκB activation analyses

5.1 Introduction

NFκB is a family of transcription factors with a pivotal role in inducing genes involved in physiological processes as well as in the response to injury and infection. NFκB exists in the cytoplasm of the majority of cells as homo or heterodimers of a family of structurally related proteins (Kopp and Ghosh, 1995). To date, five proteins belonging to the NFκB family have been identified in mammalian cells: p65, c-Rel, Rel-B, p50 and p52 (Miyamoto and Verma, 1995; Baldwin, 1996).

The translocation of NFκB to the nucleus requires the breakdown of the ligation with its inhibitor, IκB, which is achieved by phosphorylation by the enzyme Phospho-IκB-α. However in addition to phosphorylation, degradation of the IκB protein is required, otherwise the NFκB activation is blocked (Alkalay *et al.*, 1995). Nevertheless, during persistent NFκB activation, some NFκB remains sequestered in the cytoplasm but retains the ability to enter the nucleus and initiate transcription (May and Ghosh, 1998).

Thus the crucial events, which occur during NFκB activation, are:

(1) Stimulation of the cell

(2) Phosphorylation of the IκB-α

(3) Degradation of the protein IκB-α

(4) Translocation of NFκB to the nucleus

Since the early ninety's, activation and modulation of NFκB has been studied using different cell types, such as macrophages or DC, and different models of cell

stimulation. For example using murine macrophage-like cell lines, lipoarabinomannan (LAM) derived from *Mtb* was found to activate NFκB and that this activation was partially modulated by TNF-α (Brown and Taffet, 1995). LPS has also been shown to activate NFκB in the human monocyte cell line THP1, with translocation of c-Rel, p50 and p65 into the nucleus (Cordle *et al.*, 1993). Recently, some of the receptors involved in recognition of molecules such as LAM and LPS, and signalling NFκB activation have been identified as "pattern-recognition" receptors such as Toll-like receptors (TLR) (Yang *et al.*, 1999). These receptors are able to recognise a wide range of molecules associated with prokaryotic pathogens (for example TLR9 recognises CpG DNA motifs found in bacteria (Sester *et al.*, 1999; Bowie and O'Neill, 2000), emphasising the importance of NFκB activation in innate immunity.

Cytokines and chemokines are also involved in both the signalling of NFκB activation and in the consequent transcription activation response. Thus IL-12, IL-1β and TNF-α are capable of stimulating NFκB activation through their various receptors, and are up-regulated by the transcription activity of NFκB (reviewed by Baeuerle and Henkel, 1994; Gerondakis *et al.*, 1998). Thus, in addition to their role in innate immunity, members of the NFκB family also have important roles in the adaptive immune system by, for example, mediating lymphocyte activation and cytokine production, immunoglobulin isotype switching and upregulation of cytokine receptor expression.

Other cellular processes, in addition to stimulation of cells of the immune system, are also regulated by NFκB. These include cell cycle regulation, apoptosis and cell adhesion processes. For example NFκB mediates expression of Bcl-X, a gene involved in apoptosis (Kuhnel *et al.*, 2000; Chen *et al.*, 2000), and differential expression of NFκB during mononuclear phagocyte differentiation may play an

important role in determining the fate of immature mononuclear phagocytes (Ammon *et al.*, 2000). Thus the NFκB superfamily of signal transducers and transcription factors plays a central role in the rapid innate immune response, the adaptive, acquired immune response and in the differentiation and expansion of the cells of the immune system.

The results presented in Chapters 3 and 4, indicating that DC were rapidly activated by contact with *Mtb*, suggested that NFκB activation played a key role in the response of DC to mycobacteria. In this chapter the kinetics and molecular modulation of NFκB activation in this system have been studied.

5.2 Phosphorylation and degradation of IκB

Approximately $3x10^6$ cells, tsDC were infected with *Mtb* at a ratio of 5 bacteria per cell. One group of cells remained uninfected (controls). Cells were collected at various time intervals after infection and cytoplasmic extracts were tested, by immunoblotting, for the presence of the enzyme Phospho-IκB-α.

The enzyme (43 Kda) could be detected in uninfected cells. However, as early as 5 minutes after infection there was a marked upregulation of Phospho IκB-α (Figure 5.1) suggesting that phosphorylation of IκB-α was occurring.

Concurrently, immunoblotting to detect degradation of IκB-α was carried out. The degradation of the protein IκB-α (37 Kda) was detectable after 10 minutes of infection (Figure 5.2). Thus this rapid up-regulation of Phospho-IκB and the degradation of IκB indicates that NFκB activation is rapidly induced in DC exposed to mycobacteria.

5.3 Kinetics and modulation of NFκB activation

5.3.1 NFκB activation

Approximately $3x10^6$ tsDC were infected with *Mtb* at a ratio of 5 bacteria per cell. One group of cells remained uninfected (controls). Nuclear extracts from these cells were collected at various time intervals following infection, and were used in EMSA assays to detect nuclear translocation of NFκB.

By 20 minutes after *Mtb* infection there was a clear increase in the binding activity that peaked at 30 minutes post-infection. The binding activity appeared to have declined by 1 hour post-infection; there was no increase in the binding activity in the nuclear extracts from the uninfected cells (Figure 5.3). Three distinct bands could be seen. Two of them (1 and 2) were difficult to detect in uninfected cell extracts. The third band (3) was present in all extracts, but increased in the infected cells between the 20 and 30 minutes.

This experiment confirmed the translocation of NFκB to the nucleus after *Mtb*-infection, thus indicating cell activation.

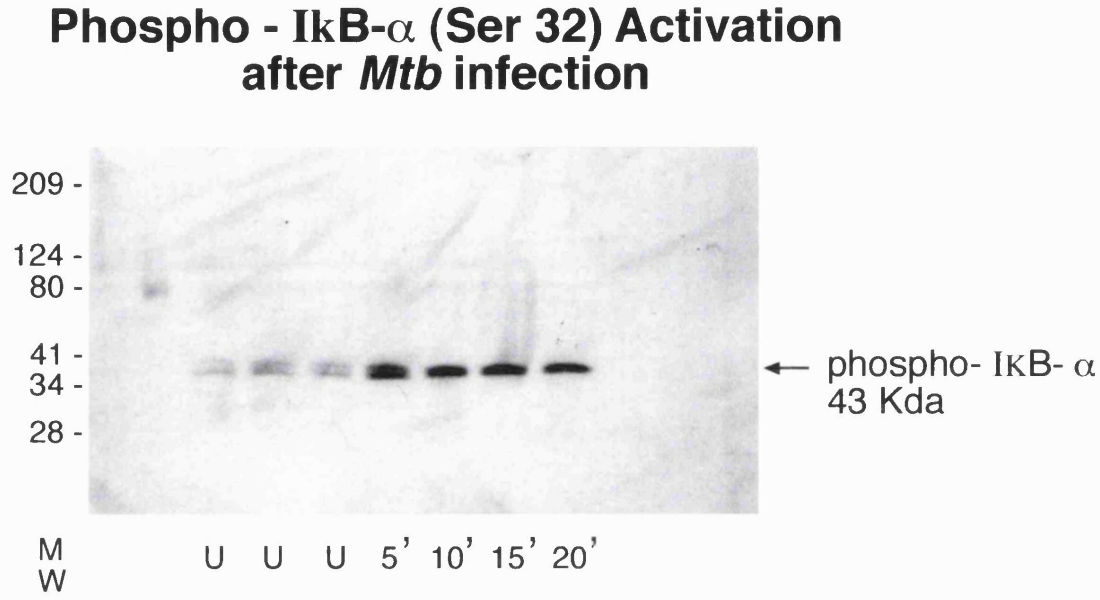

Figure 5.1 Immunoblot to detect expression of the enzyme phospho-IkB-α in tsDC uninfected controls (U) and in tsDCinfected with *Mtb* (5,10,15 and 20 minutes).

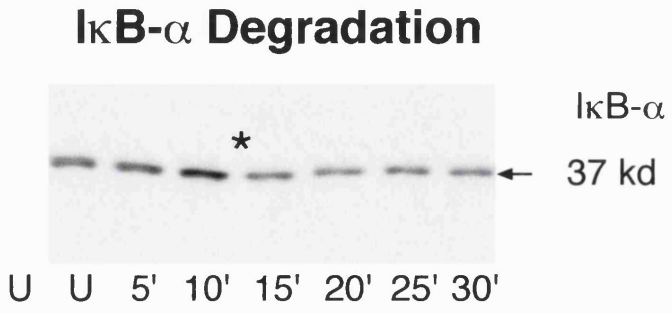

Figure 5.2 Immunoblot to detect the degradation of the protein IkB-α in tsDC uninfected controls (U) and tsDC-*Mtb* infected (5-30 minutes); *_ represents the starting point of degradation.

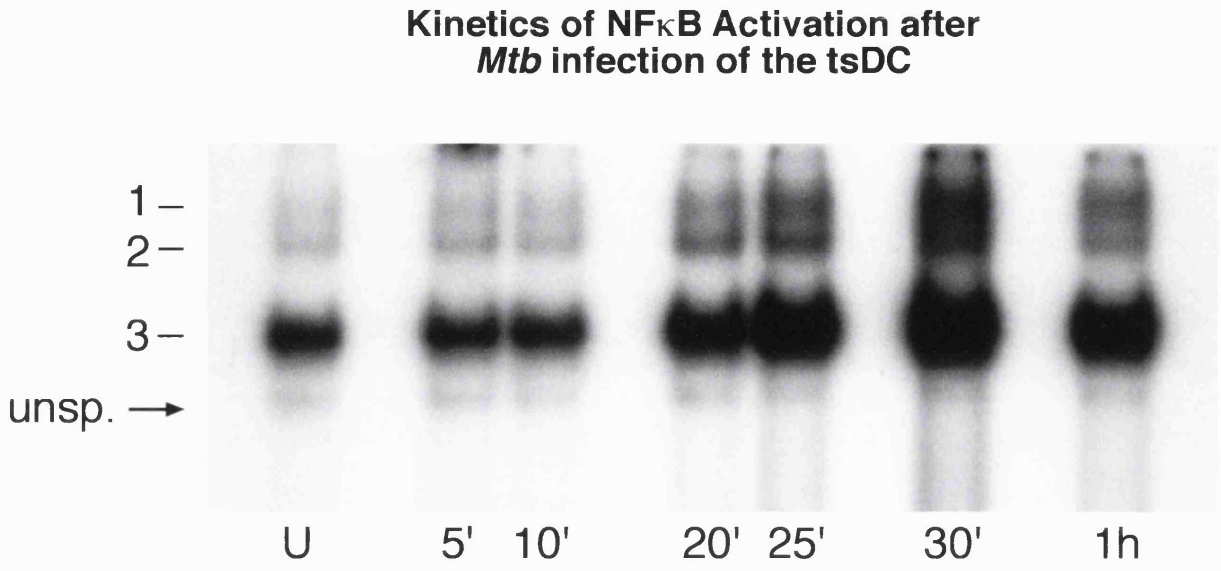

Figure 5.3 EMSA illustrating the binding activity of NFkB from 5 minutes to 1 hour post-*Mtb* infection. U=uninfected control cells

