

**TNF- α IN HOST IMMUNITY TO TUBERCULOSIS: *IN VIVO* AND *IN VITRO*
STUDIES OF PROTECTION AND PATHOGENESIS**

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"Who is wise? He who learns from all men, as it is said: From all my teachers have I gotten understanding"

Ben Zoma (1st century AD, *Ethics of the Fathers*).

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<u>Table of contents</u>		Page
Abstract		2
Abbreviations		3
Chapter 1	General Introduction..... <i>Discussion of the literature and a general overview of tuberculosis from a historical, immunologic and pathophysiologic viewpoints.</i>	4
Chapter 2	General Methods..... <i>Materials and experimental techniques used in the animal and human studies described.</i>	28
Chapter 3	The role of TNF- α in patients with severe tuberculosis..... <i>A study to examine the cause of deterioration following initiation of antibiotic therapy in patients with severe tuberculosis. The role of TNF-α in this clinical deterioration is discussed.</i>	40
Chapter 4	The effect of mycobacteria killing on the host response to infection.. <i>An experimental animal model of tuberculosis to determine whether killing of mycobacteria in vivo is associated with increased TNF-α production.</i>	49
Chapter 5	The immunopathologic effects of TNF- α in murine mycobacterial infection is dose dependent..... <i>Experiments in which strains of mice with various gene disruptions ("knock-out") are infected with recombinant BCG expressing TNF-α to study the role of TNF-α in protection against mycobacterial infection.</i>	57
Chapter 6	TNF- α and macrophage control of mycobacterial growth..... <i>Macrophages prepared from various gene disrupted mice are infected with BCG to examine the role of different cytokines in the control of mycobacterial infection.</i>	75
Chapter 7	Thalidomide induced antigen specific immune stimulation in patients with HIV and tuberculosis infection. <i>A double blind randomized control study to determine the effect of thalidomide during antituberculous therapy in patients co-infected with HIV and TB. The studies suggest a novel role for thalidomide as an immunomodulatory agent in this setting.</i>	89
Chapter 8	Concluding Remarks..... <i>Summary of the results and findings in this thesis.</i>	111
References.....		113
List of Figures and Tables.....		131
Publications arising from this work.....		133

Abbreviations

AFB	acid fast bacilli
APC	antigen presenting cell
BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guerin
BCG-TNF	BCG secreting murine tumor necrosis factor alpha
CFU	colony forming unit
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DMSO	dimethyl sulfoxide
DTH	delayed type hypersensitivity
ELISA	enzyme linked immuno absorbent assay
ENL	erythema nodosum leprosum
HIV	human immunodeficiency virus
IFN- γ	interferon gamma
IFN-KO	IFN- γ gene disrupted mouse (knock out)
IL	interleukin
iNOS	inducible nitric oxide synthase
iNOS-KO	iNOS gene disrupted mouse (knock-out)
IRF-1	Interferon regulatory factor-1
LAM	lipoarabinomannan
M-APC	monocyte APC
NF- κ B	nuclear factor κ B
PBMC	peripheral blood mononuclear cells
PPD	purified protein derivative of tuberculosis
ROI	reactive oxygen intermediates
RNI	reactive nitrogen intermediates
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	Severe combined immune deficiency
TCR	T cell receptor
TLR	toll-like receptor
TNF- α	tumor necrosis factor alpha
TNF-KO	TNF- α gene disrupted mouse (knock out)
TNF-R1	TNF- α receptor 1
TNFR-KO	TNF-R1 gene disrupted mouse (knock-out)

Chapter 1. GENERAL INTRODUCTION

“There is a dread disease which so prepares its victim, as it were, for death; which so refines it of its grosser aspect, and throws around familiar looks, unearthly indication of the coming change - a dread disease, in which the struggle between soul and body is so gradual, quiet, and solemn, and the result so sure, that day by day, and grain by grain, the mortal part wastes and withers away, so that the spirit grows light and sanguine with its lightening load, and, feeling immortality at hand, deems it but a new term of mortal life; a disease in which death takes the glow and hue of life, and life that gaunt and grisly form of death; a disease in which medicine never cured, wealth warded off, or poverty could boast exemption from; which sometimes moves in giant strides, and sometimes at a tardy pace; but, slow or quick, is ever sure and certain.”

Charles Dickens, Nicholas Nickelby, 1870

Tuberculosis has been widespread from earliest history as demonstrated by skeletal deformities suggestive of spinal tuberculosis (Potts Disease) seen in Neolithic man (5000 BC) [Sager P, Schalimtzek M and Moller-Christensen V, 1972]. The earliest written reports similar of tuberculosis probably come from Chinese writings of about 2700 BC, which mention “lung fever” and “lung cough”.

The causative organism of this disease, *Mycobacterium tuberculosis*, was discovered by Robert Koch, a German physician and self-taught microbiologist, who described it at a lecture in Berlin on March 24, 1882. Koch had used solid medium to culture pure colonies of single organisms, which he painstakingly characterized [Krause AK, 1932]. Three weeks later (!) Koch’s short paper was published in the Berliner Klinische Wochenschrift describing this work and formulating the Koch Postulates [Brock TD, 1999].

Up until the early 1900’s, the diagnosis of tuberculosis (known then as consumption) was a death knell. Dr. Edward Livingston Trudeau, described his own experience: *“Even at that early date Dr. Janeway’s great skill in physical diagnosis was recognized, and he had a class at Bellevue for physical diagnosis to which I belonged. He received me cordially and began the examination at once. When this was concluded he*

said nothing. So I ventured, "Well, Dr. Janeway, you can find nothing the matter?" He looked grave and said, "Yes, the upper two-thirds of the left lung is involved in an active tuberculous process."... "I think I know something of the feelings of the man at the bar who is told he is to be hanged on a given date, for in those days pulmonary consumption was considered as absolutely fatal" [Trudeau EL, 1916].

The Tuberculosis Epidemic in South Africa

"I consider it my duty to travel and work where I can use my scientific abilities to the best. At home, there are so many demands on my time, and controversies are so fierce, that it is virtually impossible to get any work done. Out here in Africa, one can find bits of scientific gold lying on the streets. How much have I learned and seen since I first came to Africa!"

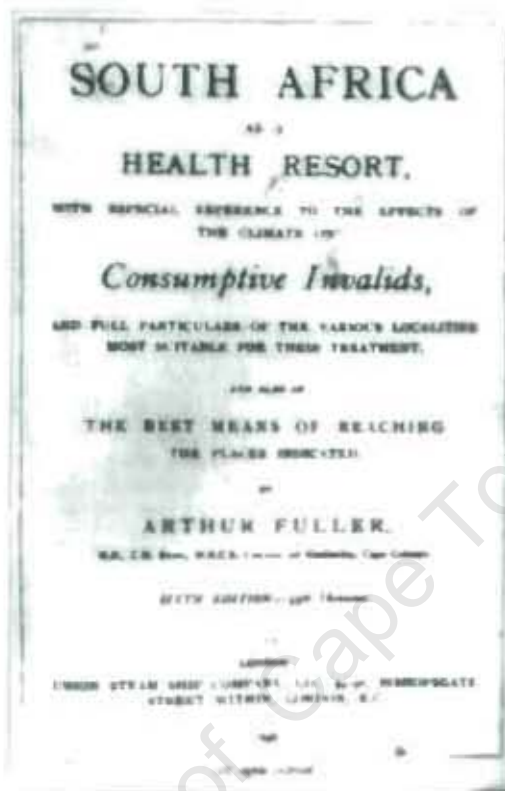
Robert Koch, 1890

More than 100 years after Koch's discovery, tuberculosis, the "Great White Plague", rages on [Dubos R and Dubos J, 1952]. The estimated annual incidence of tuberculosis is 8-10 million and about 3 million people die of the disease each year world-wide [Dye C, Scheele S, Dolin P, et al., 1999]. At present, it is estimated that 1 billion persons are infected with *M. tuberculosis*. Of those, 6-10 million are in South Africa where there is an average incidence of 340/100,000 persons and approximately 60,000 notified cases and 6,000 registered deaths in the country each year [Strebel PM and Seager JR, 1991].

The history of tuberculosis in South Africa illustrates the importance of socioeconomic, political and demographic factors in determining disease incidence [Packard RM, 1989]. Historically, although it is unlikely that tuberculosis was ever absent from the first European settlers, the pool of infection was increased by large numbers of tuberculous English and Scottish immigrants who came to South Africa in the last quarter of the 19th century hoping to be cured by exposure to the warm climate. The benefits of the South African climate for those with consumptive complaints were publicized extensively in books and articles written in this period (Figure 1-1). During

Figure 1-1

South Africa as a health resort for tuberculosis. Top: Advertisement, “South Africa as a health resort”, from 1898. Bottom: A photograph of Kimberley Sanatorium established in the late 19th century. Both reproduced with permission from *A Century of Tuberculosis, South African Perspectives*, Editors HM Coovadia and SR Benatar, Oxford University Press, Cape Town, 1991.



the 1890's the following advertisement appeared in a local paper: "*South Africa, the land of the high veldt [sic] and the Karroo [sic]! The land of soaking sunlight, and crisp dryness, and the cool night-wind! The land of elevated plains which joins the virtues of the desert, for which sick men flee to Egypt, with the virtues of the mountain, for which they seek Switzerland! The Cape which cures consumption; the Cape which is of Good Hope to all weak chests, the Cape which offers life and health and a career to the Englishmen suddenly confronted by that modern absolutist, the doctor, with the cold sentence of death or exile!*" [Scholtz W, 1897].

As a result of this publicity and selective immigration of infected individuals, the tuberculosis mortality rate in the late 19th century amongst Europeans in the Cape exceeded that of their British counterparts. Finally, in 1913 the Immigrants Regulation Act prohibited the entry of people with clinically recognizable tuberculosis. The introduction of antituberculosis drugs in the 1950's was followed by a sharp decline in tuberculosis mortality rates but incidence rates continued to increase. Recent notification figures suggest that the incidence rate is still rising and the burgeoning human immunodeficiency virus (HIV) epidemic will cause the numbers of individuals with tuberculosis to rise even further.

Specific conditions within South Africa have contributed to the inability to bring tuberculosis under control. The political and economic policies of the Nationalist Government led to disparities in tuberculosis control efforts [Packard RM, 1989; Strebel PM and Seager JR, 1991]. While the prevalence of tuberculosis among whites declined, virtually disappearing by the early 1980s, tuberculosis levels amongst the black and colored populations remained high. Factors such as urbanization and crowding, unemployment and poverty, alcoholism and poor nutrition contributed to the continued high incidence of tuberculosis in these populations. Even where local tuberculosis control programs were relatively well organized and efficient they did not succeed in controlling the epidemic [Wilkinson D, 1999]. The Global Tuberculosis Control WHO Report of 1999 notes that despite cure rates of 76% and very good adherence to directly observed therapy (DOT) in Guguletu, Cape Town, numbers remain high [WHO, 1999]. The specific underlying causes for this are not fully understood. Genetic predisposition to infection and/or development of active disease, *M. tuberculosis* strain variations, and

development of drug resistance strains as well as environmental factors may contribute to the susceptibility of the Western Cape population to infection or active clinical tuberculosis.

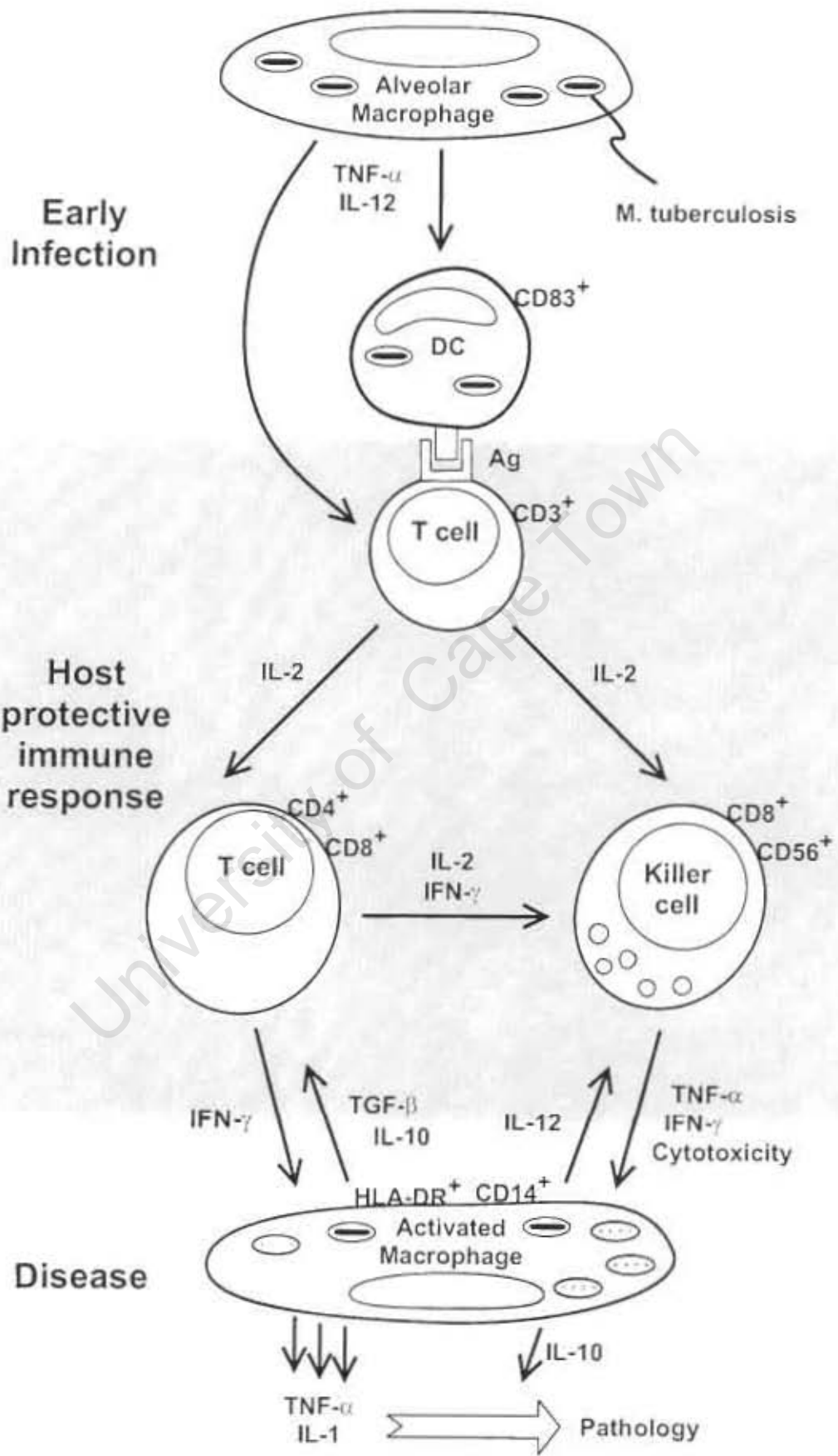
M. tuberculosis infection of the human host

Tuberculosis is an airborne infection spread from individual to individual when an infected person with acute pulmonary tuberculosis (open granulomas) releases viable organisms from the lung during coughing or speaking. Small aerosolized droplet nuclei (1-5 μ m) containing one to three viable mycobacteria are then inhaled into the mid and lower zones of the lungs of healthy individuals. The infecting organisms are then phagocytosed by resident alveolar macrophages. The organisms may proliferate in the macrophages, and the infected cells may drain into regional lymph nodes and into the lymphatic system and thus be distributed to many organs throughout the body. Soluble mediators such as cytokines produced by the infected macrophages lead to a cellular inflammatory response. Recruitment of T cells to the site of infection, T cell activation and secretion of a range of T cell cytokines lead to the development of a protective immune response which prevents further proliferation of the organisms but may damage host tissue through the mediators of inflammation [Figure 1-2]. The initial small area of infection with regional lymphadenopathy constitutes the primary or Gohn complex; its subsequent calcification is common [Benatar SR, 1991].