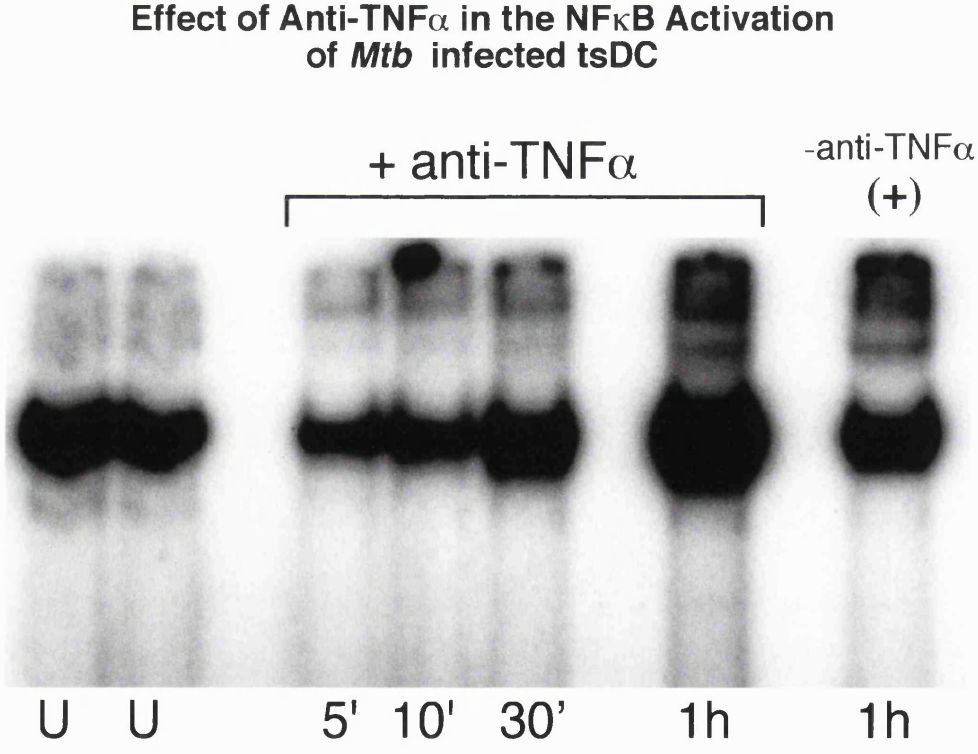

Figure 5.4 EMSA illustrating the binding activity of NFkB in the presence of the antibody anti-TNFα.U= Uninfected control cells; -anti-TNF-α= medium without the TNF-α blocking antibody.

5.3.2 TNF-α regulation

Since TNF-α was observed to be rapidly produced following activation of DC by *Mtb* (Chapter 4) it was possible that it was responsible for NFκB activation. To test this an anti-TNF-α antibody was used to block TNF-α -mediated activation of NFκB.

Using a protocol similar to the previous experiment tsDC were infected with *Mtb* in the presence or absence of anti-TNF-α antibody at a concentration (1.2μg/ml) previously shown to block TNF-α activity. Control groups consisted of uninfected tsDC, and infected tsDC without anti-TNF-α antibody.

The results shown in Figure 5.4 indicate that the binding activity of the nuclear extracts was unaffected by the presence of the blocking antibody (lanes 3,4,5 and 6), indicating that production of TNF-α was not contributing significantly to NFκB activation.

5.3.3 Phagocytosis of *Mtb* is not required for NFκB activation

The results presented in Figures 5.1-5.3 indicate that the initial stages of NFκB activation were detectable as early as 5 minutes following the addition of *Mtb* to the DC cultures. This suggests that phagocytosis of *Mtb* is not necessary for initiation of activation. In order to address this an inhibitor of phagocytosis, colchicine, was used. Colchicine inhibits intracellular polymerisation of tubulin, and prevents phagocytosis of *Mtb* (Bermudez and Goodman, 1996).

In a preliminary experiment 10 μM of colchicine was added to the tsDC cultures 30 minutes prior to infection. After 12 hours the percentage of cells which had phagocytosed *Mtb* was determined microscopically. The results (Figure 5.5) show that

in the absence of colchicine, approximately 80% of the cells contained bacteria; the presence of colchicine in the medium reduced this to 10%.

The effect of inhibition of phagocytosis by colchicine on NFκB activation was then studied. In the absence of *Mtb*, the presence of colchicine in the medium had no effect on NFκB activation as assayed by EMSA (Figure 5.6). As previously, *Mtb* infection induced NFκB activation as early as 15 minutes after infection, and this was unaffected by the presence of colchicine in the medium. Thus it appears that phagocytosis of *Mtb* is not required for activation of NFκB in DC.

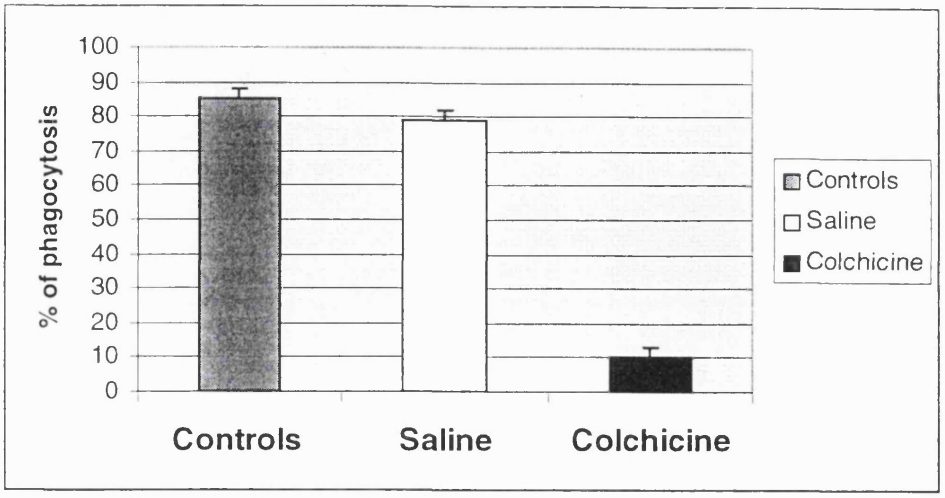

Figure 5.5 The effect of Colchicine on the phagocytosis of *Mtb* by tsDC. The cells were infected for 12 hours, washed and stained, and the percentage of cells, which had phagocytosed *Mtb*, determined. Monolayers were incubated with either no inhibitor (Controls), with an irrelevant substance (Saline) or Colchicine (10 μM). The data represent the means of the phagocytosis percentage in three separate well monolayers ± SD

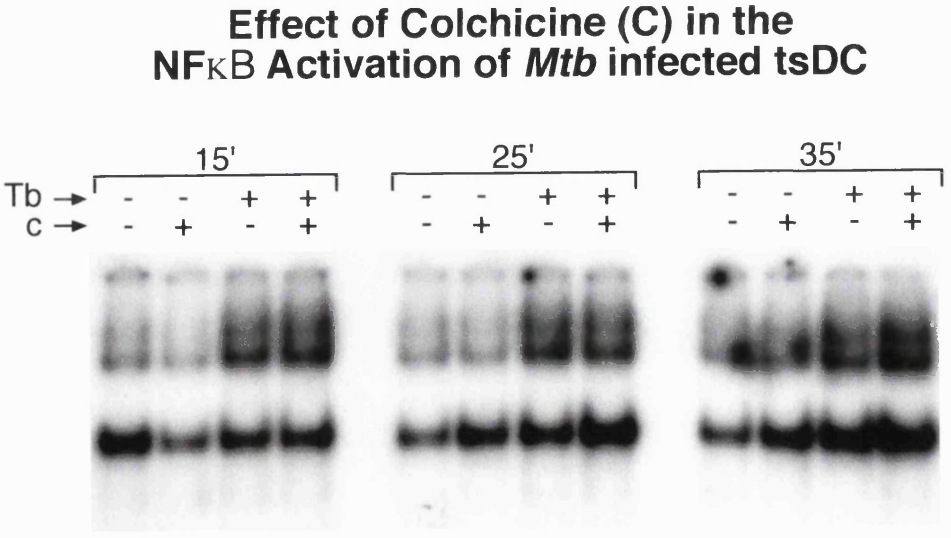

Figure 5.6 EMSA illustrating the effect of the phagocytosis inhibitor Colchicine (C) on NFkB activation in uninfected and *Mtb* infected cells at 15, 25 and 35 minutes. + (present); (-)absent

5.3.4 c-Rel and Bcl-X expression in bone marrow-derived DC (bmDC) and macrophages

The electron microscopy experiments described in Chapter 3 failed to reveal any evidence of apoptosis in *Mtb* infected DC. Subsequent experiments revealed that the fate of *Mtb* in DC was different from the fate in macrophages (see section **3.3**). Since the anti-apoptotic protein Bcl-X is known to be under the control of NFκB /c-Rel activity (Chen *et al.*, 2000), the involvement of c-Rel and Rel B was compared in bmDC and bone marrow macrophages following infection with *Mtb*. Supershift experiments on bmDC and macrophages, infected or uninfected by *Mtb*, were carried out using antibodies which recognise c-Rel and Rel-B. The results are shown in Figure 5.7.

The expression of c-Rel could be seen in both the *Mtb*-infected DC and in the macrophages using the supershift assay. In order to estimate the relative amounts of c-Rel, immunoblotting was carried out, first to compare *Mtb*-infected and uninfected bmDC (Figure 5.8a), and then to compare uninfected DC with macrophages (Figure 5.8b). The results demonstrated that c-Rel was present in both *Mtb*-infected and uninfected bmDC, at approximately equivalent levels. c-Rel could also be detected in uninfected bone marrow macrophages but appeared to be present at significantly lower levels than in bmDC (Figure 5.8b). The apparent difference in c-Rel expression in macrophages and DC suggested that the expression of proteins which are critical checkpoints of apoptosis, Bcl-2 and Bcl-xL, might differ. Bcl-2 expression was readily detectable in the mouse myeloblast cell-line M1, but could not be detected in *Mtb* infected DC or macrophages (Figure 5.8c). The anti-apoptotic protein Bcl-xL could be seen expressed at very low levels in both DC and macrophages (in this case, Hela cells were used as controls; Figure 5.8d). Thus, in spite of the apparent increased expression

of c-Rel in bmDC, there was no concomitant increased expression of Bcl-xL, and hence no evidence for a shift towards an anti-apoptotic phenotype in the *Mtb*-infected DC.

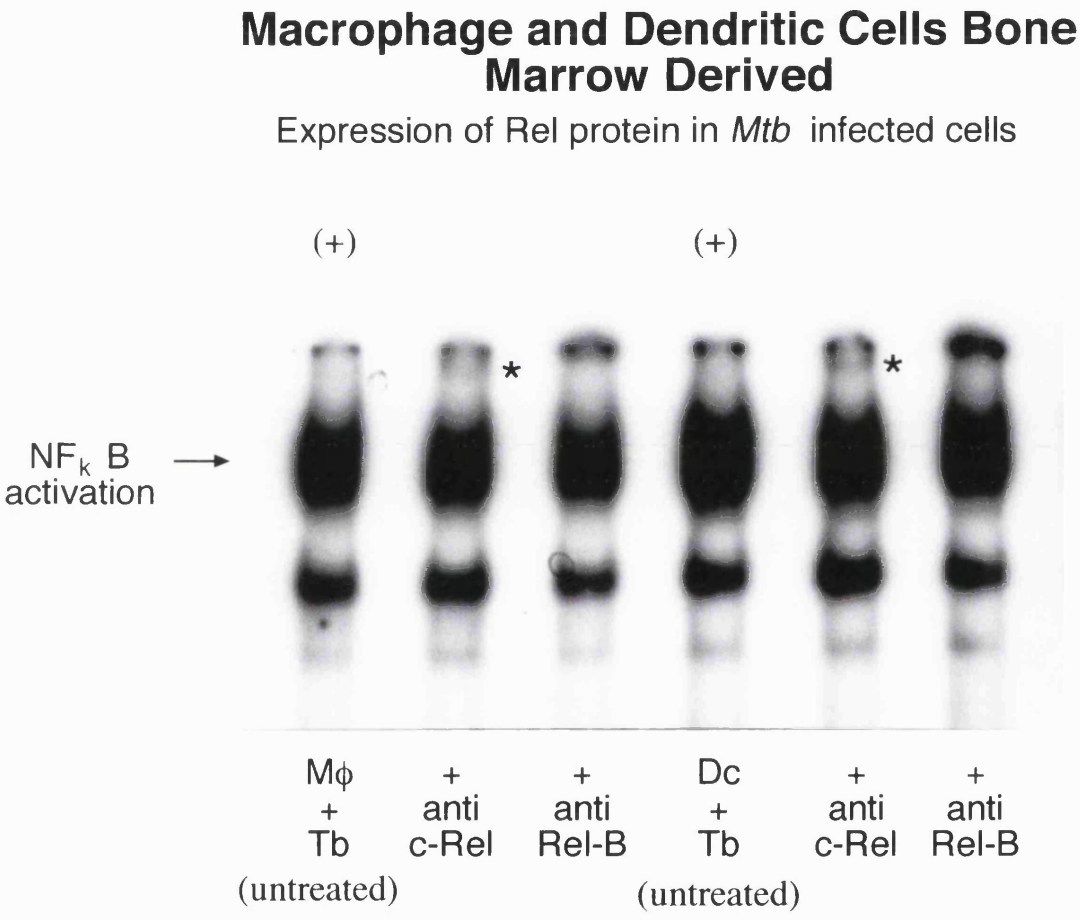

Figure 5.7 NFkB supershift to identify the rel proteins expressed in *Mtb* infected macrophages (ϕ) or bone marrow derived DC (DC). Mϕ +TB and DC+TB are untreated controls.
*- supershifted bands

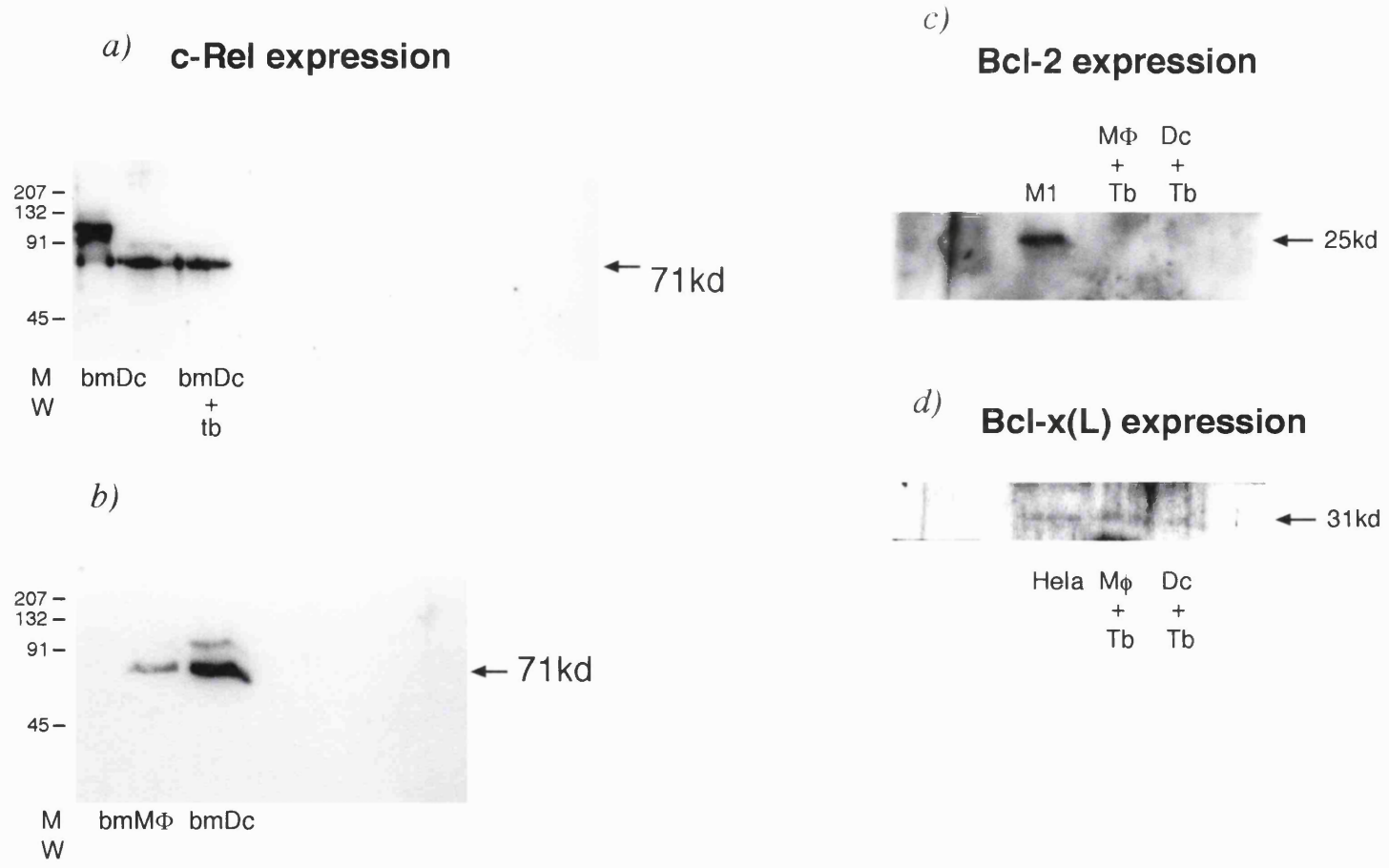

Figure 5.8 Immunoblottings for c-Rel expression on
a) bone marrow derived dendritic cells uninfected or *Mtb* infected (control)
b) bone marrow derived Mϕ and dendritic cells

c) Immunoblotting for Bcl-2 expression in *Mtb* infected Mϕ and DC (M1-positive control)
d) Immunoblotting for Bcl-x(L) expression in *Mtb* infected Mϕ and DC (Hela-positive control)

5.5 Discussion

In this chapter the role of the transcription factor NFκB in the activation of DC by *Mtb* was investigated. The activation of the phosphorylating enzyme Phospho IκB-α and the degradation of IκB in the cytoplasm were detectable as early as 5-10 minutes following addition of *Mtb* to DC. Translocation of NFκB to the nucleus was detectable by 20 minutes. Thus activation of the NFκB signalling pathway was extremely rapid. This activation did not require TNF-α production and was independent of phagocytosis of *Mtb*. At the start of these experiments these results were extremely surprising. However over the last 2 to 3 years the role of pattern recognition receptors such as TLRs in detecting the presence of bacterial molecules and signalling an inflammatory response through the NFκB pathway (Yang *et al.*, 1999) has emerged. It would be of interest to block receptors known to be involved in *Mtb* recognition and uptake (TLR, Fc, complement and mannose receptors) and investigate the effect on the NFκB activation pathway.

The c-Rel protein was found to be expressed in both macrophages and DC. However, there was no difference in the expression of the apoptosis marker proteins Bcl-2 and Bcl-xL, nor there was increased expression of Bcl-xL in *Mtb*-infected DC. These results are consistent with a model in which NFκB could act in conjugation with other transcription factors to control the expression of Bcl-xL depending on the cell type and /or activating stimuli (Kuhnel *et al.*, 2000).

Overall the immunological modifications observed in chapter 4 were confirmed at the molecular level by these results, which also indicated that detection of *Mtb* and signalling for cytokine gene transcription occurs following surface contact between *Mtb* and DC.

Chapter 6
Results

Chapter 6

Priming of T cell responses in vivo by *Mtb* infected DC and the induction of protective immunity

6.1 Introduction

Over the past decade there has been considerable interest in producing DC-based vaccines for immune prophylaxis or immunotherapy against tumours (Dhodapkar and Bhardwaj, 2000) or infectious diseases like influenza, malaria or HIV (Guy *et al.*, 2001; Naik *et al.*, 2000; Granelli-Piperno *et al.*, 2000). The ability of antigen-pulsed DC to prime protective immune responses in a weakly immunogenic syngeneic tumour model system (Celluzzi *et al.*, 1996) has emphasised the potential of this approach.

Studies using murine models of tuberculosis have demonstrated a role for both MHC Class II-restricted, CD4+ T cell responses and MHC Class I-restricted, CD8+ T cell responses. For example mice which lack β2-microglobulin, and hence lack CD8+ T cell responses are more susceptible to infection with *Mtb* than wild-type mice (Flynn *et al.*, 1992). Similarly, MHC Class II knockout mice, which lack CD4+ T cells are also more susceptible to *Mtb* infection (Tascon *et al.*, 1998).