The development of immunity is associated with development of a positive delayed type hypersensitivity (DTH) skin reaction to tuberculin within 4-6 weeks of infection. Approximately one third of close contacts of tuberculosis patients are found to have evidence of infection (positive tuberculin skin test). This relatively low infectivity suggests that fairly prolonged close contact is necessary for transmission. Indurations of 5 to 10mm following tuberculin injection are considered indicative of infection in geographic areas free of other mycobacterial infections. Tuberculin testing cannot in itself sensitize a non-infected person but can stimulate established weak hypersensitivity. Skin test responses may also be positive after *M. bovis* bacillus Calmette-Guerin (BCG) vaccination [Holt PG, 1979]. DTH is associated with but is not synonymous with

Figure 1-2

Cells and cytokines involved in the immune response to mycobacterial infection.



protective immunity. Up to one fifth of patients with newly diagnosed tuberculosis have negative skin test responses and up to 50% of patients with extrapulmonary tuberculosis are similarly non-responsive [Daniel TM, Oxtoby MJ, Pinto E, et al., 1981]. A negative DTH response in the face of known tuberculosis infection may be due to anergy resulting from processes that interfere with the cellular immune response such as HIV infection, malnutrition, glucocorticoid therapy and tuberculosis itself.

It is estimated that approximately 5-15% of patients infected by the tubercle bacillus will ultimately develop tuberculosis. This suggests that the majority of infected humans mount an immune response which is essentially protective. The asymptomatic untreated infected person carries a 10% risk of progressing to tuberculosis during their lifetime; development of active disease can occur at any stage after primary infection. The factors determining who will develop disease are as yet not understood. However, a greater percentage of infected individuals in adverse living circumstances go on to develop clinical disease.

Tuberculosis in HIV infected individuals

Since the early 1980s a variety of factors have exacerbated the global tuberculosis problem in both developing and industrialized nations. The most important contributor has been the emerging epidemic of HIV infection and AIDS [Barnes PF, Bloch AB, Davidson PT, et al., 1991]. In the world today, 33 million people have AIDS or the virus that causes it. At least 70% of documented cases are African, and the number of HIV-infected individuals is increasing more rapidly in South Africa than in any other country in the world [UNAIDS Report, 1999].

HIV infection accelerates the clinical course of tuberculosis by destroying the CD4⁺ T cells that are responsible for cell mediated immunity. HIV coinfection is the most profound risk factor for progression from tuberculosis infection to active tuberculous disease. For immunocompetent individuals infected with *M. tuberculosis*, the lifetime risk of developing clinical disease is 10% [Comstock GW, 1982]. In contrast, individuals coinfecting with *M. tuberculosis* and HIV have a 5-15% annual risk of disease even in low prevalence areas [Selwyn PA, Hastel D, Lewis VA, et al., 1989]. In some areas in Cape

Town, a high tuberculosis prevalence area, the annual risk of developing active tuberculosis in advanced HIV infection is about 30% [Wood R and Hudson C, 1997]. In addition, HIV infected individuals are more likely to develop progressive tuberculosis following primary infection and are more likely to reactivate a previously acquired latent infection. Tuberculosis in HIV infected individuals often pursues an unusual course, resembling the fulminant primary disease seen in infants or resulting in extrapulmonary manifestations with dissemination [Post FA, Wood R and Pillay GP, 1997]. The deadly partnership between HIV and *M. tuberculosis* is mutually synergistic as active tuberculosis seems to enhance HIV replication and accelerate progression to AIDS [Goletti D, Weissman D, Jackson RW, et al., 1996]. *M. tuberculosis* infection may stimulate HIV replication by triggering the release of cytokines such as tumor necrosis factor alpha (TNF- α) which induces viral transcription, and by activating the CD4⁺ T cells that support viral replication [Fauci AS, 1996].

Cellular immunity and the macrophage granulomatous response in tuberculosis

Cellular immune function can be subdivided into two components: 1) Natural or innate immunity, mediated by phagocytic cells and natural killer cells that recognize and interact with cell wall structures of the pathogens; 2) Specific or acquired immunity, predicated upon T and B lymphocytes that utilize specific rearranged receptors to recognize and respond to specific antigens of the invading pathogen. The elaboration by the cells of protein mediators collectively termed "cytokines" is a feature of both innate and acquired immunity. Acting in concert with one another, cytokines regulate the function of the cellular components of the innate immune system and co-ordinate activation of the specific immune response. Thus, the two responses are interwoven and interdependent. Cytokines are frequently categorized based on sequence homology, or according to the type of receptors they engage. Hence, some belong to the chemokine family, others are denoted "growth factors". One of the most important and best studied cytokine families is that which encompasses TNF- α and TNF- β (lymphotoxin).

As mentioned above, it is the resident macrophages of the lung that first encounter and phagocytose the inhaled infective tubercle bacilli. These cells are relatively permissive for the growth of intracellular mycobacteria. For the macrophage to control

the growth of the organisms or even to kill them, the cells must be activated [Dannenberg AM Jr, Ando M and Shima K, 1972]. Following phagocytosis of mycobacteria, macrophages release a variety of soluble mediators including cytokines, which in turn interact with receptors on the surface of the cells to induce changes in their physiology and bactericidal capacity (activation). The major cytokines produced in response to mycobacterial infection by the monocytes/macrophages are TNF- α , interleukin-1 beta (IL-1 β), IL-6, IL-10, and IL-12 [Rook GA, 1988]. These molecules play a central role in inflammation as well as the developing immune response. Exposure of macrophages to these cytokines contributes to the functional regulation and activation of the cells. For sustained macrophage activation to occur, antigen specific lymphocyte cytokines are also required. Interferon gamma (IFN- γ) is the principle T cell mediator of macrophage activation and expression of antituberculosis resistance. Various *in vitro* studies in human and murine systems have shown that mycobacterium reactive CD4+ T cells are potent IFN- γ producers [Kaufmann SH and Flesch I, 1986]. IFN- γ is also produced by mycobacterium-specific CD8+ T cells [De Libero G, Flesch I and Kaufmann SH, 1988].

The protective immune response to *M. tuberculosis* infection is characterized by the formation of the granuloma or the tubercle. *"The tubercle is composed of a collection of phagocytes mesodermic in origin, which move towards the spot where the bacilli are situated and englobe them. The phagocytes retain their condition of epitheloid cells or are transformed into giant cells"* [Metchnikoff E, 1891]. The cells of the granuloma surround the infected focus (alveolar macrophages containing viable bacilli) and prevent dissemination by containment of the organisms within this physical barrier.

For optimal granuloma formation, blood derived monocytes and lymphocytes must be activated to differentiate into a well-organized interactive multi-cellular structure. It is the interaction of the cells with the infecting organism and its components that drives this immunologic activation. A number of cytokines are involved. The role of TNF- α in granuloma formation in mice infected with BCG was demonstrated in a number of studies. Infected animals were given anti TNF- α antibodies resulting in dramatically delayed development of granulomas [Kindler V, Sappino A-P, Grau GE, et al., 1989]. In these studies it was suggested that TNF- α released from macrophages in the microenvironment of developing granulomas is involved in a process of

autoamplification. TNF- α may act in an autocrine or paracrine way, enhancing its own synthesis and release, thus favoring further macrophage recruitment and activation leading to bacterial elimination. More recently Bean et al. have confirmed the importance of TNF- α in granuloma formation [Bean AG, Roach DR, Briscoe H, et al., 1999].

Inside the granuloma the infecting organisms are localized within the macrophages where they can survive and replicate in the phagocytic vacuoles of the cell. *M. tuberculosis* enters macrophages via specific binding to cell surface receptors. The success of *M. tuberculosis* as an obligate intracellular pathogen is dependent on its ability to modify its intracellular environment in order to persist within the host phagocytes. Usually, microorganisms sequestered within the phagosomes of macrophages are subject to degradation by the various lysosomal digestive enzymes transferred into this subcellular compartment as a result of phagolysosomal fusion [Cohn ZA, 1963]. However, in the case of mycobacteria, the phagosomes containing the tubercle bacilli acquire markers for the endosomal pathway but fail to fuse with lysosomes [Russell DG, Sturgill-Koszycki S, Vanheyningen T, et al., 1997]. In addition the pH within the phagosomes appears to be of importance for mycobacterial survival. Gomes et al showed that when macrophages infected with *Coxiella burnetii*, an intracellular pathogen which maintains an acidified vacuole, were coinfecting with *M. tuberculosis*, the growth of *M. tuberculosis* was reduced in the acidified vacuoles [Gomes MS, Paul S, Moreira AL, et al., 1999]. *M. tuberculosis* appears to produce ammonia and this might induce an increase in intralysosomal pH that might be protective against some of the cytotoxic mechanisms of macrophages [Gordon AH, Hart PD and Young MR, 1980]. The precise mechanisms by which the macrophage controls the growth of or kills mycobacteria are unknown. Via et al. have shown that the T cell cytokine IFN- γ enhances phagolysosomal fusion and leads to restricted growth of the mycobacterium in the phagosomal compartment in murine macrophages [Via LE, Fratti RA, McFalone M, et al., 1998].

During phagocytosis the macrophage generates reactive oxygen intermediates (ROI) as antimicrobial mediators. However, mycobacteria have evolved mechanisms to evade these oxidative toxins and experimental data indicate that *M. tuberculosis* can interfere with the toxic effect of ROI. Some mycobacterial compounds including the glycolipids and LAM downregulate the effects of ROI by scavenging reactive

intermediates [Chan J, Xing Y, Magliozzo RS, et al., 1992] and by inhibiting protein kinase C needed for the activation of the oxidative burst [Gennaro R, Florio C and Romeo D, 1985]. In addition, it has also been shown that isoniazid resistant strains of *M. bovis* that lack catalase are more sensitive to the H₂O₂ produced by the oxidative burst and are less virulent [Laochumroonvorapong P, Paul S, Manca C, et al., 1997], indicating that the neutralization of ROI is important for survival of mycobacteria.

Cytokine-activated macrophages also produce reactive nitrogen intermediates (RNI) that mediate potent antimycobacterial activity [Xie QW, Cho HJ, Calaycay J, et al., 1992] through nitric oxide (NO) and related RNI generated from the substrate L-arginine via the action of the inducible form of the enzyme nitric oxide synthase (iNOS) [Nathan CF and Hibbs JB Jr, 1991]. Studies have demonstrated an association between the activation of the L-arginine-dependent cytotoxic pathway and the antimycobacterial effect in cytokine activated murine macrophages [Chan J, Xing Y, Magliozzo RS, et al., 1992]. The synergistic effect of IFN- γ and TNF- α in inducing macrophage antimycobacterial function via RNI production underscores the importance of these cytokines in defense against *M. tuberculosis*. Experimental data in mice suggest that the production of RNI may be the principle effector mechanism responsible for killing or inhibiting the growth of virulent *M. tuberculosis* [Nathan CF and Hibbs JB Jr, 1991]. The role of RNI in defense against pathogens has not been established in humans, and there is difficulty in activating human macrophages *in vitro* to generate reactive nitrogen intermediates.

The role of T cells in the protective response to tuberculosis

T lymphocytes are essential contributors to protection. They do not act alone but must interact with other cells of the immune system to achieve optimum responses. All T cell subsets appear to contribute to protection. The central role of T lymphocytes has been demonstrated by experiments showing that nude and SCID mice (mice that are unable to produce T cells) develop more severe experimental *M. tuberculosis* and BCG infections than their control counterparts [Izzo AA and North RJ, 1992]. The presentation of antigen to T cells is necessary for their activation, and requires both the binding of the antigen-HLA or antigen-CD1 complex with the T cell antigen receptor and additional costimulatory signals delivered by the antigen-presenting cells (APC). Dendritic cells are

specialized APCs that express high levels of costimulatory molecules and are uniquely capable of activating naïve T cells in lymph nodes. After enzymatic processing in the endosomes of macrophages and dendritic cells, the antigens are bound to antigen-presenting molecules, and the resulting complex is expressed on the cell surface for presentation to T cells [Banchereau J and Steinman RM, 1998].

T cells expressing an α/β -T-cell receptor (TCR) constitute more than 95% of post thymic T cells in peripheral organs and blood. In contrast, γ/δ T cells are a minority at these sites but more common in mucosal surfaces such as lung. α/β T cells can be further divided into CD4+ T cells which recognize antigenic peptides in the context of MHC class II molecules, and CD8+ T cells, which respond to peptides presented by the MHC class I gene products. Antigen specific CD4+ T lymphocytes have been identified consistently in experimental and human tuberculosis [Barnes PF, Mistry SD, Cooper CL, et al., 1989]. In mice adoptive protection against *M. tuberculosis* and BCG largely depends on transfer of selected CD4+ T cells [Orme IM, 1987]. Also, mutant mice deficient in MHC class II and therefore missing functional CD4+ T cells die of BCG infection and *M. tuberculosis* infection [Flynn JL, Goldstein MM, Triebold KJ, et al., 1992]. This is consistent with HIV infection where patients more readily contract clinical tuberculosis as CD4+ T cells become progressively depleted [Wood R and Hudson C, 1997].