Reconstitution of athymic mice with either CD4+ or CD8+ T cells, or a combination of both, revealed that both subsets are able to confer similar levels of protection; in addition, this protection was only conferred by T cells which were capable of producing IFNγ (Tascon *et al.*, 1998). This would suggest that, early during the experimental infection of mice, at least in this model system, CD8+ T cells contribute to protective immunity by producing effector cytokines rather than by cytotoxic mechanisms. Other studies have indicated that CD8-mediated cytotoxicity might be

important in the later stages of infection (Laochumroonvorapong *et al.*, 1997; Cooper *et al.*, 1997; Sousa *et al.*, 1999), whereas IFNγ production is important in the earlier stages (Kaplan, 1994; Feng *et al.*, 1999; Tascon *et al.*, 1998). In any event, immunisation, which stimulates both specific CD4+ and CD8+ T cell responses, would appear to be optimal for effective protection against *Mtb* and antigen-pulsed DC have been shown to be effective in stimulating both CD8+ and CD4+ T cell responses (Wong *et al.*, 1998).

A number of studies have shown that DC, but not macrophages can efficiently phagocytose apoptotic antigen presenting cells and cross-present viral, tumor, and self-antigens to CD8+ T cells (Albert *et al.*, 1998a; Albert *et al.*, 1998b; Inaba *et al.*, 1998). This *in vitro* pathway corresponds to the *in vivo* phenomena of cross-priming and cross-tolerance (Albert *et al.*, 1998b). Thus, DC can use unique pathways for the phagocytosis, processing, and presentation of antigen derived from apoptotic cells and this mechanism enables antigen presentation in association with Class I and Class II MHC molecules. The trafficking of exogenous antigen by immature DC in this cross-priming pathway is poorly understood, but presentation to T cells *via* this pathway is remarkably efficient (Kurts *et al.*, 1998; Kurts *et al.*, 1997). MHC Class I – restricted cross priming of CD8+ T cells is thought to be dependent on CD4+ helper T cells (Bennett *et al.*, 1997).

Cross-presentation of antigens and CD8+ T cell priming has been demonstrated in tumour models (Robinson *et al.*, 1999; Nouri-Shirazi *et al.*, 2000) and in immunity to the infectious agent *Listeria monocytogenes* (Tvinnereim and Harty, 2000).

In the experiments described in this chapter the activation and cross-priming of naïve T cells by activated DC was studied. The cross-presentation of mycobacterial antigens to T cells by DC was also studied. Furthermore, the ability of *Mtb*-activated DC to

confer protective immunity against challenge with viable *Mtb* was evaluated, and compared to the protective immunity conferred by the current vaccine, BCG.

6.2 Priming of T cell responses *in vivo* by *Mtb* infected DC

6.2.1 Functional activation of DC in response to *Mtb* infection

In previous experiments (see chapter 4) we have shown that infection of DC with *Mtb in vitro* results in the activation of DC to produce cytokines and up-regulate the cell-surface expression of accessory molecules, including MHC Class II. We now wished to demonstrate that such activation *in vitro* results in functional activation of DC and priming of T cell responses.

Primary bone marrow-derived DC were generated from male C57/BL6 mice. The bmDC were activated after infection with *Mtb in vitro* (uninfected DC and LPS activated DC were used as controls). $5x10^6$ DC were transferred intra-peritoneally into naïve female MHC Class II knockout mice. The DC immunisation was repeated after an interval of 4 weeks and 4 weeks later T cells ($3x10^5$) extracted from the spleens of the recipient mice were incubated with $3\text{-}5x10^4$ irradiated spleen cells from male or female mice with the same background as the recipients-C57/BL6 (Figure 6.1). Supernatants were removed after 48 hours and assayed for IFNγ and IL-2.

By using male *Mtb* activated DC as donor cells and female mice as recipients we were able to test the ability of *Mtb*–activated DC to prime immune responses to an unrelated, endogenous antigen, the male antigen (Chen and Silvers, 1991; Desquenne-Clark *et al.*, 1992). By using MHC Class II knockout mice as recipients, it was possible to detect if the priming of MHC Class I-restricted CD8+ T cells had occurred in the absence of CD4+ T cell help, since the recipient mice lack CD4+ T cells. The results are shown in Figure 6.2.

T cells from MHC Class II knockout, female mice, which had received male *Mtb*-activated DC and which were then stimulated *in vitro* with male irradiated spleen cells produced high levels of IFNγ and IL-2 while T cells from mice which received uninfected DC did not respond to male or female spleen cells. Mice which received LPS-activated DC also produced IFNγ and IL-2 in response to male, but not female, irradiated spleen cells. However the LPS-activated DC were not as effective as *Mtb*-infected DC at priming T cell responses to male antigen in MHC Class II knockout female mice. This experiment demonstrates that *Mtb*-infected DC are functionally activated and capable of priming T cells *in vivo*. It also suggests that *Mtb*-infected DC could prime MHC Class I restricted T cells.

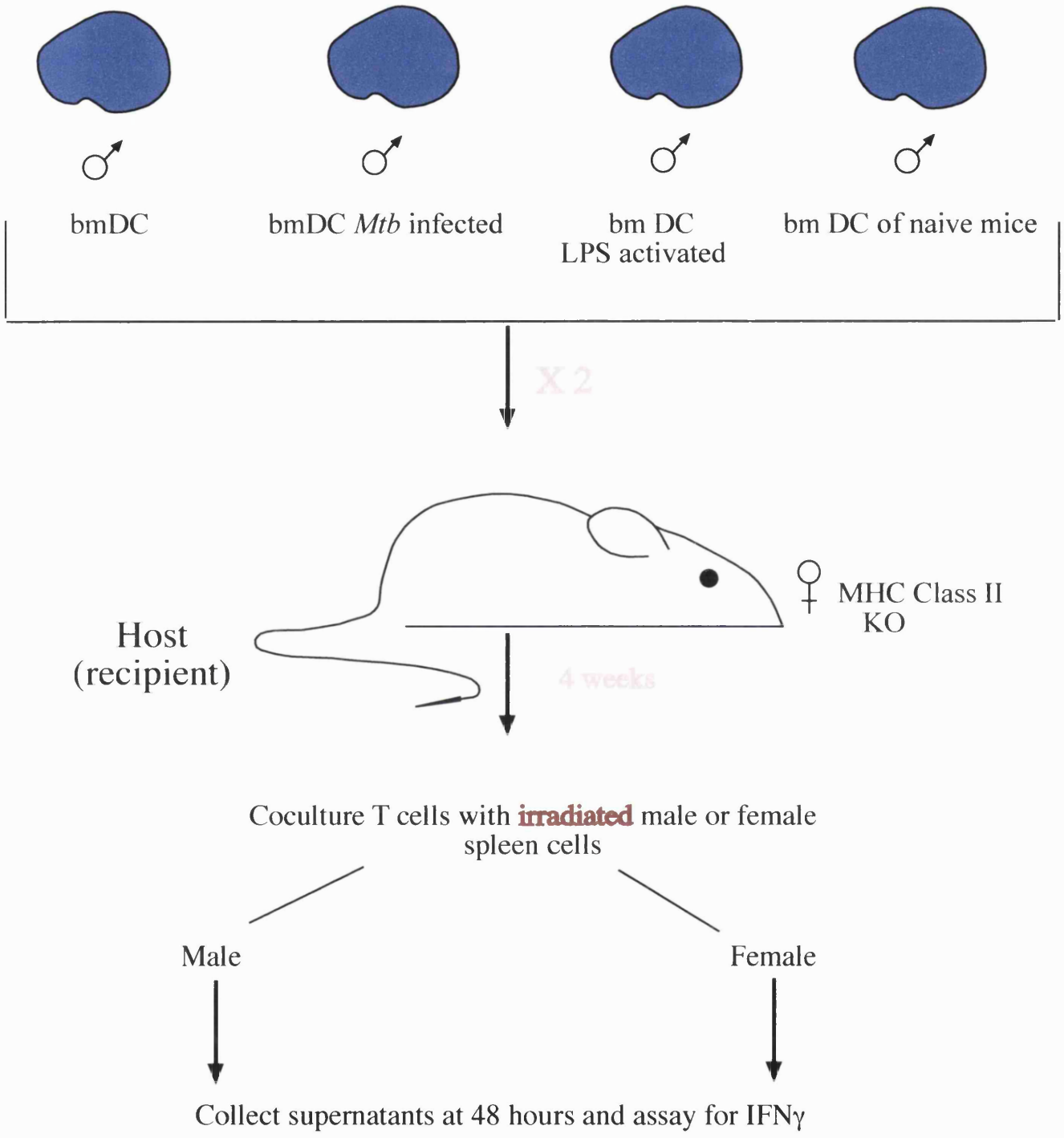

Figure 6.1 Illustration of the experimental murine model protocol used to test dendritic cell activation after *Mtb* infection.

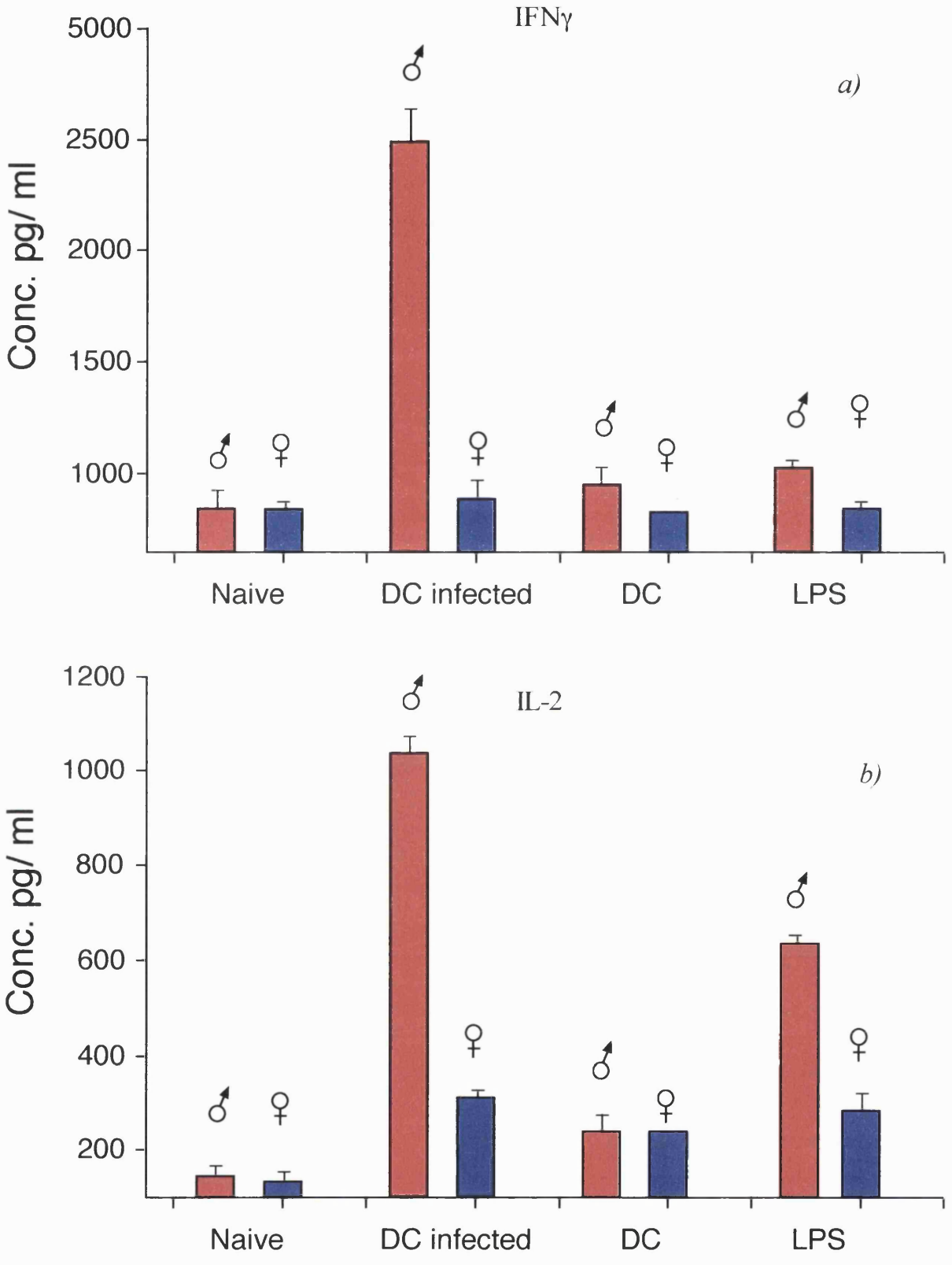

Figure 6.2 IFNγ (a) and IL-2 (b) production by T cells belonging to the four groups of immunised mice (naive, DC *Mtb*- infected, DC and LPS) stimulated with male and female spleen cells. Supernatants were collected at 48 hours and analysed by ELISA.For stastitical analysis it was used Students t-test and when $p<0.01$ differences were considered significant.

6.2.2 Cross priming of T cell responses specific for *Mtb*

To study the ability of DC to cross-prime T cell responses to mycobacterial antigens, immunologically normal C57/BL6 female mice were immunised intraperitoneally with DC from MHC Class II knockout female C57/BL6 mice which had been infected *in vitro* with *Mtb* and subsequently irradiated, and with DC from immunologically normal C57/BL6 mice which had been infected and irradiated (see Figure 6.3). The recipient mice received cells 3 times at 3-4 weeks intervals. T cells from these recipient mice were incubated with bmDC *Mtb*-infected or uninfected obtained from C57/BL6 mice; After 48 hours, supernatants were assayed for IFNγ. In addition CD4+ T cells were analysed for intracellular IFNγ by FACS, following 6 days of *in vitro* stimulation. The results for IFNγ production in supernatants are shown in Figure 6.4.

Cells cultured with uninfected DC did not produce IFNγ, irrespective of whether the mice had received infected or uninfected DC; however cells cultured with *Mtb*-infected DC produced IFNγ if mice had received *Mtb*-activated DC *in vivo*. This was true if the donor DC were derived from normal mice or from MHC Class II knockout mice. FACS analysis for intracellular IFNγ was consistent with the results for IFNγ in the supernatants; for mice receiving DC from immunologically normal mice the frequency of IFNγ-producing CD4+ T cells in mice receiving *Mtb*-infected DC was approximately 5.0% compared with 2.3% if uninfected DC were used (Figure 6.5a). For mice receiving DC from MHC Class II knockout mice, the frequency of IFNγ-producing CD4+ T cells in mice receiving *Mtb*-infected DC was approximately 4.0% compared to 0.5% if uninfected DC were used (Figure 6.5b).

This experiment demonstrates that transfer of *Mtb*-activated DC could prime *Mtb*-specific CD4+ T cell responses in recipient mice. The fact that MHC Class II knockout DC were capable of effectively inducing *Mtb* specific CD4+ T cell responses

suggests that antigen presenting cells in the recipient mice had acquired antigen from the donor DC and that cross-presentation had occurred, since the donor DC would have been incapable of stimulating CD4+ T cells.

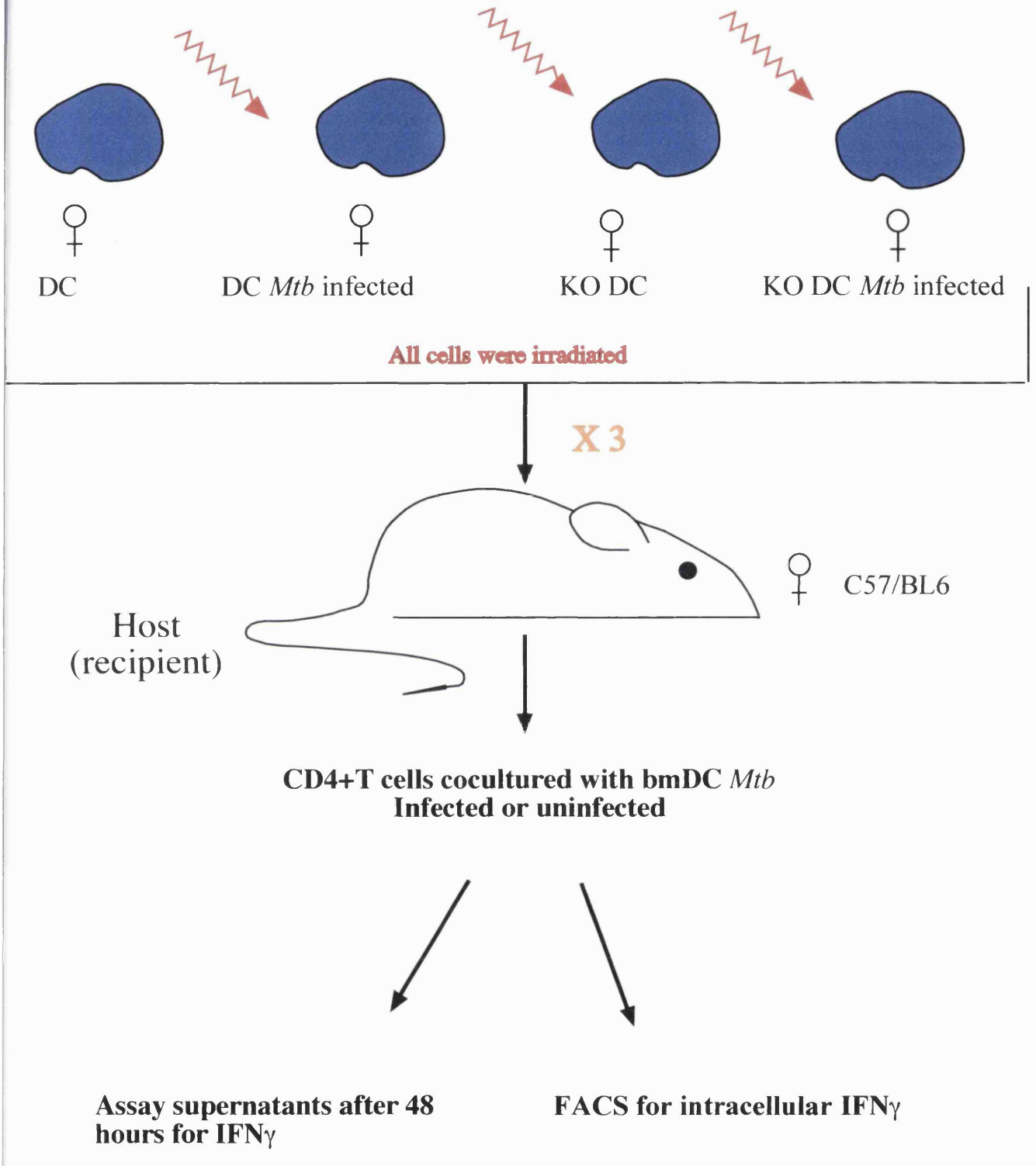

Figure 6.3 Illustration of the experimental murine model protocol used to test the ability of infected dendritic cells to cross-prime T cells specific for *Mtb*