A role for CD8+ T cells in protection against tuberculosis has also been clearly shown. Flynn first used a $\beta 2$ microglobulin deficient mouse model which lacks surface expression of MHC class I molecules and therefore the mice are also functionally devoid of CD8+ T cells. These mice die relatively rapidly from *M. tuberculosis* infection [Flynn JL, Goldstein MM, Triebold KJ, et al., 1992]. More recently the same group of investigators followed the kinetics of murine CD4+ T cell and CD8+ T cell traffic into the lungs after aerosol tuberculosis infection. Both T cell subsets displaying activated phenotype were present in the lungs as soon as 1 week post infection. By 2 weeks numbers had increased with twice as many CD4+ T cells. Staining for IFN- γ production by these cells indicated that although early in infection the majority of IFN- γ is produced by CD4+ T cells, the cytokine was also produced by CD8+ T cells triggered by the appropriate stimuli [Flynn JL, 1999]. Although previously it was thought that

mycobacterium specific CD8+ T cells were not important in the immune response to tuberculosis in humans [Rees A, Scoging A, Mehlert A, et al., 1988] this has also now been shown not to be the case [Smith SM, Malin AS, Lukey PT, et al., 1999].

A number of *in vitro* studies have shown that in both human and murine systems CD4+ and CD8+ T cells are potent IFN- γ producers. They also express specific cytolytic activities, i.e. they lyse macrophages primed with mycobacterial antigens or infected with BCG or *M. tuberculosis* [De Libero G, Flesch I and Kaufmann SH, 1988]. A mechanism for granule dependent CD8+ T cell killing of mycobacteria has been proposed by Stenger, et al. Granulysin, a protein found in granules of cytotoxic T lymphocytes (CTLs), reduced the viability of a broad spectrum of pathogens. It directly killed extracellular *M. tuberculosis*, altering the membrane integrity of the bacillus, and in combination with perforin, decreased the viability of intracellular *M. tuberculosis* [Stenger S, Hanson DA, Teitelbaum R, et al., 1998]. This group has gone on to describe the mechanisms of host cell lysis by CTLs in mycobacterial infection. They have identified two mechanisms of apoptosis or programmed cell death induced by CTLs: the granule exocytosis pathway and a second pathway utilizing Fas-Fas ligand interactions between the CTL and macrophage. The same group also described a series of human CD1- restricted T cell lines of 2 distinct phenotypes. Both CD4 (-) CD8 (-) (double negative) T cells and CD8+ T cells efficiently lysed macrophages infected with *M. tuberculosis*. The cytotoxicity of CD4 (-) CD8 (-) T cells was mediated by Fas-FasL interaction and had no effect on mycobacterial viability. The CD8+ T cells lysed infected macrophages by a Fas-independent, granule dependent mechanism that resulted in killing of the mycobacteria [Stenger S, Mazzaccaro RJ, Uyemura K, et al., 1997].

That γ/δ T cells also contribute to protection is suggested by indirect evidence. In mice γ/δ T cells accumulate at the site of BCG infection [Augustin A, Kubo RT and Sim GK, 1989]. Also, the slowly progressing BCG infection in SCID mice compared to nude mice and mice depleted of both CD4+ T cells and CD8+ T cells has been taken as evidence for a role of γ/δ T cells [Izzo AA and North RJ, 1992]. Although there are few γ/δ T cells they are more efficient producers of IFN- γ than CD4 T cells [Tsukaguchi K, de Lange B and Boom WH, 1999]. Thus there is a coordinated interplay between macrophage activation, T cell activation, production of various cytokines and lysis of

infected cells, all of which are necessary for optimum protection against *M. tuberculosis* [Kaufmann SH, 1988].

TNF- α and its receptor

TNF- α is a principal mediator of innate or natural immunity and a regulator of the inflammatory response. It has both proinflammatory and cytotoxic effects. In the 1980's TNF was cloned, purified, and crystallized, and its tertiary and quaternary structure described [Beutler B and Cerami A, 1986; Beutler B and Cerami A, 1988]. The cytokine has subsequently been shown to play a central role in the host response to viral, parasitic, fungal and bacterial infections [Elkon KB, Liu CC, Gall JG, 1997; Nashleenas M, Kanaly S and Scott P, 1998; O'Brien DP, Briles DE, Szalai AJ, et al., 1999]. The TNF cytokine family co-evolved with a family of receptors consisting of the TNF- α receptor (TNF-R1) (p55) and TNF-R2 (p75). Two TNF molecules are known: TNF- α and TNF- β or lymphotoxin. Lymphotoxin and TNF- α both bind to both receptors, giving considerable flexibility in the interactions between ligands and receptors. Both receptors are present as preformed dimers on the cell surface of virtually all cell types except for red blood cells [Hohmann HP, Remy R, Brockhaus M, et al., 1989]. TNF-R1 appears to be responsible for the majority of biologic actions of TNF. TNF-R1 is the sole mediator of TNF-mediated cytotoxicity and cytostasis, and a major mediator of neutrophil and endothelial activation, whereas TNF-R2 by itself does not seem to be sufficient to stimulate these functions [Barbara JA, Smith WB, Gamble JR, et al., 1994]. It has also been shown that TNF-R1 controls intercellular and vascular adhesion molecules, MHC class I upregulation, secretion of other cytokines, cell proliferation and NF- κ B activation [Mackay F, Rothe J, Bluethmann H, et al., 1994]. Direct signaling through TNF-R2 occurs less and appears to be confined mainly to cells of the immune system. Soluble TNF receptors retain the capacity to bind TNF and act to neutralize or modulate TNF activity [Aderka D, Engelmann H, Maor Y, et al., 1992].

TNF has been shown to be produced by numerous immune cells. In macrophages, TNF- α synthesis is induced by biological, chemical or physical stimuli. The biosynthesis of TNF- α is tightly controlled so that it is present in vanishing small quantities in quiescent cells, but it is one of the major secretory factors of activated cells [Beutler B,

Greenwald D, Hulmes JD, et al., 1985]. The TNF- α gene is one of the immediate early genes induced by a variety of stimuli and TNF- α mRNA levels increase strikingly within 15-30 minutes suggesting that the factors necessary for the induction of TNF- α expression preexist in unstimulated cells. The regulation of TNF- α synthesis is cell type specific and at the transcriptional level regulation is upstream of the TNF- α gene, and includes some nuclear factor- κ B (NF- κ B) sites.

TNF- α is essential for protection against murine tuberculosis. Flynn et al. have shown that TNF-R-p55 deficient mice are more susceptible to tuberculosis infection than wild type mice. When TNF- α was neutralized *in vivo* by monoclonal antibodies, impaired protection against mycobacterial infection was observed [Flynn JL, Goldstein MM, Chan J, et al., 1995; Kindler V, Sappino A-P, Grau GE, et al., 1989]. More recently Bean et al. have shown that TNF- α is necessary for the generation of well organized granulomas in murine tuberculosis infection [Bean AG, Roach DR, Briscoe H, et al., 1999].

Although TNF- α is crucial to the protective immune response it also contributes to the pathogenesis of both infectious and autoimmune diseases. Increased concentrations of TNF- α have been shown to trigger the lethal effects of septic shock syndrome [Waage A and Steinshamn S, 1993]. TNF- α has also been implicated in the development of cachexia, the state of malnutrition and weight loss that complicates the course of chronic infections and many cancers [Tracey KJ and Cerami A, 1992].

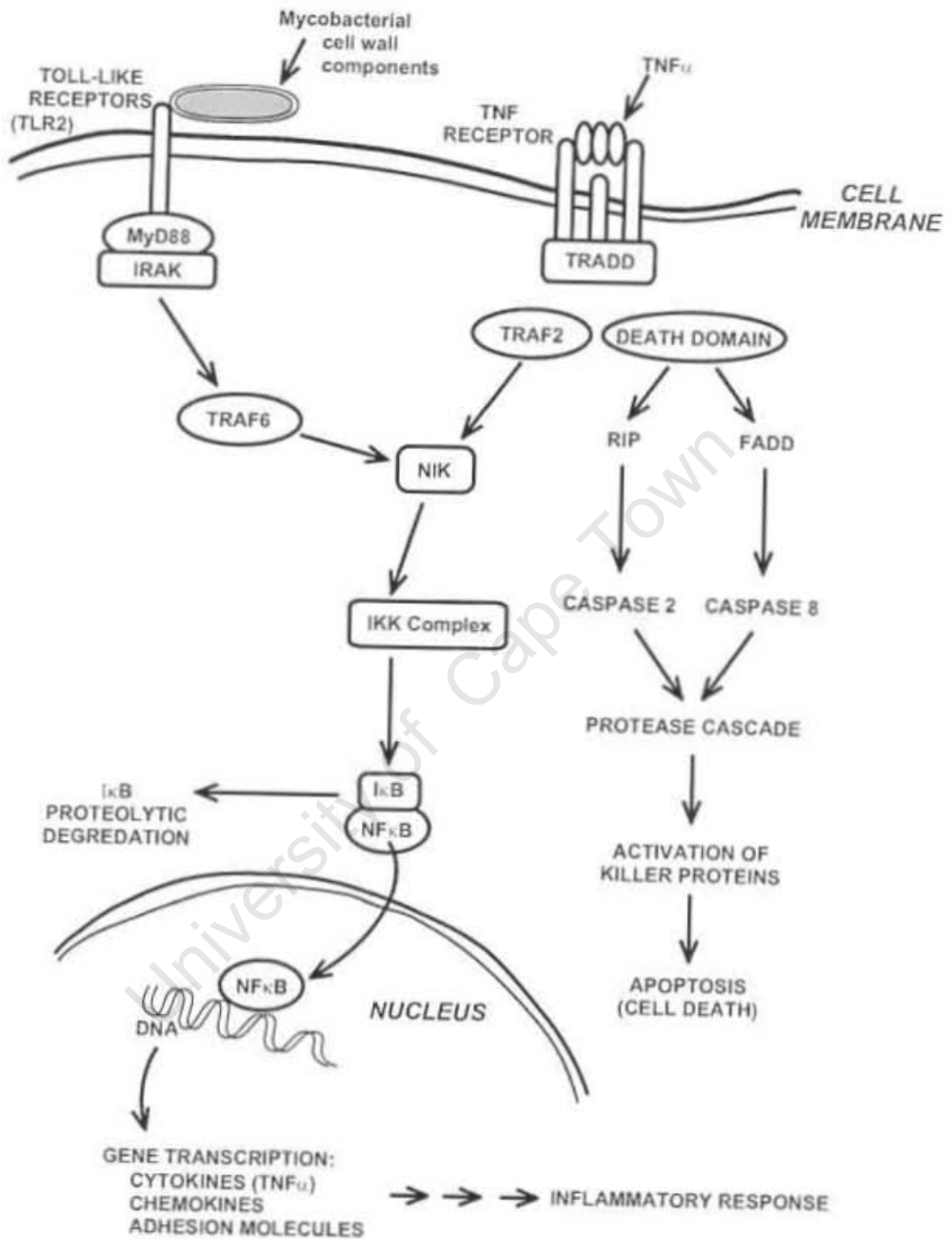
The mechanisms of TNF- α action are in the process of being worked out. When TNF- α binds to TNF-R1, a downstream signaling molecule called TRADD (TNF R1-associated death domain protein) is recruited to the TNF- α -TNF receptor complex and activated (Figure 1-3). TRADD, in turn, recruits FADD (or MORT-1), becomes fixed and thereby activates an enzyme called MACH (or FLICE), a member of the caspase family of proteases, which in turn cleaves other caspases to their active form. Downstream, the cleavage of poly-ADP-ribosyl polymerase (PARP) to its active moiety begins a process of NAD⁺ consumption that leads to cell death when all NAD⁺ is depleted. It appears that the TNF-R1-TRADD signaling cascade bifurcates at TRADD, thereby initiating two different pathways leading to either apoptosis or to protection from apoptosis via TRAF 2 (TNF receptor associated factor). The TRAF 2 route results in activation of NF- κ B leading to

Figure 1-3

Signaling pathways for TNF- α receptor and toll-like receptor (TLR-2). Activation through both receptors culminates in NF- κ B dependent gene transcription and the production of inflammatory mediators. In addition, signaling via the TNFR can result in cell apoptosis.

TLR-2 binds mycobacterial products and initiates signaling by recruiting MyD88, an adaptor protein also used by type 1 IL-1R. This leads to the recruitment of IL-1 receptor associated kinases (IRAK) 1 and 2, also known as innate immune kinases. TNFR associated factor 6 (TRAF 6) is recruited to this complex, which then activates NF- κ B inducing kinase (NIK). NIK is similarly activated by TRAF 2, which is recruited to the signaling complex of the TNFR and its adapter proteins. TNFR-associated death domain (TRADD) and receptor-interacting protein (RIP) alter ligand binding to the receptor. At this point the signaling from TRADD bifurcates and can either go via TRAF2 to the inflammatory pathway or via FADD and RIP to the apoptotic pathway.

In the inflammatory pathway, NIK phosphorylates the IKK complex, leading to phosphorylation of I κ B and NF- κ B, when then frees NF- κ B to migrate to the nucleus.



inflammation instead of apoptosis (Figure 1-3) [Malinin NL, Boldin MP, Kovalenko AV, et al., 1997].

The signaling pathways mediated by TNF-R2 are much less clearly defined. Recent evidence indicates that TNF- α may act via TNF-R2 to downregulate human and murine activated T cells by inducing apoptosis [Zheng L, Fisher G, Miller RE, et al., 1995]. The lack of a death domain in TNF-R2 indicates that TNF-R2 may use a distinct signaling pathway to induce apoptosis.