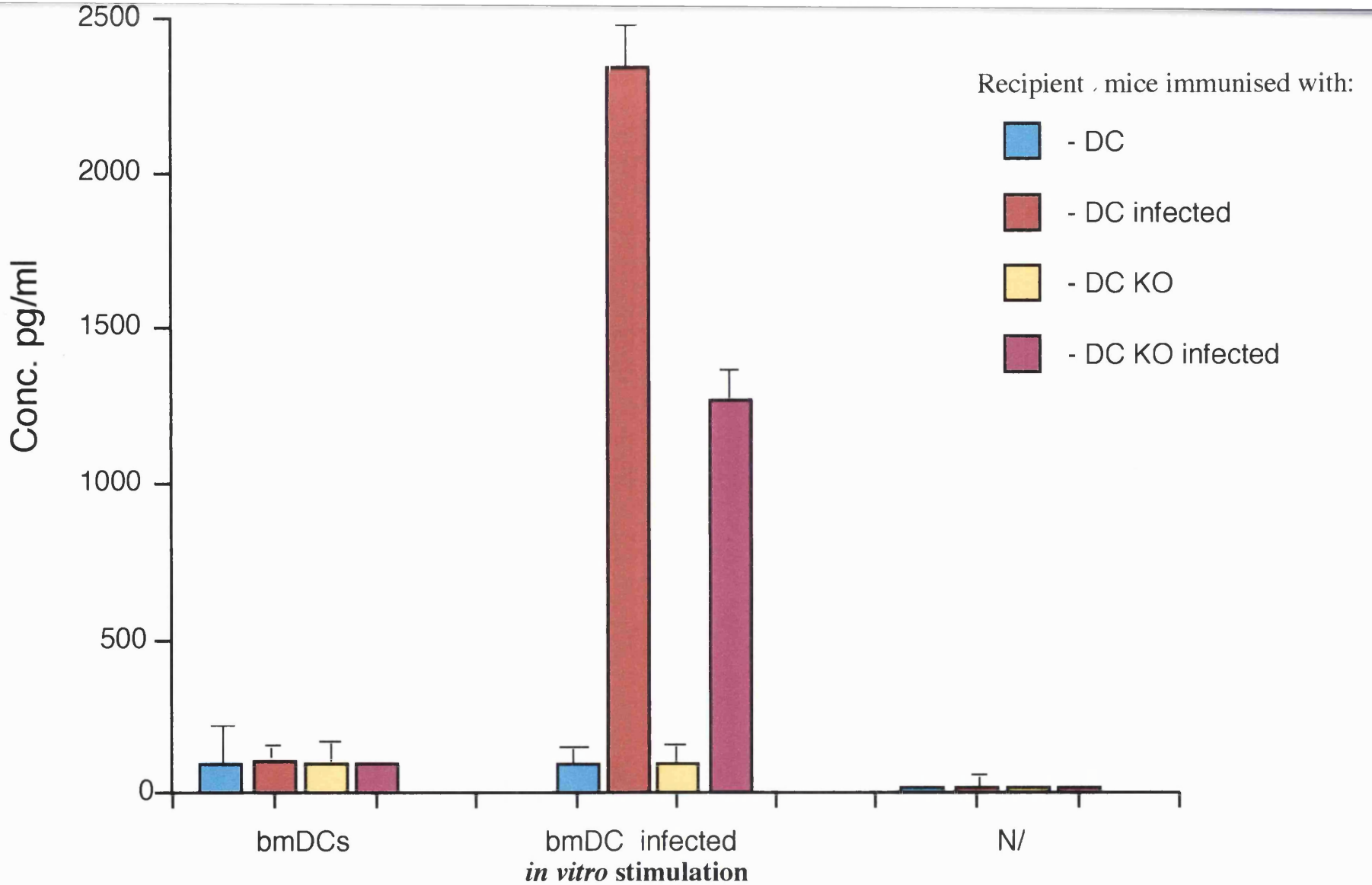

Figure 6.4 IFNγ production 48 hr after stimulation with bmDC uninfected and bmDC *Mtb* infected. N/ (non stimulated). All the values are the means of triplicate determinations, measured by ELISA. Differences between groups were considered significant, using Students t-test, if $p<0.01$.

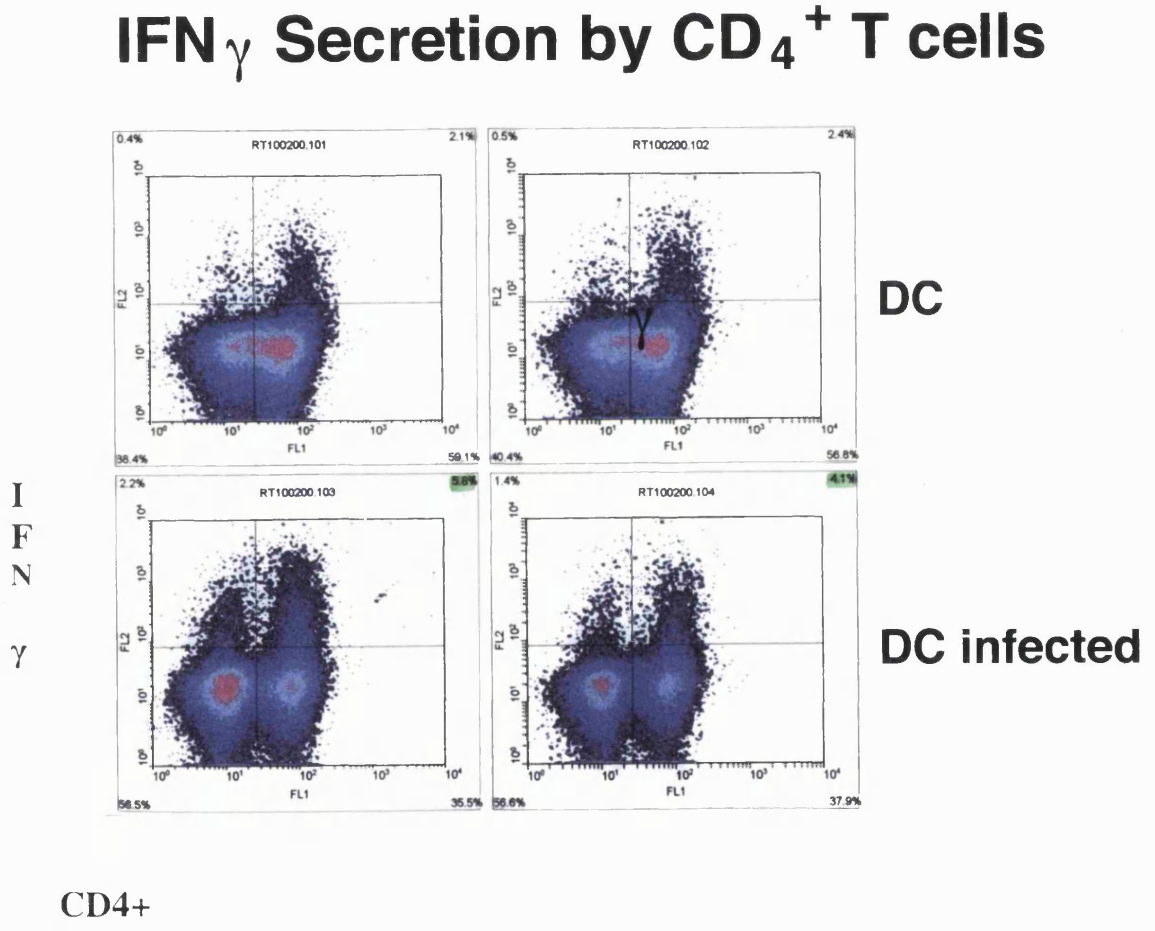

Figure 6.5 a) FACS analysis of the intracellular IFNγ-production by CD4+T cells.
The top two panels show duplicate analyses of CD4+T cells from mice which receive uninfected DC. The two lower panels show duplicate analyses of CD4+T cells from mice which received DC- *Mtb* infected.T cells from both groups of recipient mice were cultured *in vitro* with *Mtb* activated bmDC

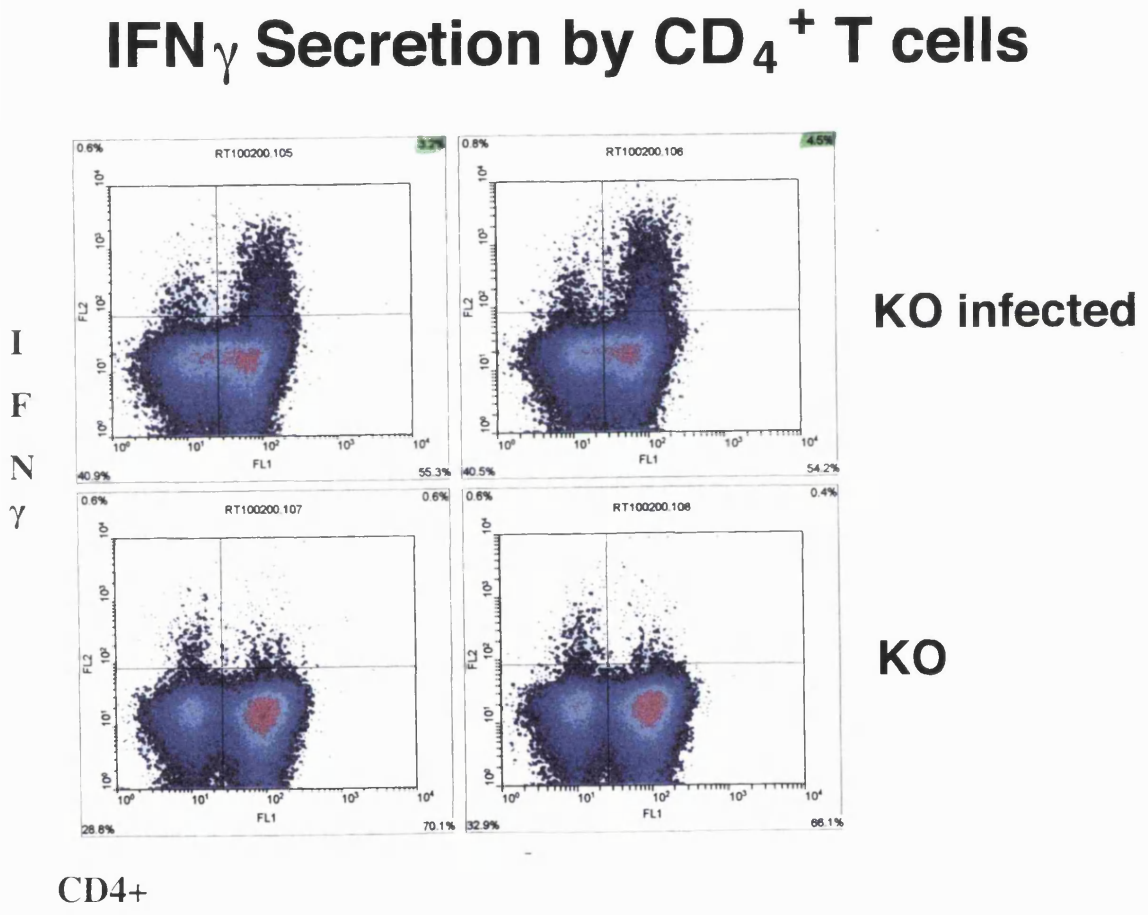

Figure 6.5 b) FACS analysis of the intracellular IFNγ-production by CD4+T cells.
The top two panels show duplicate analyses of CD4+T cells from mice, which received MHC Class II knockout DC infected with *Mtb*. The two lower panels show duplicate analyses of CD4+T cells from mice which received uninfected DC from MHC Class II knockout mice. T cells from both groups of recipient mice were cultured *in vitro* with *Mtb* activated bmDC

6.3 Protective immune responses conferred by *Mtb*-activated DC

(NB These experiments were carried out in collaboration with Mr. Evangelos Stavropoulos and Dr. M.J. Colston, who carried out the challenge infection).

The previous experiments had demonstrated that the transfer of *Mtb*-activated DC into recipient mice was capable of priming T cell responses in the recipient mice. The experiments also indicated that cross-presentation and cross-priming had occurred. We now wished to investigate whether we could successfully transfer immunity capable of protecting mice against an infectious challenge.

In the first experiment, tsDC were used to confer immunity; in the second experiment bmDC were used (see Figure 6.6).

In the first experiment CBA mice received:

i) uninfected tsDC (DC controls),

ii) a single intraperitoneal injection of 1-4x10^6 *Mtb* infected tsDC which had been irradiated prior to injection,

iii) two intraperitoneal injections of 1-4x10^6 *Mtb*-infected tsDC which had been irradiated,

iv) irradiated *Mtb* (TB IRR),

v) a single intradermal injection of BCG given 6 weeks before challenge.

The mice were challenged intravenously with viable *Mtb*, and lungs were removed six weeks later, homogenised and CFU counts performed. The results are shown in Figure 6.7A.

Counts in naïve mice, DC control mice and mice immunised with irradiated *Mtb* (TB IRR) all showed essentially identical results. There was a small but significant protective effect seen in the BCG immunised group and in the group immunised with two injections of *Mtb*-infected DC. The latter group showed significantly better protection than the BCG vaccinated group.

In the second experiment C57/BL6 mice received:

i) three intraperitoneal injections of uninfected bmDC (BMDC controls),

ii) three intraperitoneal injections of 1-4x10^6 *Mtb*-infected bmDC,

iii) three intraperitoneal injections of irradiated *Mtb* (TB IRR),

iv) a single intradermal injection of BCG given 12 weeks before challenge

The mice were challenged intravenously with *Mtb* and the lungs removed six weeks later, homogenised and CFU counts performed. The results are shown in Figure 6.7 B. Once again, no protection was seen in mice given irradiated *Mtb* or uninfected bmDC (TB IRR and bmDC controls). *Mtb* infected bmDC and BCG gave significant levels of protection.

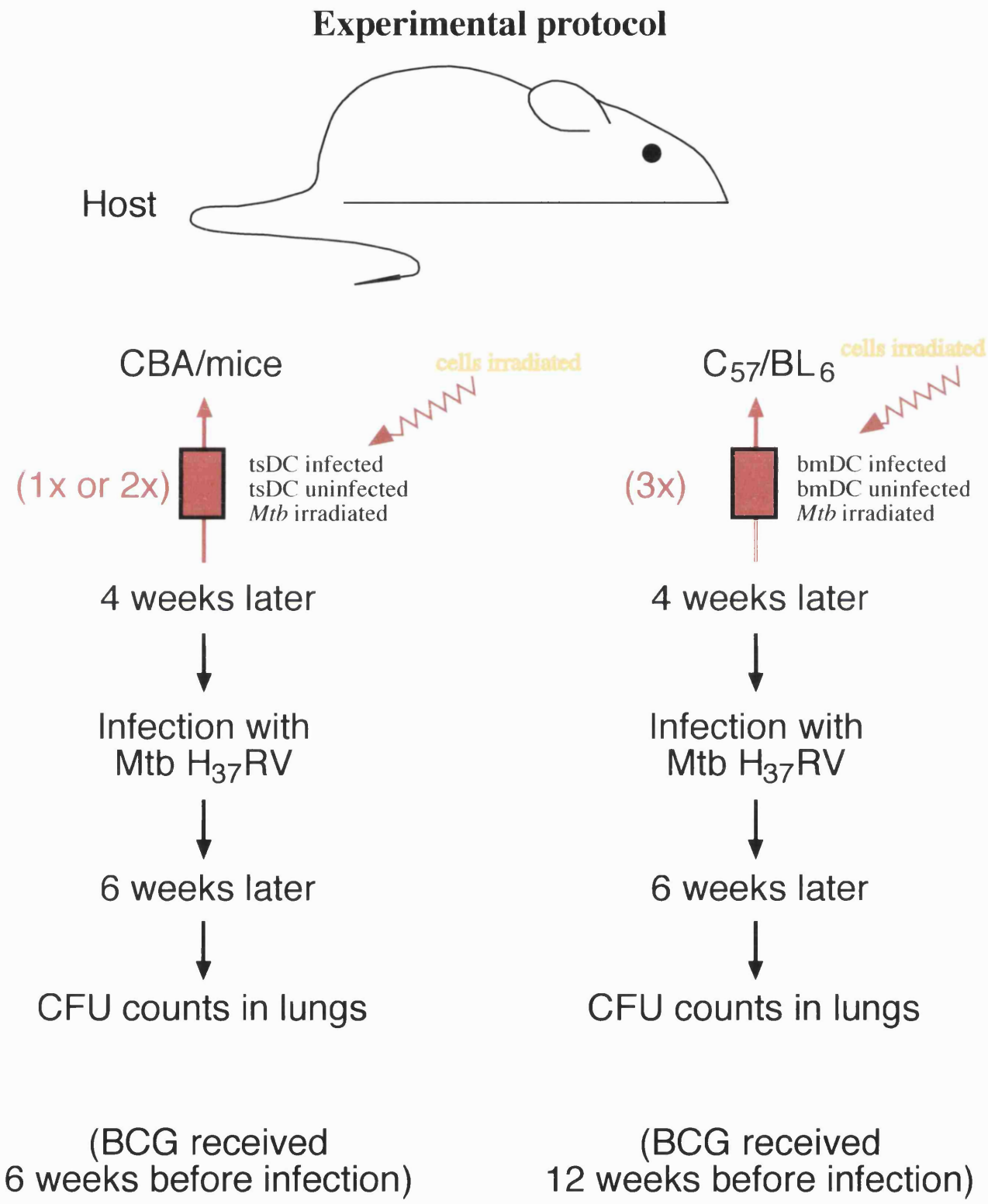

Figure 6.6 Illustration of the experimental murine model protocols used to test the protective immune response againt *Mtb* using tsDC (CBA mice) and bmDC (C57/BL6 mice).

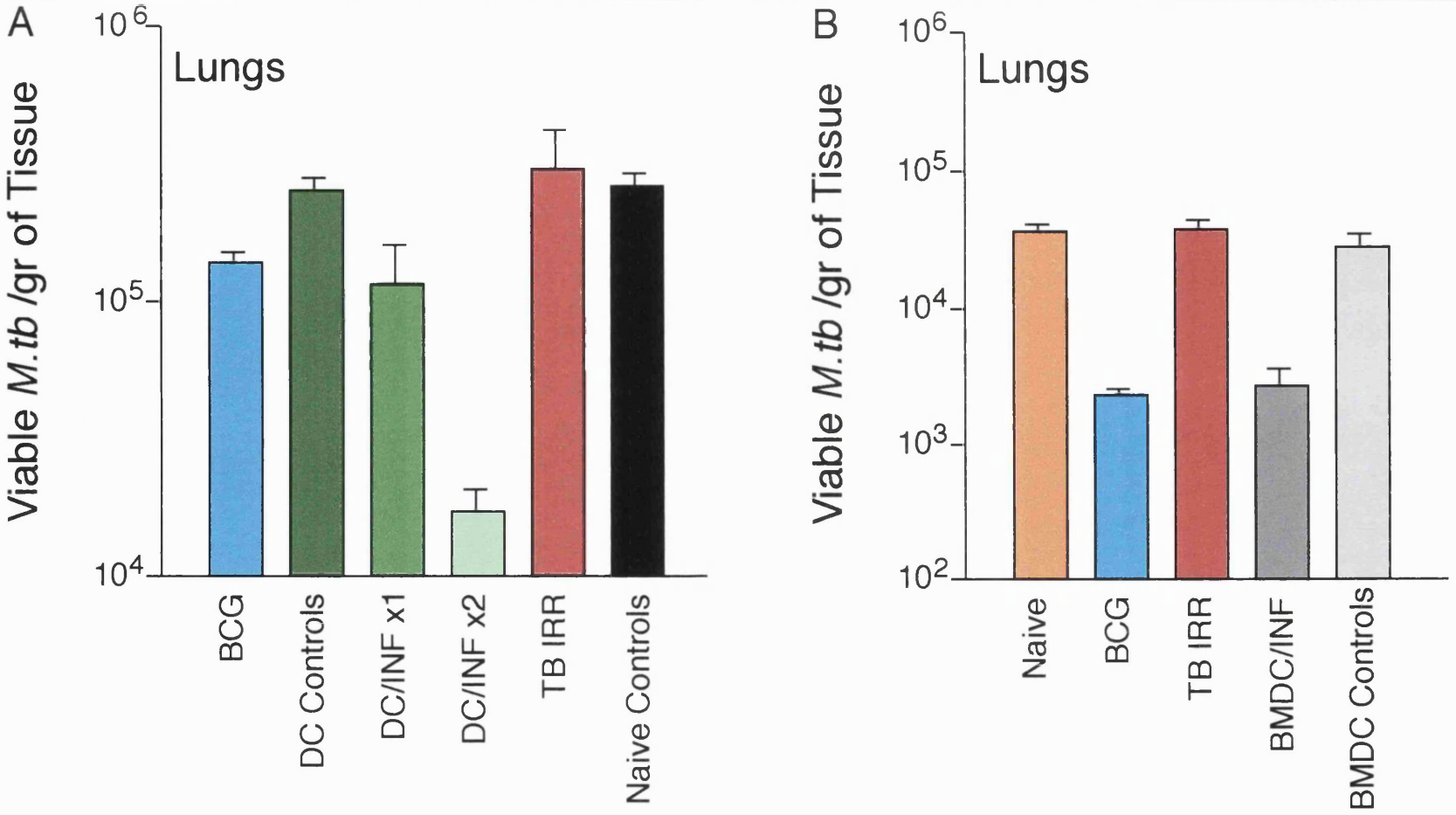

Figure 6.7 Protective immunity induced by *M.tuberculosis* infected DC. **A**) CBA mice received either tsDC uninfected (controls), tsDC *Mtb* infected -1 or 2x injections (DC INF) or irradiated *Mtb* (TBIRR) and 4 weeks after the last injection they were infected with *Mtb* H37Rv. One additional group received one injection of BCG, 6 weeks before infection. **B**) C57/BL6 mice received either bmDC uninfected (controls), bmDC-*Mtb* infected (BMDC INF) or irradiated *Mtb* (TB IRR) and 4 weeks after the last injection they were infected with *Mtb* H37Rv. One additional group received one injection of BCG 12 weeks before infection. 6 weeks later, in A and B, the number of live bacteria (mean ±SD) was assessed in the lungs. Differences between the groups were compared using Students t-test and, for stastis tical analysis p values <0.01 were considered to be significant.