When TNF- α is administered experimentally to animals it causes shock and tissue injury similar to that seen in human septic shock syndrome. There is hypertension, metabolic acidosis, decrease in peripheral vascular resistance, falling cardiac output and loss of intravascular volume through capillary leakage [Tracey KJ and Lowry SF, 1990]. Endothelium-derived nitric oxide (NO) induced by TNF- α has been implicated in decreases in peripheral vascular tone and cardiac function [Finkel MS, Oddis CV, Jacob TD, et al., 1992]. TNF- α induced loss of tight-junctions may be responsible for the capillary leakage syndrome [Brett J, Gerlach H, Nawroth P, et al., 1989].

Because TNF- α production may have deleterious effects, efforts began in 1990 to block TNF by various mechanisms. One of the first attempts was to develop a monoclonal antibody that neutralizes TNF- α , which has since been clinically tested in rheumatoid arthritis, Crohns Disease, and septic shock with varying responses. Some of the difficulties have been poor pharmacokinetics and the development of antibodies to the foreign protein. Other approaches have involved designing a macromolecule to prevent activation of the TNF receptor. Using an adenoviral vector such a molecule has been expressed in mice where it has proved to be active and allowed investigators to set up an animal model free of TNF- α [Kolls J, Peppel K, Silva M, et al., 1994]. This work is still in progress.

Drugs have also been used to inhibit TNF- α production. Corticosteroids inhibit the transcription of TNF- α and other cytokines by stimulating I κ B α production which in turn results in the inactivation of NF- κ B [Auphan N, DiDonato JA, Rosette C, et al., 1995]. Pentoxifylline and other protein kinase C inhibitors have also been reported to downregulate TNF- α and other cytokine production through inhibition of activation of

NF- κ B [Biswas DK, Ahlers CM, Dezube BJ, et al., 1994]. Thalidomide has been found to be a selective inhibitor of TNF- α in leprosy patients with erythema nodosum leprosum. In this clinical setting, thalidomide partially inhibits TNF- α production and leads to reduced plasma TNF- α levels [Sampaio EP, Kaplan G, Miranda A, et al., 1993].

Cytokine modulation of the host response to tuberculosis: use of thalidomide

About 30 years ago it was discovered that administration of thalidomide as a sedative to leprosy patients with erythema nodosum leprosum (ENL) resulted in healing of the inflammatory skin lesions and the elimination of the systemic symptoms that characterize ENL [Sheskin J, 1965]. It was then discovered that the efficacy of thalidomide in ENL is associated with a rapid reduction in the circulating level of the proinflammatory cytokine TNF- α [Sampaio EP, Kaplan G, Miranda A, et al., 1993]. In further studies it was demonstrated that thalidomide exerts a specific inhibitory effect on the production of TNF- α by lipopolysaccharide-stimulated monocytes *in vitro* [Sampaio EP, Sarno EN, Galilly R, et al., 1991]. These initial observations suggesting that thalidomide can inhibit TNF- α in ENL led to investigations of the immune modulatory effects of the drug in other infectious diseases. Thalidomide appears to be effective in some conditions when conventional immunosuppressive therapy (e.g. corticosteroids) has failed [Jacobson JM, Greenspan JS, Spritzler J, et al., 1997]. It has been shown that in tuberculosis patients with or without HIV infection, short-term thalidomide treatment reduces plasma TNF- α levels and causes accelerated weight gain in patients while on thalidomide therapy [Tramontana JM, Utaipat U, Molloy A, et al., 1995]. These effects have, until recently, been attributed to the effect that thalidomide has on production of TNF- α . The mechanism by which thalidomide reduces TNF- α production is still unclear. The drug seems to inhibit TNF- α production by human monocytes *in vitro* in association with enhanced degradation of TNF- α mRNA. It also inhibits the activation of the nuclear transcription factor, NF- κ B. This factor is a promoter for the transcription of TNF- α as well as HIV-1 [Moreira AL, Sampaio EP, Zmuidzinas A, et al., 1993].

An exciting recent new development is the discovery that thalidomide can act as a costimulatory signal for T cell activation, resulting in increased production of IL-2 and

IFN- γ , and enhanced T cell proliferation. Optimal T cell activation requires 2 signals [Mueller DL, Jenkins MK and Schwartz RH, 1989]. Signal 1 is delivered by clustering of the T cell antigen-receptor-CD3 complex through engagement of specific foreign peptides bound to MHC molecules on the surface of an APC. Signal 1 can be mimicked by cross-linking the TCR complex with anti-CD3 antibodies. Signal 2 (termed costimulation) is antigen independent and may be provided by cytokines or by surface ligands on the APC that interact with receptors on the T cell. Costimulatory signals are essential to induce maximal T cell proliferation and secretion of cytokines such as IL-2 which then drive clonal expansion of T cells. In the absence of costimulatory signals, antigenic stimulation leads to T cell anergy or apoptosis. Thalidomide appears to act as a costimulator to T cells that have received signal 1 via the TCR [Haslett PAJ, Corral LG, Albert M, et al., 1998]. The drug is not mitogenic in itself nor does it have any effect on T cell TNF production.

In addition, thalidomide has been shown to induce the upregulation of CD40L expression on activated T cells [Corral LG, Haslett PAJ, Muller GW, et al., 1999]. CD40 signaling occurs early in antigen presentation and results in a stimulatory feedback loop in which the activated APC amplifies the T cell response. These effects on T cells have also been observed *in vivo* in HIV patients (with or without tuberculosis) treated with thalidomide [Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999].

A recent, exciting and important effect of thalidomide has been seen in refractory myeloma. Singhal et al., reported favorable results in reduction of tumor load in myeloma patients given thalidomide [Singhal S, Mehta J, Desikan R, et al., 1999]. The mechanism of drug action in this disease is unclear. Thalidomide may alter the secretion and biologic activity of cytokines such as IL-6, IL-1 β , and TNF- α which modulate growth and survival of myeloma cells and/or may induce the secretion of IFN- γ and IL-2 by CD8⁺ T cells [Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999]. The immune modulating effects of thalidomide in patients may thus be attributed to a balance between the drug-induced inhibition of production of monocyte cytokines including TNF- α and the drug's apparent adjuvant effect on T cell responses. This may explain the paradoxical clinical effects the drug may have in some clinical situations. For example, in the management of Behcet's Disease, thalidomide is very effective in healing the orogenital ulceration (anti-inflammatory effects) while simultaneously inducing or exacerbating erythema nodosum

(possible T cell effect) in these patients [Hamuryudan V, Mat C, Saip S, et al., 1998]. Exactly how the drug works, and determining which mechanisms mediate the activity of thalidomide in a range of clinical disorders, is critical in defining its therapeutic utility.

It has recently been noted that thalidomide also affects another important cytokine, IL-12. IL-12 is a proinflammatory cytokine which synergizes with low doses of IL-2 to increase production of IFN- γ , T cell proliferation, and cytolytic activity of both natural killer cells and CTLs [Gately MK, Wilson DE and Wong HL, 1986; Trinchieri G, 1996]. Thalidomide has been reported to inhibit IL-12 production by LPS stimulated monocytes [Moller DR, Wysocka M, Greenlee BM, et al., 1997]. *In vivo* however, thalidomide treatment has been found to increase IL-12 levels in the plasma of tuberculosis and HIV infected patients (see Chapter 7). While thalidomide inhibits LPS-induced monocyte T cell-independent IL-12 production, the drug stimulates the production of this cytokine in CD40L-CD40 T cell dependent systems. Corral et al. have examined the effect of thalidomide on the expression of CD40L on T cells stimulated by anti-CD3. Thalidomide induced a dose dependent and significant increase in CD40L expression which paralleled the increases in IL-12 production induced by anti-CD3. Thus the effect of the drug on IL-12 production varies according to the nature of the stimulus and the cell type being stimulated [Corral LG, Haslett PAJ, Muller GW, et al., 1999].

Animal models for the study of tuberculosis

The use of animal models to study tuberculosis began with the work of Max Lurie, a Lithuanian who immigrated to the United States in 1908. As a medical student he diagnosed his own case of tuberculosis. Because a number of family members had died of tuberculosis, Lurie felt strongly that there must be a genetic predisposition to the disease. While a patient at the National Jewish Hospital in Denver, Lurie began investigating the experimental pathology of tuberculosis. His work set the stage for widespread use of inbred laboratory animals to elucidate genetic aspects of disease. Lurie chose bovine tuberculosis infection of rabbits for his studies, because he considered it to be the animal model that best approximated the pathogenesis of tuberculosis in humans. He undertook his studies with inbred families of rabbits. When infected with the virulent Ravenel strain of *Mycobacterium bovis*, the "resistant" rabbit families developed cavitary tuberculosis

resembling that found in adult immunocompetent humans. The “susceptible” rabbit families developed haematogenously disseminated tuberculosis resembling that found in infants and immunocompromised individuals [Lurie MB, 1964]. Unfortunately, all of Lurie’s rabbit families have been extinct for about 25 years because of infertility due to inbreeding. Nowadays most commercially available rabbits are outbred and show intermediate resistance to tuberculosis. Some will develop cavities when infected with virulent *M. bovis*, but some will die early from haematogenous spread of the disease. The rabbit is now being used as a model of cavitory disease and tuberculous meningitis [Tsenova L, Bergtold A, Freedman VH, et al., 1999].

A number of other animal species can be productively infected with *M. tuberculosis*. Mice and guinea pigs, as well as some primate species have been used for experimental tuberculosis. In addition tuberculosis can occur naturally in a wide range of domestic and wild animals [Thoen CO, 1994]. Although this wide range of experimental models appears to be an advantage, it has also led to much debate in the field as to which is more suited to study tuberculosis infection. The guinea pig and the mouse represent two ends of the spectrum: the guinea pig is exquisitely sensitive to low dose pulmonary infection, and the mouse is relatively resistant to such exposure. Therefore, the guinea pig may be useful to model the natural history of rapidly progressive tuberculosis while the mouse is more useful in defining and mirroring the protective immune response that occurs in individuals exposed to the bacillus who do not develop disease. The mouse has thus come to be regarded as a good model of the acquired cellular response in the “resistant” host [Orme IM, Andersen P and Boom WH, 1993]. Because the guinea pig also develops strong dermal delayed type hypersensitivity reactions and tissue granulomas, the guinea pig is an excellent model for studies of pathogenesis and vaccine induced resistance to tuberculosis [Smith DW and Wiegshaus EH, 1989]. However, the relative paucity of species-specific reagents with which immunologic studies might be performed has hindered the use of this model. Only recently have commercial monoclonal antibodies for a few lymphocyte phenotypic makers come available and purified cytokines and their antibodies have not yet been developed [McMurray DN, Collins FM, Dannenberg AM Jr, et al., 1996]. As research in tuberculosis is focused increasingly on the immunologic and immunopathologic mechanisms of disease, the lack

of fundamental information about the immune system of the guinea pig and the lack of immune reagents have made it a less popular experimental model.

For the mouse, on the other hand, there is a vast array of immunologic reagents with which to study the expression of immunity to *M. tuberculosis* infection. In addition there are a multitude of inbred strains available. During the past decade there have been an enormous number of studies on the immune responses of the mouse to tuberculosis infection. Mice are also relatively inexpensive, easy to handle and breed, and easily contained in Bio-Safety Level 3 (BSL3) facilities, which are required for experiments with *M. tuberculosis*. Mice, in addition do not cough and therefore pose less of an aerosol risk to laboratory workers. There are several ways to infect mice with *M. tuberculosis*. A number of aerosol generating devices have been manufactured to enable low dose aerosol exposure which is thought to more closely resemble the human infection. Alternatively, intravenous infection is usually given in much higher dose via the lateral tail vein. Another important fact that has emerged in the development of the murine model is that not all mouse strains are alike. North and colleagues have repeatedly emphasized the need to identify and characterize the inbred strain being used in a particular study and to be aware of differences in resistance and susceptibility [Medina E and North RJ, 1998].

Another use of the mouse model which has recently emerged is the exploitation of new technologies of gene transfection and specifically targeted gene disruption to dissect immunoregulation in mice. For instance, using CD8 gene disrupted mice it was demonstrated [Flynn JL, Goldstein MM, Triebold KJ, et al., 1992] that CD8+ T cells may play an important role in resistance to tuberculosis. This knowledge will have bearing on the design of new vaccines against tuberculosis. Other studies in mice in which various cytokine genes have been disrupted have elucidated which cytokines are essential to control tuberculosis infection. Murine models have also been set up to more closely resemble clinical scenarios, hence the various models to examine 'latent' or 'dormant' tuberculosis infection [McCune RM, Tompsett R and McDermott W, 1956].

Finally, the last word in this section should be the recognition that an experimental model is just that and nothing more. The relevance of the experimental model to the human disease is variable and in the past, too little importance has been placed on this. It is difficult to produce a model that accurately reflects human disease

totally although we may mimic some aspects of the disease, as for example the protective immune response seen in mice. But mice do not display the usual human symptoms of tuberculosis. In addition, there has been concern that the experimental gene deletions in mice now used widely may cause other profound changes to immune networks so that a simple interpretation of experimental data may suffer from oversimplification.

In this thesis I have utilized the murine tuberculosis model, with and without specific genetic deletions and I have drawn a number of conclusions with the above caveat in mind.

Rationale for thesis studies

Tuberculosis has waxed and waned several times in the course of human history, but at the dawn of the new millennium many developing countries including South Africa continue to face “The Great White Plague”. It is a major threat to public health and a cause of massive morbidity and mortality. The fact that 90% of the world’s HIV epidemic is occurring in sub-Saharan Africa together with the tuberculosis epidemic makes the situation one of utmost global importance.

The fight against tuberculosis must be carried along two independent approaches: the treatment and prevention of the spread of the bacilli through directly observed therapy and case finding and a better understanding of the host pathogen interaction. We need to have shorter more effective treatment regimes, better vaccines, improved monitoring of clinical response to antibiotic treatment, and more conclusive assessment of cure after therapy. We also need to know who is susceptible to infection and who will develop active disease and finally what immune determinants constitute host protection.

This body of work examines some aspects of the host response. In particular, I have looked at the role of an important inflammatory cytokine, TNF- α in tuberculosis infection. In patients with severe tuberculosis TNF- α contributes to morbidity and possibly mortality. I also used the mouse model of tuberculosis to further define the role of TNF- α containment versus immunopathology in tuberculosis. Then to further explore the mechanism whereby TNF- α exerts its effects in tuberculosis infection, I have examined the response of murine macrophages infected *in vitro*.