6.4 Discussion

The experiments described here demonstrated that the infection of DC with *Mtb* is sufficient to induce activation of DC and as a consequence of this activation the cells acquire the ability to prime CD8+ T cell responses *in vivo*. The model system that was used requires some comment: first the male antigen used had no known cross-reactivity with environmental antigens (Keene and Forman, 1982). Secondly, CD8+ T cells that recognize the H-Y male antigen are usually helper-dependent (Guerder and Matzinger, 1992) and finally, responses can be easily distinguished because T cells from normal naïve female mice respond *in vitro*, only if they were first primed *in vivo* with APC (Fuchs and Matzinger, 1992).

Mtb activation of DC resulted in efficient priming of T cells specific for *Mtb*; this priming of T cells was demonstrated using DC derived from MHC Class II knockout mice, suggesting that cross-presentation and priming had occurred. Further experiments analysing the molecular basis for the effective induction of activation of DC in response to *Mtb* infection may help in future vaccine design.

In the experiments addressing the cross-presentation *in vivo* using bmDC derived from Class II KO mice effective priming of CD4+ T cells was demonstrated. However effective cross priming of CD8+ T cells needs to be investigated to complement our studies; in addition the phenotype of the cross-presenting DC cells, in this model, needs to be studied further.

Our results demonstrated that *Mtb*-activated DC could not only prime immune responses *in vivo*, but could also confer protective immune responses against a live infectious challenge with *Mtb* in mice. BCG-activated DC transferred to the lungs of mice which are subsequently infected with *Mtb* by the aerosol route, have also been shown to confer protection (Demangel *et al.*, 1999). In our experiments, two or three

immunisations with *Mtb*-activated DC were more effective than a single injection; the immunological basis for this is unknown.

Overall, these *in vivo* experiments confirm the *in vitro* results presented in Chapters 4-6, and support the development of antimycobacterial vaccine strategies which target DC *in vivo*.

Chapter 7
General discussion

Chapter 7

General Discussion

Although DC are recognised to be the most important cells involved in antigen presentation, their role in priming T cells against mycobacterial antigens, and how this influences the subsequent course of infection is not understood in any detail. The overall aim of this study was to investigate several aspects of the interaction of DC with the human pathogen *Mtb*, with a view to establishing model systems which would assist future studies in this area.

The initial experiments were aimed at establishing the intracellular fate of *Mtb* which had been phagocytosed by DC. Although no attempt was made to carry out a detailed ultrastructural analysis of the various molecules involved in uptake and intracellular processing of *Mtb*, the electron microscopy studies described in **Chapter 3** illustrate that the model would be an ideal experimental system for such analysis. The process of phagocytosis was clearly evident, with bacilli being taken up both individually and in small clumps. The phagocytic capacity of the tsDC used was at least equivalent to that seen with macrophages. Following phagocytosis, the bacteria were seen in large cytoplasmic vesicles, possibly representing a retention compartment for antigen processing and loading of MHC Class II molecules, as observed previously by DC in lymphoid organs (Kleijmeer *et al.*, 1995). A detailed examination of these vesicles using intracellular staining techniques, confocal microscopy, immunoelectron microscopy etc would provide an ideal system for investigating antigen processing of *Mtb* in more detail. There was no evidence of apoptotic cell death of *Mtb*-infected DC, at least by ultrastructural criteria, and the DC appeared to be able to inhibit growth of

Mtb more effectively than peritoneal macrophages. The results presented in **Chapter 3** (section **3.3**) suggest that the mechanism involved in this was multifactorial involving the production of ROI and RNI. Recent studies (Gonzalez-Juarrero and Orme, 2001; Bodnar *et al.*, 2001), using primary DC showed that *Mtb* was capable of intracellular multiplication; this may indicate that DC, like macrophages, can exhibit different levels of antimycobacterial activation.

Following exposure to *Mtb*, DC rapidly produced cytokines, mostly proinflammatory cytokines (**Chapter 4**), which reflects changes seen *in vivo* at the site of granuloma formation (Robinson *et al.*, 1994). This rapid cytokine response probably plays a role in providing the appropriate local milieu for cell recruitment and differentiation. Thus, the production of IL-12 has a key role in *Mtb* infection as a regulatory cytokine which drives the production of IFNγ-producing Th1 cells and promotes cell-mediated immunity (Gazzinelli *et al.*, 1993), reviewed by (Trinchieri and Gerosa, 1996). In the experiments described in **Chapter 4**, cytokine production tended to be higher in *Mtb* infected bmDC than in tsDC, possibly reflecting regulatory changes and other factors such as accumulation of necrotic cells in the long-term cultured cells. The observation that *Mtb*-infected bmDC cultures produced small but significant levels of IL-10, emphasises that there is a cytokine balance which might shift in vivo; IL-10 decreases IL-12 production and promotes a shift towards a Th2 response (Fiorentino *et al.*, 1991; D'Andrea *et al.*, 1993). It was not clear, from the experiments presented here, whether the IL-10 was produced by bmDC or by contaminating cells such as macrophages or NK cells. Purification of the DC by cell sorting might help to resolve this.

The differentiation of DC, following exposure to *Mtb*, towards an APC phenotype is underlined by the upregulation of costimulatory molecules and MHC Class II expression at the cell surface. This would represent an *in vivo* DC which had acquired

antigen in the periphery and migrated to lymphoid tissue for T cell contact. It is notable that the upregulation of MHC Class II seen in our experiments was weak, a point which has also been reported for primary DC stimulated with *Mtb* (Gonzalez-Juarrero and Orme, 2001; Bodnar *et al.*, 2001). Whether DC-stimulation by *Mtb* is suboptimal is not clear at the moment, but is currently being investigated as it could represent a mechanism by which *Mtb* limits the induction of an optimal protective response.

The ultrastructural and immunological changes in *Mtb*-infected DC reported in Chapters 3 and 4 are further supported by the demonstration of the activation of the signalling cascade resulting in NFκB activation reported in **Chapter 5**. The rapidity with which it was possible to detect phosphorylation and degradation of the inhibitory protein IκB-α (5 minutes) and the subsequent nuclear translocation of NFκB (20 minutes), was at first surprising and suggested that it was initiated by cell surface contact rather than by an intracellular event following phagocytosis. Some studies have suggested that phagocytosis is required for NFκB activation in *Shigella* and *Listeria* infections (Dyer *et al.*, 1993; Hauf *et al.*, 1994). The use of the phagocytosis inhibitor colchicine confirmed that NFκB activation was not dependent on phagocytosis, supporting earlier studies with *Mycobacterium avium* (Giri *et al.*, 1998). The signal for activation is probably by receptors such as TLRs; however communication between DC, and a role for DC:DC contact may also be involved.

The role of NFκB activation in pro- or anti apoptotic development is poorly understood. The expression of c-Rel is thought to play a role in driving an anti-apoptotic response in several types of cells (Chen *et al.*, 2000). We observed increased expression of c-Rel in bmDC compared to macrophages, but were unable to establish a relationship with Bcl-2 expression.

The molecular activation of DC following exposure to *Mtb* was shown to result in functional activation, with *Mtb* activated DC being able to prime T cell responses to an unrelated antigen, following transfer into syngeneic mice (Chapter 6). Previous studies have shown that antigen-pulsed DC are able to activate T cells (Celluzzi *et al.*, 1996). Our results suggest that such priming could take place in the absence of T cell help, as suggested previously (Rock *et al.*, 1990). Our results also suggested that there was cross priming of CD8+ T cells as described previously (Bennett *et al.*, 1997).

The most important finding with regard to the functional activation of DC by *Mtb* arose from the experiments investigating protective immune responses against challenge with live *Mtb*. A single transfer of *Mtb*-activated DC resulted in small, but significant levels of protection, while two or more transfers resulted in protection which was significantly greater than that seen with BCG. Most attempts to achieve protection that is greater than that seen with BCG in small rodent models have failed to do so. This approach now provides us with an opportunity to dissect the immunological requirements for a protective immune response which is greater than that seen in BCG. For example, is it simply a quantitative effect, generating more effector cells? Or is there a qualitative difference between the response generated by *Mtb*-activated DC and that generated with BCG? The use of newly evolving techniques such as the analysis of global gene expression, or ELISPOT techniques to quantify responses can now be used to address these questions. By understanding the immunological consequences of DC-generated protection, it is possible that new strategies which target DC activation *in vivo*, could be developed into new therapeutic and prophylactic approaches to TB.

The results described in this work demonstrated that the infection of DC with *Mtb* is sufficient to functionally switch an immature DC into an antigen presenting DC capable of activating naïve T cells. This T cell activation resulted in the generation of significant levels of protective immunity against challenge with viable *Mtb* in mice (published work: Tascon, Soares, Ragno, Stavropoulos, Hirst and Colston. Immunology, 2000, 99, 473-480).

Other groups have used similar models to investigate interactions between *Mtb* and murine DC *in vitro*. For the most part, results are similar to those reported here, in that similar changes in the expression of cytokines, accessory molecules and MHC Class II molecules were reported (Bodnar *et al.*, 2001). One significant difference between our findings and those of Bodnar *et al* involves the ability of DC to restrict growth of *Mtb* following phagocytosis. In **Chapter 3** we report that the number of viable *Mtb* which could be recovered from tsDC declined with time, whereas an identical infection of peritoneal macrophages was followed by an increase in recoverable *Mtb* (Fig. 3.14). In contrast, Bodnar *et al* (2001) found that *Mtb* grew similarly in bmDC and in macrophages. There are a number of possible explanations for this:

- In our experiments we used a DC cell line (tsDC) and peritoneal macrophages, whereas Bodnar et al used primary bmDC and bone marrow derived macrophages from C57BL/6 mice.
- The cells used by Bodnar et al appeared to have a reduced ability to phagocytose *Mtb* compared to those used in this study.
- The multiplicity of infection (MOI) and the time scale used to assess the viability of *Mtb* within the cells differed between the two studies.

In repeated experiments we were able to detect a small, transient production of NO following infection of DC with *Mtb* opposite to higher amounts of NO produced by

Mtb infected macrophages seen in previous works; we were also able to detect a rapid, transient up-regulation of iNOS expression using RT-PCR (data not shown). This suggests that, at least in the tsDC model system that we used, the control of bacterial replication may differ from that seen in macrophages.

The experiments describing the priming of CD8+ T cells responses by *Mtb*-infected DC in the absence of classical CD4+ T cell help **(Chapter 6)**, are novel and demonstrate for the first time that infected DC are able to directly prime CD8+ T cells. The mechanisms involved in this are the subject of current investigation within the laboratory. The levels of protective immunity generated by immunisation with *Mtb*-infected DC were surprisingly high, and could be significantly greater than those seen with BCG immunisation. In most experimental systems it has been difficult to demonstrate immunity greater than that seen with BCG and hence we feel that further exploration of the mechanisms involved should provide important information for the development of antimycobacterial vaccines.

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