Finally, recognizing the importance of TNF- α and the immune cascade in generating specific cell mediated immunity for the control of tuberculosis I have looked at the effects of immune modulation in patients co-infected with tuberculosis and HIV. I used thalidomide as an immunomodulatory agent because of the drug effects both on TNF- α production and T cell stimulation, and explored the possibility of using an adjuvant therapy that may assist conventional therapy to result in more favorable outcomes.

University of Cape Town

Chapter 2. GENERAL METHODS

Mycobacteria cultures and bacillary enumeration

Mycobacterium tuberculosis. *M. tuberculosis* strain Erdmann was provided as multiple stock vials by Dr. J. Belisle, Colorado State University, Fort Collins, CO. All stocks were stored at 10^7 - 10^8 bacilli/ml and kept at -70°C until use. Before use, the mycobacteria were grown for 7 days in Middlebrook 7H9 medium (Difco, Detroit, MI) containing 0.05% Tween 80 (Sigma, St. Louis, MO) at 37°C with daily agitation.

Recombinant BCG: Recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) strain Montreal secreting murine TNF- α (BCG-TNF) or containing the vector only (BCG-vector) were a kind gift from Dr. Richard Young of the Whitehead Institute, Cambridge, MA. Murine cDNA for TNF- α was cloned into the plasmids pRBD3 and pRBD4, as described [O'Donnell MA, Aldovini A, Duda RB, et al., 1994]. The expression vectors contained a kanamycin-resistance gene. The BCG-TNF and BCG-vector were grown to mid log phase in Middlebrook 7H9 medium (Difco) containing kanamycin (18 $\mu\text{g}/\text{ml}$) (Sigma) with minimal agitation for 7 days and kept frozen in aliquots until use [Murray PJ, Aldovini A and Young RA, 1996].

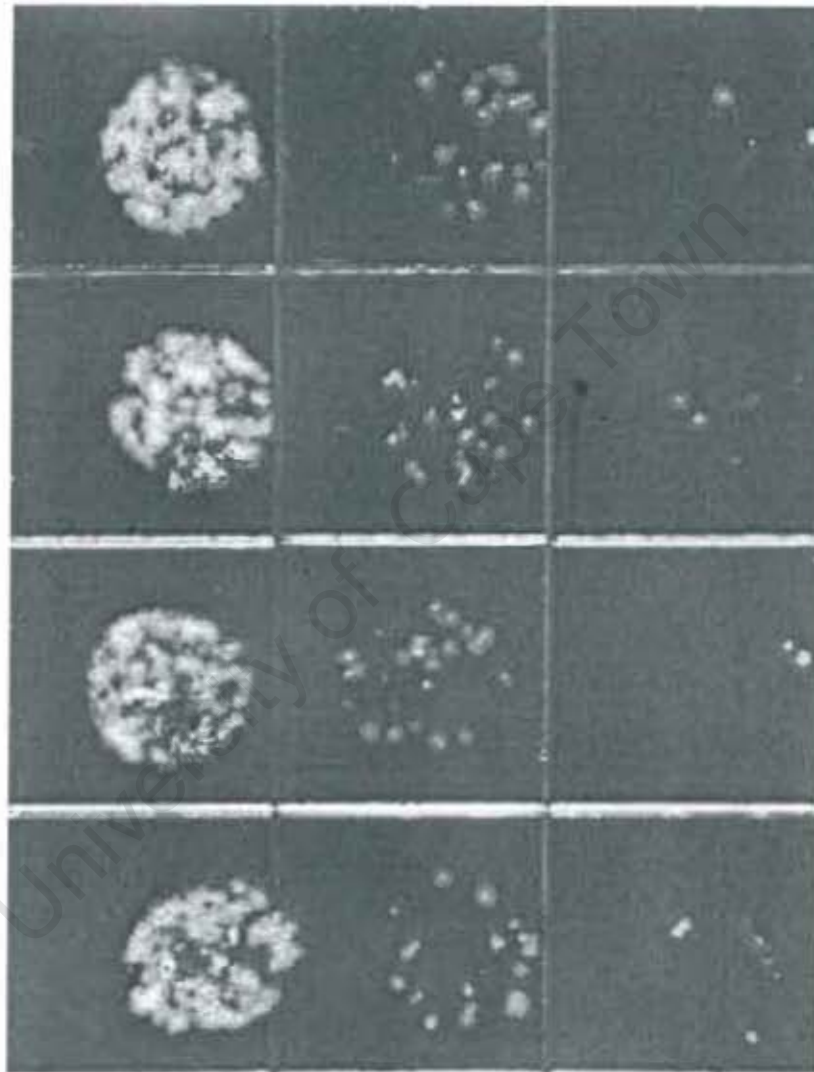
Colony forming units (CFU) assay: The number of viable mycobacteria in 7H9 culture medium, or in macrophages infected *in vitro*, or from the lungs, liver and spleen of infected mice, were evaluated. Ten-fold serial dilutions of culture supernatants or of organ homogenates were used. Infected macrophage monolayers were probe sonicated (model 60 Sonic Dismembrator, Fisher Scientific, Springfield, NJ) for 4 x 5 sec pulses per well to release intracellular bacilli. Bacterial suspensions were serially diluted and plated on kanamycin (18 $\mu\text{g}/\text{ml}$) supplemented 7H11 agar plates. The number of viable bacilli was evaluated by counting individual colonies using a magnifying microscope after 2-3 weeks of growth at 37°C (Figure 2-1). Each dilution was done in multiples of 6 and a mean colony count obtained. Organ homogenates from mice infected with recombinant mycobacteria were plated onto 7H10 agar plates as well as 7H10 agar plates supplemented with 18 $\mu\text{g}/\text{ml}$ of kanamycin (Sigma). The plates were incubated at 37°C

Figure 2-1

Photograph of the colony forming unit assay. Serial ten fold dilutions of mycobacterial suspensions were plated on solid agar culture giving rise to individual colonies for enumeration of viable bacilli.

Colony Forming Unit Assay

Dilution: 1 : 1 1 : 10 1 : 100



for 3 weeks. Organisms were enumerated as CFU as described previously [Moreira AL, Tsenova-Berkova L, Wang J, et al., 1997].

Mice

Inbred strains of mice: 8-10 week old C57BL/6 and F1 C57BL/6x129 mice were obtained from the breeding stock at the University of Cape Town, Cape Town, South Africa or from Jackson Laboratory, Bar Harbor, Maine, and used for the *in vivo* infection as well as a source of macrophages for *in vitro* infection experiments.

Gene disrupted mouse strains: 8-10 week old homozygous TNF- α receptor 1 (p55TNF- α R1) gene disrupted mice (TNFR-KO) on a C57BL/6 genetic background [Rothe J, Lesslauer W, Lotscher H, et al., 1993] were used. TNF- α gene-disrupted (TNF-KO) and iNOS gene-disrupted (iNOS-KO) mice on a C57BL/6x129 genetic background were also used [Marino MW, Dunn A, Grail D, et al., 1997]. In addition, IFN- γ gene-disrupted (IFN-KO) mice on a BALB/c genetic background were used. Mice were obtained from the University of Cape Town breeding stock or from Jackson Laboratories. The mice were kept under specific pathogen-free conditions at the University of Cape Town or The Rockefeller University animal facility, until infection.

Infection of mice with mycobacteria

Aerosol infection of mice: Infection was carried out according to a protocol developed in this laboratory [Moreira AL, Tsenova-Berkova L, Wang J, et al., 1997]. Briefly, mice were inoculated via the respiratory route by exposure to an aerosolized suspension of mycobacteria generated by a Lovelace nebulizer using a nose-only exposure apparatus (In-Tox Products, Albuquerque, NM) [Tsenova L, Moreira AL, Party E, et al., 1997]. To achieve the appropriate initial infecting inoculum, bacterial suspensions were diluted to a concentration of $1.5 \times 10^7/\mu\text{l}$ viable organisms. This suspension implants approximately 300 organisms into the lungs of mice as confirmed by plating lung homogenates 3 weeks after infection.

Intravenous infection of mice: To monitor the course of intravenous infection of mice two inocula were used: a high dose of 1×10^8 organisms or a lower dose of 2×10^5 organisms. Recombinant bacilli in a volume of 200 μl saline were injected into the tail

vein of both gene disrupted and control wild type mice for comparison. The initial infecting load was assayed by plating liver, spleen and lung homogenates 6 hours after infection. Thereafter, groups of infected animals were sacrificed at different time points and their organs evaluated as indicated. Because mice infected with higher inocula were likely to die quickly, experiments with high inocula were terminated earlier (40 days). Mice were first anesthetized with a solution containing 44 mg/kg of ketamine (Aveco Co., Fort Dodge, IA) and 5 mg/kg of xylazine (Rompum, Mobay Corp., Shawnee, KS). Blood was collected by cardiac puncture and serum prepared and kept frozen at -80°C until assay. Lungs, liver and spleen were collected aseptically immediately after cardiac puncture, weighed and used for evaluation of bacillary load, cytokine mRNA expression levels and histology. Serum and organs from uninfected mice were used for determination of baseline cytokine and cytokine mRNA levels.

For survival experiments in TNF-KO mice, the mice were infected with either 5×10^6 organisms in 200 μl or a high dose of 2×10^9 organisms in 200 μl . TNF-R1 genetically deficient mice (TNFR-KO) were infected with 5×10^6 or 1×10^8 organisms in 200 μl . The infecting loads in the organs were confirmed by CFU assay 6 h post infection.

All protocols were approved by the University of Cape Town Animal Ethics committee and/or The Rockefeller University's Institutional Animal Care and Use Committee.

Antibiotic treatment of infected mice

Isoniazid (Nydrasid Injection, Apothecon; Bristol-Myers Squibb, Princeton, NJ) was administered at a dose of 20mg/kg/day as an intraperitoneal injection. Rifampicin (Rifadin; Merrell Dow Pharmaceuticals, Kansas City, MO) was administered orally at a dose of 20mg/kg/day in a suspension of saline via a gavage needle. Orange coloration of urine confirmed rifampicin absorption.

Murine cytokine and cytokine mRNA levels

Determination of TNF- α levels in plasma of infected mice: At the time of sacrifice mice were bled by cardiac puncture into EDTA-containing tubes. Plasma was

stored at -80°C until assay. TNF- α levels in plasma were evaluated by commercial ELISA kits as described by the manufacturer (Endogen Inc., Woburn, MA).

Determination of TNF- α levels in the bacterial culture supernatants: The ability of the recombinant BCG to secrete murine TNF- α was verified by measuring by ELISA (Endogen) the concentration of the cytokine in the bacterial culture supernatants. Only mycobacteria that continued to produce the cytokine were used for infection. To ensure that the recombinant BCG-TNF were still secreting the cytokine during the infection, mycobacteria recovered from the lungs of the infected mice (giving rise to colonies in the CFU assay) were grown in 7H9 medium supplemented with 18 ug/ml of kanamycin for one week, and the supernatants tested for TNF- α concentrations by ELISA.

Determination of cytokine levels in macrophage culture supernatants: Macrophage culture supernatants were removed and stored at -70°C for cytokine analysis by ELISA. TNF- α , IL-12, IL-10 and IFN- γ were measured in the culture supernatant using commercial kits (Endogen) according to the manufacturer's specifications.

Cytokine mRNA levels in the infected lung: Total cellular RNA was prepared at 14, 28 and 45 days from lungs, spleens and livers of mice infected intravenously either with BCG-vector or BCG-TNF. Tissues were homogenized in 3 ml of RNeasyTM (Qiagen, Crawfordsville, IN) and RNA was extracted according to the manufacturer's instructions. The reverse transcription polymerase chain reaction (RT-PCR) was carried out as previously described [Laochumroonvorapong P, Wang J, Liu CC, et al., 1997]. Briefly, 1 μg of RNA was reverse transcribed using a murine Moloney leukemia virus reverse transcriptase and amplified with *taq* polymerase according to procedures given in the GeneAmp RNA PCR kit (Perkin Elmer, Branchburg, NJ). Primers for TNF- α , IL-10, IFN- γ , IL-12 and β -actin were used as described [Moreira AL, Tsenova-Berkova L, Wang J, et al., 1997]. Densitometry of the amplified bands was carried out using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Results were normalized to the density of β -actin.

Morphology of murine tissues and cells

Histopathology: Lungs and liver of mice were fixed in 10% buffered formalin, paraffin embedded and processed for histology. Sections were stained with hematoxylin-eosin and Ziehl-Neelsen for histologic evaluation and photography.

Immunohistology: Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated through graded alcohols. Antigen retrieval was accomplished by boiling the slides in 10 mmol/l of citrate buffer pH 6.0 for 20 min. The staining of the sections was performed in an automated immunostainer (Ventana, Tucson, AZ) using a polyclonal rabbit anti-mouse iNOS [Kristof AS, Goldberg P, Laubach V, et al., 1998] (1:300) (Calbiochem, La Jolla, CA) and for cell apoptosis using the TUNEL stain [Perry SW, Epstein LG, Gelbard HA, et al., 1997].

Morphometric evaluation of granuloma size: Morphometry of the lesions was performed using Microcomp, a computer based image analysis system (Southern Micro Institute, Atlanta, GA). A calibration micrometer (μm^2) slide was used to determine the area evaluated.

Electron microscopy: Peritoneal or alveolar macrophages from both TNF-KO and wild type control mice were seeded into wells containing plastic coverslips. Cell monolayers were infected either with BCG-TNF or BCG-vector at an MOI of 1:1. Coverslips were removed on day 1 after infection, fixed in 1.0% glutaraldehyde (Polysciences, Warrington, PA) in 0.1M cacodylate buffer (pH 7.4) for 1 hr at 37°C and post fixed in 1.0% osmium tetroxide (Polysciences) in 0.1M cacodylate buffer (pH 7.4). After washing in buffer the cells were stained *en bloc* in 2.0% uranylacetate, and then subjected to ascending dehydration in alcohol and embedding in Epon. Sections were examined and photographed in a JEOL electron microscope.

In vitro murine macrophage culture

Peritoneal macrophages: Peritoneal macrophages were obtained by lavage of the peritoneal cavity using a standard procedure [Cohn ZA and Benson B, 1965]. Briefly, the skin overlying the abdomen was dissected away and the peritoneal cavity was flushed with 5ml of cold Dulbecco's medium (DMEM), (GibcoBRL, Life Technologies, Grand

Island, NY) using a heparinized syringe and a 26 gauge needle. The lavage fluid was removed with a sterile glass pasteur pipette.

Alveolar macrophages: Alveolar macrophages were obtained by a modification of the method described by Holt [Holt P, 1979]. Briefly, after a lethal dose of anesthetic the animal was pinned onto a cork dissection board and the skin area overlying the trachea was dissected away. Overlying muscles and membranes were removed carefully by blunt dissection taking care to avoid lateral neck vasculature. Once the trachea was fully exposed it was cannulated using a sterile disposable 24 gauge intravenous cannula (0.7mm diameter) (Introcan, B Braun, Melsungen, Germany). The inner needle was removed and the cannula sutured lightly into place using 4.0 silk suture on a round needle (Clinisut, Sasurel, Port Elisabeth, SA). The dissection board was then tilted slightly so that the animal was in the head up position. Initially 0.8ml of DMEM supplemented with lidocaine hydrochloride (Sigma) (lavage fluid) was instilled into the lung via the cannula. The fluid was left *in situ* for 3 minutes and then gently aspirated. The process was repeated 5 times with an increase in volume of lavage fluid to 1ml. The lavage fluids from repeated washes were pooled.

Macrophage culture: Both bronchial and peritoneal lavage fluids were centrifuged for 8 minutes at 4°C. Cells were washed at 4°C in cold DMEM and then suspended in DMEM supplemented with 10% fetal calf serum (Gemini, Calabasas, CA), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (GibcoBRL) (complete medium) and plated in 48 well plates at 5×10^5 cells per well. After 2 hours of incubation at 37°C to allow adherence the cells were washed with warm complete medium and then incubated overnight. Before infection cells were washed with complete medium without antibiotics. Since lymphocytes are nonadherent, most will be washed away in this step.

Mycobacterial infection of macrophages: BCG-vector or BCG-TNF were suspended in complete medium without antibiotics at 37°C and added to 18h macrophage cultures at a multiplicity of infection of 1 bacillus per cell (MOI of 1:1). Bacilli were phagocytosed fully by 6 h after infection as evaluated in pilot studies. The cultures were therefore not washed after addition of the mycobacteria to the cells.

Nitroblue tetrazolium staining of murine macrophages

Oxygen free radical production: Macrophages were seeded on sterile glass coverslips in culture wells and infected with recombinant BCG as described above. Coverslips were removed at daily intervals, medium was washed off, and the cells were treated with phorbol myristate acetate, (PMA) (100ng/ml) (Sigma) followed by nitroblue tetrazolium (NBT) (Sigma)-saturated serum free medium (100 μ l) at 37°C for 1 hr. The number of cells with blue color was counted by microscopy and expressed as percentage blue stained macrophages.

Nitrite levels in murine culture supernatants

Nitrite production: The production of nitrite by peritoneal macrophages was measured by the Griess assay in culture supernatants [Granger DL, Taintor RR, Boockvar KS, et al., 1996]. One hundred microliters of culture supernatants were mixed with 100 μ l of modified Griess reagent (Sigma). Absorbance was measured after 10 mins at 570 nm in an ELISA microreader (Opsys MR, Dynatech, Chantilly, VA). A standard curve of NaNO₂ was used to establish the NO₂⁻ concentration in the samples.

iNOS inhibition: iNOS activity was inhibited using aminoguanidine (AMG) (Sigma). A stock solution of AMG (1M) in sterile normal saline was prepared. AMG was added to each well at a final concentration of 1mM at the time of infection with recombinant BCG [Misko TP, Moore WM, Kasten TP, et al., 1993].

Immunoblotting for proteins in murine cell lysates

Cell lysates were prepared from peritoneal macrophage monolayers at 8h, 24h and 48h post infection with recombinant BCG. Cell lysates were then electrophoresed on a 4-15% SDS PAGE gel and transferred to nitrocellulose. Immunoblots were probed for IRF-1 (48 kDa) using a rabbit anti-IRF-1 antibody (at 1:2000) dilution purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and for iNOS (130 kDa) using a monoclonal anti-iNOS antibody (at 1:1000) from Transduction Laboratories (Lexington, KY). In addition cyclooxygenase-2 (cox-2) was probed to assess the extent of reactive oxygen metabolism occurring in the macrophage cultures. HRP-conjugated anti-rabbit or anti-mouse

secondary antibodies (Pierce Chemical Co., Rockford, IL) were used to visualize the bands by chemiluminescence [Kamijo R, Harada H, Matsuyama T, et al., 1994].

Cytokine and cytokine receptor levels in human plasma

Blood samples from tuberculosis patients collected in EDTA were used for these studies. Blood samples were collected at about 8 a.m. from all patients to avoid diurnal variation in cytokine levels. The following cytokines and soluble markers of T cell activation were measured in human plasma by ELISA, in accordance with each manufacturer's instructions: Total TNF- α (unbound and bound to soluble TNF- α R) (Medgenix, Fleuris, Belgium) (mean normal 6 pg/ml); TNF- α R (Medgenix) (mean normal 1.2 ng/ml); IFN- γ (Medgenix) (mean normal 0.2 IU/ml); IL-6 (Medgenix) (mean normal 8.5pg/ml); IL-10 (Medgenix) and soluble IL-2R (sIL-2R) (mean normal 1250 pg/ml); (Genzyme, Cambridge, MA); IL-12 (this assay measures both p40 and p70 subunits of IL-12) (Endogen) (mean normal 81 pg/ml); and soluble CD8 (sCD8) antigen (Endogen). For each batch of cytokine kits the range of expected values for normal control is provided by the manufacturer and was confirmed in our laboratory.

Human peripheral blood mononuclear (PBMC) and lymphocyte assays

PBMC: PBMC were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation as described previously [Boyum A, 1968] and resuspended at a density of 2×10^6 /ml in RPM1 1640 (Gibco) supplemented with 10% human AB+ serum (Biocell, Carson, CA), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Gibco) (R10).

Lymphocyte proliferation assay: Aliquots of 100 μ l of PBMC suspensions in R10 were added to triplicate wells of 96 round bottom tissue culture plates (Costar, Corning, NY). R10 medium (unstimulated control) or R10 medium containing PPD (Statens Seruminstitut, Copenhagen, Denmark) (stimulus) were added to obtain a final concentration of 20 μ g/ml of PPD per well. Plates were incubated for 5 days at 37°C in 5% CO₂. On day 5, 1 μ ci of tritiated thymidine [(³H)-TdR] (New England Nuclear, Boston, MA) was added to each microwell for the last 18 h of culture. DNA was then harvested onto fiber mats, which were dried and immersed in scintillation fluid for

counting β emission. Data are presented as stimulation index (SI) calculated as: cpm in presence of PPD/cpm in the culture medium alone.

Human T Cell subsets: T lymphocyte subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$) in PBMC were enumerated by flow cytometry (FACSCOUNT, Becton Dickinson, San Jose, CA) at days 0, 21, 42 and 56.

In vitro analysis of thalidomide effect on antigen specific T cell responses

Monocyte antigen-presenting cells (M-APC): PBMC (3×10^6) from HIV infected individuals were incubated in 1 ml RPMI 1640 containing 2% pooled human serum and penicillin/streptomycin (R2) in 12-well polystyrene tissue culture plates (Costar) for one hour at $37^{\circ}C$. Subsequently, non-adherent cells were washed off with warmed RPMI. The remaining adherent cells (M-APC) were reincubated in R2 overnight with or without antigens (see below). M-APC were then detached by incubation on ice for 20 minutes followed by vigorous pipetting, then washed three times in saline and resuspended.

Dendritic cells (DC): DC were prepared from progenitor cells derived from PBMC by an adaptation of the method of Bender et al. [Bender A, Sapp M, Schuler G, et al., 1996]. Briefly, PBMC were incubated with neuraminidase-treated sheep red blood cells (SRBC) on melting ice for 40 minutes to remove T cells. Non SRBC-rosetting cells were subsequently isolated by density centrifugation. The latter cells were then plated at 10^6 cells in 1 ml/well in 12-well tissue culture plates in RPMI containing 1% autologous plasma containing 10mM HEPES buffer and penicillin/streptomycin. Recombinant human IL-4 (Genzyme Corp., Cambridge, MA) and GM-CSF (Immunex Corp., Seattle, WA) at 1000 U/ml each were added and the cultures incubated for seven days. Cells thus derived exhibited the characteristic stellate morphology of immature DC and were strongly HLA-DR+ and CD14- by flow cytometry. The DC were "pulsed" with antigens for 4 hours while maintained in the cytokine supplemented media. Subsequently, DC were harvested and washed three times. Each preparation of DC was divided into two aliquots: one was added directly (live DC) to autologous purified $CD4^+$ T cells, and the other was fixed for 30 seconds in 0.05 % glutaraldehyde as described previously [Haslett

PAJ, Corral LG, Albert M, et al., 1998], followed by three additional washing steps, before T cell coculture.

CD4⁺ and CD8⁺ T cells: CD4⁺ and CD8⁺ T cells were purified from PBMC by positive selection employing antibody-coated magnetic beads (Dynal, A.S., Oslo, Norway), in accordance with the manufacturer's instructions. Positively selected cells were separated from the beads by a further incubation with a secondary antibody which competes for the binding site of the primary antibody ("Detachabead", Dynal). Flow cytometric analysis revealed that cells isolated in this way were from 95.3 to 99.4 % CD3⁺CD4⁺ and 97.0 to 99.0% CD3⁺ CD8⁺.

APC incubation with antigens: M-APC and DC were incubated with the following antigens: PPD (Statens Seruminstitut) 10 µg/ml; recombinant baculovirus-derived HIV-1 p24 gag antigen, or a control baculovirus protein (Protein Sciences Corp., Meriden CT) 10 µg/ml; tetanus toxoid (Statens Seruminstitut) 10µg/ml; cytomegalovirus antigen (1/100 dilution of CMV infected cell line culture supernatant (Biowhittaker Inc., Walkersville, MD), or control antigen (diluted supernatant of uninfected culture); no antigen (negative control).

Antigen presenting cells were added to purified autologous CD4⁺T cells in triplicate cocultures in 96-well plates, so that each well contained 10⁵ T cells and M-APC (ratio of T:M-APC = 3:1) or DC (T:DC = 10:1) in a total volume of 200µl of R10 culture medium. Thalidomide (Celgene Corp., Warren, NJ) was dissolved in dimethyl sulfoxide (DMSO) to give a stock solution of 20 mg/ml, and immediately added to the cultures in serial dilutions to achieve the final concentrations indicated in the results. Dilutions were made so that all culture conditions contained the same concentration of DMSO. Cultures were treated daily with fresh thalidomide DMSO in 50% volume exchanges.

Effect of thalidomide on HIV activation of human T cells *in vitro*

PBMC or T cells were stimulated by cross-linking CD3, a component of the TCR complex. Forty-eight well flat-bottomed tissue culture plates were coated with mouse anti-human CD3 monoclonal antibody (a kind gift of Dr. Robert Zivin, Orthobiotech, Raritan, NJ) at a coating concentration of 1-10µg IgG/ml as previously described [Haslett PAJ, Corral LG, Albert M, et al., 1998]. For each experimental condition, duplicate

cultures were set up at $0.5-1.0 \times 10^6$ cells per well (either purified T cells or PBMC) in 48-well plates. Cultures were treated daily with thalidomide at various concentrations as described above. At different time points culture supernatant were collected and stored at -70°c for assay of HIV p24 antigen by ELISA (Immunotech Inc., West Rook, ME) in accordance with the manufacturer's instructions.

Delayed type hypersensitivity response to PPD

Skin test for delayed-type hypersensitivity (DTH) was conducted at baseline and on day 42. Five units of PPD (Connaught Laboratories Limited, Willowdale, Ontario, Canada) were injected intradermally into the volar aspect of the left forearm and induration in two perpendicular diameters measured 48h later. An induration $\geq 5\text{mm}$ in diameter was considered positive [CDC, 1997].

Ethical considerations

All patient studies described herein were approved by the necessary regulatory agencies. These include the Medical Ethics Committee of the University of Cape Town, South Africa, the Institutional Review Board of the Rockefeller University, New York, and the U.S. Food and Drug Administration. Signed informed consent was obtained from all patients participating in the studies. The identities of all study patients were protected.

Statistical analysis

Student's paired t test was used for continuous variables when comparing follow up results to baseline results within a treatment group. To analyze differences between the two treatment groups, student t test was used. Chi-square analysis was used for comparing proportions. If data were not normally distributed, log transformations of data were performed. Linear regression analysis for associations between two variables were carried out using Excel spread sheets and data analysis software for these calculations (Microsoft Corp., Richmond, WA).

In vitro data was analyzed using an independent t -test when indicated.

Chapter 3. THE ROLE OF TNF- α IN PATIENTS WITH SEVERE TUBERCULOSIS

Introduction

The initiation of antibiotic therapy in patients with severe tuberculosis is often associated with a paradoxical clinical deterioration before the patients begin to show improvement. The clinical deterioration may be characterized by an increase in the size of lymph nodes and of intracranial tuberculomata, worsening of pulmonary disease or dissemination of tuberculosis with development of the adult respiratory distress syndrome, or unexpected death [Newcombe JF, 1971; Lees AJ, MacLeod AF and Marshall J, 1980; Onwubalili JK, Scott GM and Smith H, 1986]. It is possible that the release of mycobacterial components, including lipoarabinomannan (LAM), and the 30kD alpha antigen during mycobacterial destruction by antibiotics may be responsible for an inflammatory host response and production of TNF- α leading to this paradoxical deterioration [Moreno C, Taverne J, Mehlert A, et al., 1989; Aung H, Toossi Z, Wisnieski JJ, et al., 1996]. Indeed, when PPD is injected into animals presensitized to mycobacterial antigens, a systemic reaction characterized by fever, malaise and shock occurs [Youmans GP, 1979]. This is similar to the 'tuberculin shock' described by Koch in 1890 following injection of crude tubercle extracts into consumptive patients [Koch R, 1890].

To understand the underlying mechanism for the transient therapy-induced clinical deterioration, we monitored the clinical status of patients with newly diagnosed severe tuberculosis for the first 42 days of treatment. We examined plasma cytokine and cytokine receptor levels to determine whether changes in clinical status were associated with changes in levels of these protein mediators of the host immune response.

Study Design

Sixteen HIV negative adults with newly diagnosed severe (Karnofsky score of ≤ 70) pulmonary tuberculosis were enrolled into the 6 week pilot study carried out in Cape Town [Karnofsky DA and Burchenal JH, 1949]. Patients receiving immunomodulatory therapy (corticosteroids) were excluded. All consenting patients admitted to the hospital over a period of 4 months were recruited into the study. The initial evaluation involved clinical

assessment including Karnofsky score, chest radiography, temperature, weight and blood sampling for serum lactate determination (in fluoride coated tubes) and cytokine and cytokine receptor assays (collected in EDTA vacutainers; plasma stored at -70°). After commencing standard short course therapy (isoniazid 5 mg/kg, rifampicin 10 mg/kg, pyrazinamide 20-35 mg/kg and ethambutol 15-25 mg/kg) clinical evaluation (always performed by the same physician) and blood collection (between 8am and 12 noon) were carried out on days 0, 3, 7 and weekly for up to 42 days. Chest radiographs were assessed and scored by noting the number of affected zones and the presence or absence of cavitation.

Results

Clinical status and response to antituberculosis therapy: Sixteen patients (10 males and 6 females) were enrolled in the study (Table 3-1). Sputum microscopy was positive for acid fast bacilli at baseline in 15/16 patients. *M. tuberculosis* sensitive to isoniazid and rifampicin was isolated from these patients. One patient (#2) was sputum negative but tuberculosis was diagnosed on the basis of clinical history, a miliary pattern on chest radiograph and Pott's lesion on thoracic spinal radiograph. This patient refused invasive procedures to confirm the diagnosis, but improved following antituberculosis therapy. Patient weights at baseline ranged from 40-91 kg with a mean of 51.5 kg. Mantoux skin testing was positive in 14/16 patients (Table 3-1). Of the two patients who did not show ≥ 10 mm induration at the site of the skin test, one had disseminated tuberculosis (#2) and the other was an alcoholic and was undernourished (#13). When retested six weeks after initiating therapy both patients had become skin test positive.

Nine patients completed the full study period (Table 3-1). There were 2 deaths on day 12 after commencement of treatment (#10 and 11). One patient (#3) developed drug hepatitis on day 28. All subsequent blood and clinical results from this patient were excluded from analysis. Four patients (#5, 6, 7 and 9) were discharged (days 7 or 14) to ambulatory clinic follow-up and were not available for further study by us. The acute bed requirement at the study hospital prompted the discharge of these patients rather than any specific clinical characteristics.

Table 3-1 Patient demographics, clinical status and response to treatment.

Pt #	Age/Sex	Lactate (mmol/l)	Karnofsky Score	Weight (kg)	Mantoux	Chest* x-ray	Outcome
1	38/F	2.4	70	80	+	2	improved
2	54/M	1.5	50	44.5	-	6	improved
3	51/M	1.8	20	42	+	1	hepatitis day 28
4	43/M	1.5	30	42	+	5	improved
5	23/F	1.5	40	40	+	4	discharged day 14
6	37/M	Nd	60	57	+	4	discharged day 7
7	45/M	Nd	40	50	+	6	discharged day 14
8	37/M	1.6	40	56	+	4	improved
9	49/M	2.0	30	47	+	2	discharged day 7
10	72/M	1.9	30	44	+	5	died day 12
11	68/F	2.0	30	45	+	5	died day 12
12	53/F	1.8	40	91	+	1	improved
13	35/M	1.7	40	43	-	5	improved
14	27/F	0.7	50	44	+	3	improved
15	40/F	2.5	40	48	+	4	improved
16	26/M	1.1	40	50.5	+	5	improved

*Radiographic extent of lung involvement as graded out of 6 zones; nd - not done.

Following initiation of antituberculosis treatment, a significant decrease in the Karnofsky score was observed by day 3 ($p < 0.001$) which continued to decrease to day 7 ($p < 0.001$). By day 14, the Karnofsky score had increased to almost baseline levels and continued to increase until the end of the study (Figure 3-1A). The median Karnofsky score for the population on day 7 was 20 compared to a median of 40 at baseline. During the treatment period, the patients initially lost weight (Figure 3-1B). By day 3 significant weight loss was observed ($p = 0.02$) which persisted to day 7 ($p = 0.03$) and returned to baseline by day 14. Thereafter, patients continued to gain weight. Mean maximal weight loss over the course of the study was 1.5 kg (paired t test $p < 0.001$).

In 3 patients (#4, 7, and 15), the sputa became smear negative by day 7 and culture negative by day 14. Sputum could not be produced by day 7 in 5 patients (#2, 3, 13, 14 and 16) and by day 14 in 2 patients (#1 and 8). Patient #12 converted to sputum negative on day 28. The mean baseline temperature of the study cohort was 38.2°C and showed a steady downward trend in all patients, returning to normal in all patients evaluable by day 21 (median 14 days) after initiation of treatment.

Serum lactate was monitored at baseline in 11 patients (mean 1.7 mmol/l). After commencement of therapy, 9 patients were reevaluated and all showed an increase in serum lactate to mean peak levels on day 7 of 2 mmol/l (range 1.1-3.6) ($p = 0.06$) (Figure 3-2A). The two patients (#10 and 11) with the highest peak lactate levels died on day 12. The mean increase in serum lactate levels relative to the baseline value over the entire period of measurement was 1.36 mmol/l (paired t test $p < 0.001$).

Cytokine levels and response to therapy: Plasma cytokine and cytokine receptor levels were assessed at entry and throughout the treatment period. TNF- α levels at baseline ranged from 2.5pg/ml to 325pg/ml with a median of 28pg/ml and a mean of 47pg/ml (mean levels in normals 6pg/ml). TNF- α concentrations increased after initiating antibiotic treatment in 14/16 patients (Figure 3-2B). The two patients who did not show an increase in TNF- α were the two patients who had died by day 12. One of these patients had a relatively high TNF- α level at the initiation of therapy (113pg/ml). Figure 3-1A shows the mean TNF- α concentration relative to baseline over time. An increase in relative TNF- α levels was apparent by day 3 ($p = 0.06$), continued to day 7 ($p = 0.04$) and peaked by day 14 ($p = 0.05$).

Figure 3-1

Effect of anti-tuberculosis therapy on patient responses: **A.** Karnofsky score (open circles) and plasma TNF- α levels (closed circles), expressed as mean response \pm SEM relative to baseline. **B.** patient weight expressed as mean percent change in weight \pm SEM normalized to baseline. p values were calculated using a paired *t* test for changes in values at the time indicated relative to baseline. The statistical significance of the values compared to baseline are denoted on the Figure as follows: (a) $p=0.06$; (b) $p=0.05$; (c) $p=0.04$; (d) $p=0.03$; (e) $p=0.02$; (f) $p<0.001$.

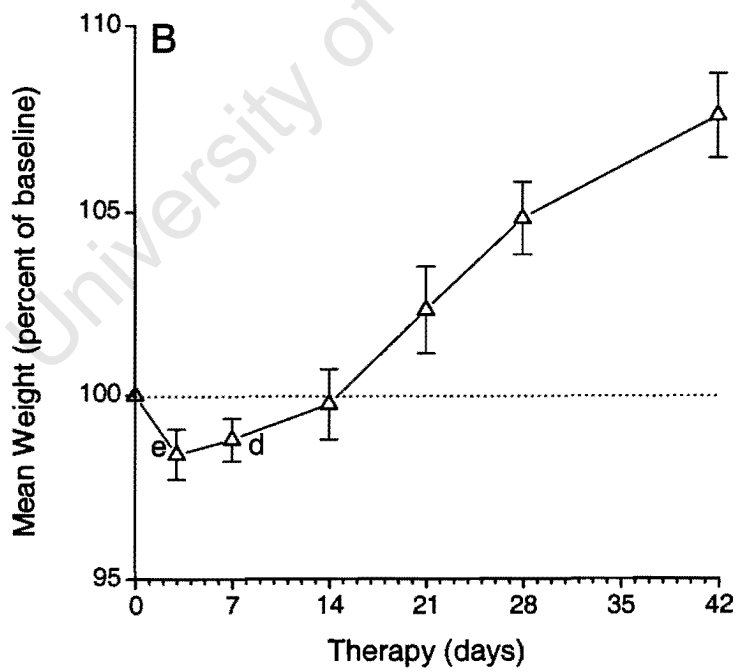
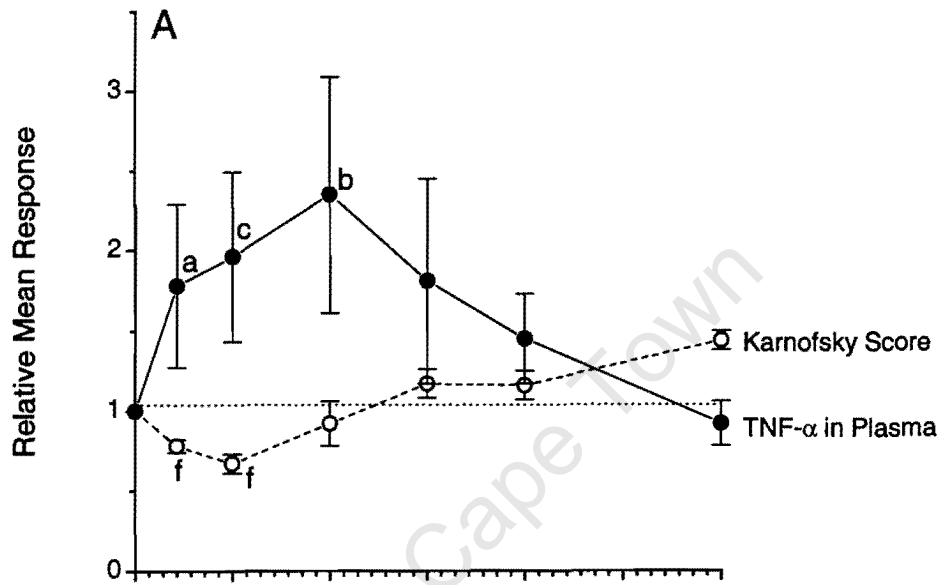
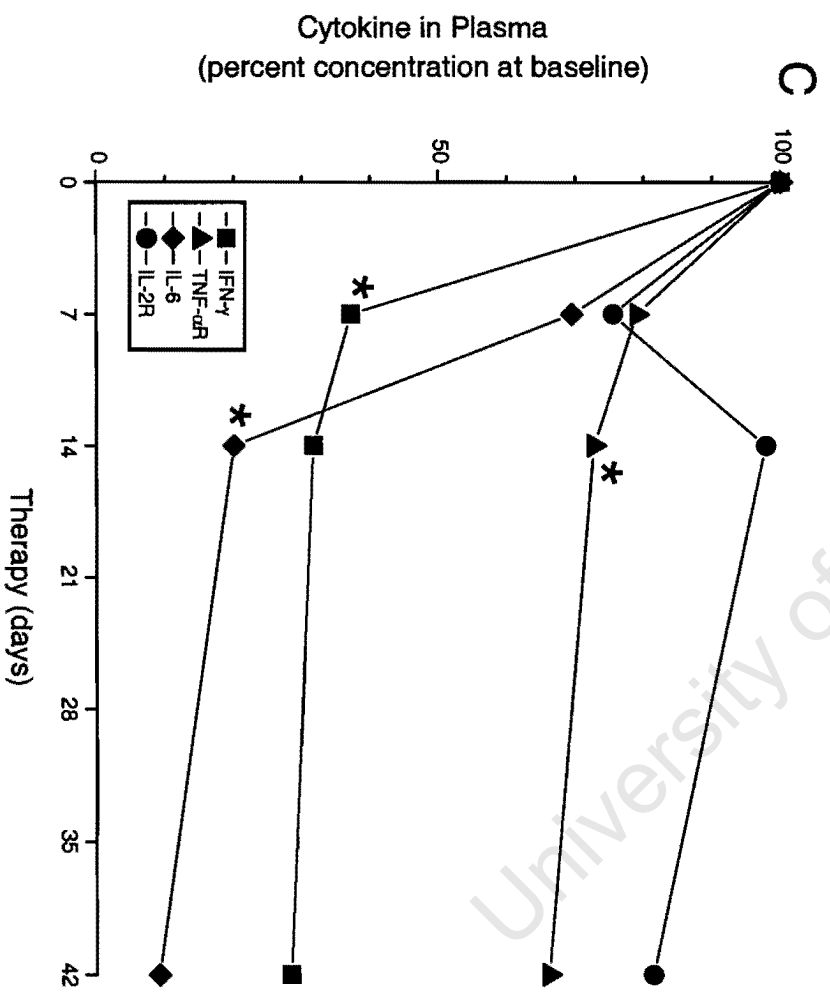
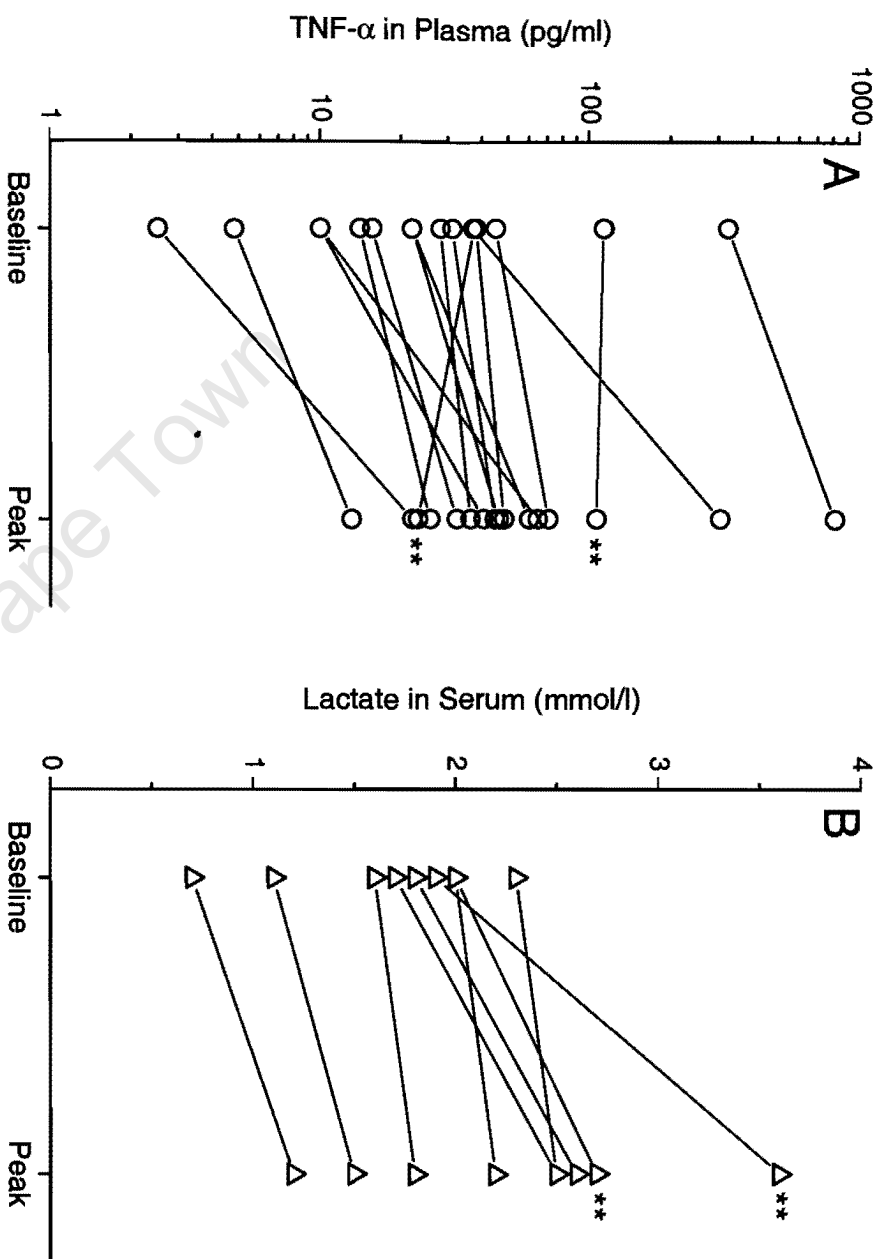


Figure 3-2

Mean lactate, cytokine and cytokine receptor levels in plasma for all patients tested during the study period. **A.** TNF- α levels in plasma at baseline and at peak for each patient. **B.** Lactate levels at baseline and at peak for each patient. **C.** Cytokine levels expressed as percent concentration at baseline. SEMs for the results shown are: IFN- γ 8-11%; TNF- α R 11-14%; IL-6 4-24%; sIL-2R 11-13%. * $p < 0.05$. **Denotes the 2 patients that died.



At baseline there was no correlation between TNF- α levels and radiographic extent of disease, presence of cavitation, or severity of illness as assessed by Karnofsky score or serum lactate concentration. However, day 3 absolute TNF- α levels correlated significantly with the reduction in the Karnofsky score relative to baseline ($r=0.61$; $p=0.02$).

IFN- γ and IL-6 were detected in the plasma of all patients at baseline. Mean IFN- γ concentration at baseline was 1.7 ± 1.1 IU/ml (mean normal 0.2 IU/ml). The mean IL-6 concentration was 230 ± 115 pg/ml (mean normal 8.5 pg/ml). Thereafter, plasma levels of these cytokines decreased (Figure 3-2C). In all plasma specimens assayed, neither IL-10 nor IL-4 were detected. TNF- α R was detected in all plasma samples at baseline (mean 6.5 ± 2.9 ng/ml; mean normal 1.2 ng/ml). Thereafter TNF- α R levels did not follow the TNF- α concentrations but were reduced over the study period (Figure 3-2C). sIL-2R levels at baseline were high (mean 4750 ± 1868 pg/ml; mean normal 1250 pg/ml) and declined slowly over time (Figure 3-2C).

Discussion

We observed a selective transient increase in plasma TNF- α after the initiation of antituberculosis therapy in patients with severe tuberculosis. This was accompanied by concomitant transient weight loss and clinical deterioration as assessed by a decrease in Karnofsky score and an increase in lactate levels. TNF- α has been shown to be protective during the host response to *M. tuberculosis* infection by enhancing the development of granulomas and inhibiting intracellular growth of mycobacteria [Kindler V, Sappino A-P, Grau GE, et al., 1989; Bermudez LEM and Young LS, 1988]. However, TNF- α also has immunopathologic effects, causing fever, weight loss, tissue necrosis and shock [Moreno C, Taverne J, Mehlert A, et al., 1989; Beutler B and Cerami A, 1987]. The transient increase in TNF- α levels observed in these patients following initiation of therapy may contribute to the temporary clinical deterioration as well as the weight loss seen.

The cachetic effects of TNF- α have been observed in a number of clinical situations. Patients with advanced cardiac failure lose body weight and muscle bulk and may even have low grade fevers. Mononuclear cells isolated from these patients have elevated TNF- α [Zhao SP and Xu TD, 1999]. In another example, rabbits infected with *Trypanosome brucei* demonstrate weight loss and wasting. It was this observation that led

to the discovery of the serum protein cachetin, which was later shown to be TNF- α [reviewed by Vassalli P, 1992].

The 30kD alpha antigen of *M. tuberculosis*, the mycobacterial 65kD heat shock protein, and one of the mycobacterial cell wall components including LAM, have been reported to induce an inflammatory cytokine response, characterized by production of TNF- α [Moreno C, Taverne J, Mehlert A, et al., 1989; Friedland JS, Shattock R, Remick DG, et al 1993; Aung H, Toossi Z, Wisnieski JJ, et al., 1996]. LAM is structurally analogous to lipopolysaccharide (LPS) which is released from Gram-negative bacilli after exposure to certain antibiotics. If mice infected with Gram negative bacteria are treated with antibiotics which induce release of LPS, the animals undergo physiologic deterioration, shock, and death [Bucklin SE and Morrison DC, 1995]. It is therefore possible that in our study TNF- α production was induced by LAM and/or the 30kD alpha antigen released from mycobacteria killed by antibiotics. We therefore investigated whether antibiotic therapy induces increased disease progression in mice infected with *M. tuberculosis* (see below).

While TNF- α levels in the plasma increased following initiation of therapy, other monocyte proteins including IL-6 and TNF- α R decreased. The mechanism underlying this dissociation between production of TNF- α , and IL-6 and TNF- α R in tuberculosis patients is unknown. It is of interest that the levels of plasma IFN- γ , the central protective TH1 type cytokine, were also reduced after the initiation of antituberculosis therapy. This observation confirms our previous report in which levels of IFN- γ in plasma and levels of IFN- γ mRNA in peripheral blood mononuclear leukocytes were highest in tuberculosis patients with the most acute symptoms, and decreased during antituberculosis therapy combined with adjunctive recombinant IL-2 treatment [Johnson BJ, Ress SR, Willcox P, et al., 1995]. sIL-2R is a marker of immune activation and has been shown previously to correlate with severity of clinical manifestations in other chronic inflammatory diseases [Mangge H, Kenzian H, Gallistl S, et al., 1995]. In the present study sIL-2R levels did not increase over baseline and were therefore not predictive of the clinical deterioration.

It is notable that increased TNF- α levels were observed in all patients except the two patients (#10 and #11) who died during the study. This may be similar to the absence of TNF- α production in terminal AIDS patients compared to HIV⁺ asymptomatic patients who have high TNF- α levels [Thea DM, Porat R, Nagimbi K, et al., 1996]. Alternatively, an

increase in TNF- α may have been missed in the two patients because of the timing of blood collection. Although the patients in this study had severe disease, their baseline plasma TNF- α levels (mean 47pg/ml; median 28pg/ml) were not very striking. However, following the initiation of antibiotic therapy, the mean maximal plasma TNF- α level was 110pg/ml (median 46pg/ml) which was accompanied by a significant clinical deterioration.

The causative relationship between initiation of anti-tuberculosis therapy and transient clinical deterioration cannot be fully established in humans since it is ethically impossible to compare the responses of treated to those of untreated patients. We have therefore initiated experiments in mice infected by aerosol with virulent *M. tuberculosis* to directly examine the host response to initiation of antibiotic therapy (see below).

Chapter 4. THE EFFECT OF MYCOBACTERIA KILLING ON THE HOST RESPONSE TO INFECTION.

Introduction

In the studies described above we observed a transient clinical deterioration in patients after the initiation of antituberculous therapy. However, the causative relationship between initiation of therapy and the clinical deterioration could not be established in humans because it is ethically impossible to compare the responses of treated and untreated patients. We therefore carried out experiments in mice infected with virulent *M. tuberculosis* to directly examine the host response to initiation of antibiotic therapy. In order to investigate the hypothesis that mycobacterial cell wall components may be responsible for the cytokine induction, mice were also infected with viable *M. tuberculosis* mixed with killed *M. tuberculosis*.

Results

The early effects of antibiotic treatment on *M. tuberculosis* induced cytokine production in the infected murine lung were examined. BALB/c mice were infected by aerosol with *M. tuberculosis* Erdman strain as described in methods. After 21 days of infection mice were treated with antibiotics (rifampicin and INH) for 6 days. Infected lungs from groups of mice were evaluated at different time points after initiation of treatment for bacillary load and TNF- α mRNA levels. As shown in Figure 4-1, antibiotic treatment reduced the numbers of viable bacilli (CFU) by about 100 fold. Concurrently, a transient small increase in TNF- α mRNA levels in the infected lungs was noted (Figure 4-1). TNF- α was not detected in the plasma at any time.

To test whether dead and fragmented mycobacteria could cause the increase in cytokine mRNA levels in the lungs, mice were infected with either a standard dose of viable organisms or a similar dose of viable organisms mixed with a dose of heat killed organisms (1:1). While the presence of heat killed organisms had no effect on the bacillary load (CFU) in the tissues of infected mice (Figure 4-2), the presence of dead organisms did induce a small increase in the levels of cytokine mRNA in the lungs of the mice (Figure 4-3). TNF- α and IL-6 mRNA levels were transiently increased on day 21

Figure 4-1

Effect of anti-tuberculous therapy on murine mycobacterial infection and cytokine response in the lungs. Bacillary load (Top) and TNF- α mRNA levels (Bottom) in the lungs of mice infected with *M. tuberculosis* and treated with placebo (open triangle) or antibiotics (closed triangles). Antibiotic treatment: INH given intraperitoneally and rifampicin given by gavage both administered daily. Results are from 4 mice per group per time point. TNF- α mRNA levels at the start of antibiotic treatment were designated as 100% and results for the other time points were normalized accordingly. Results are expressed as means \pm one SEM.

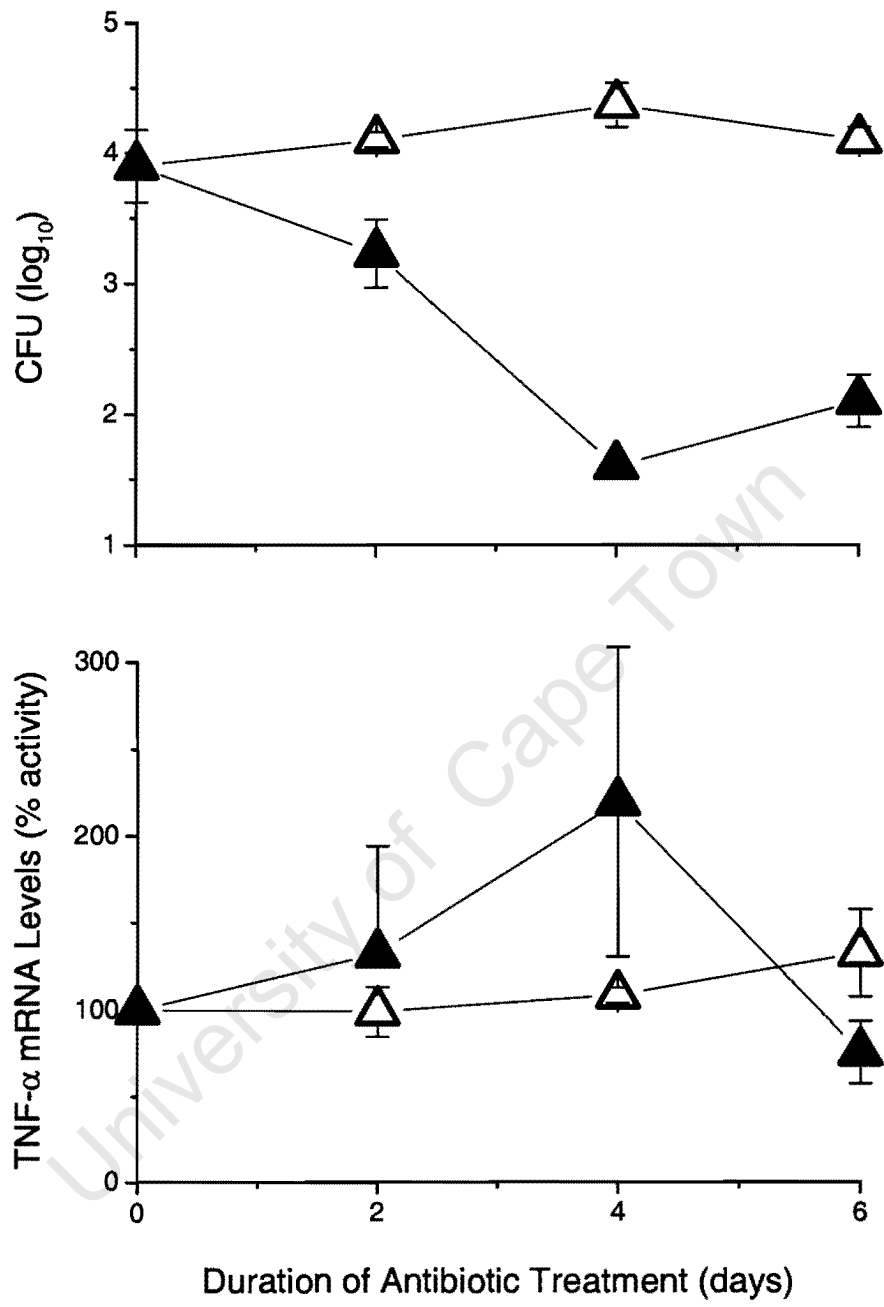
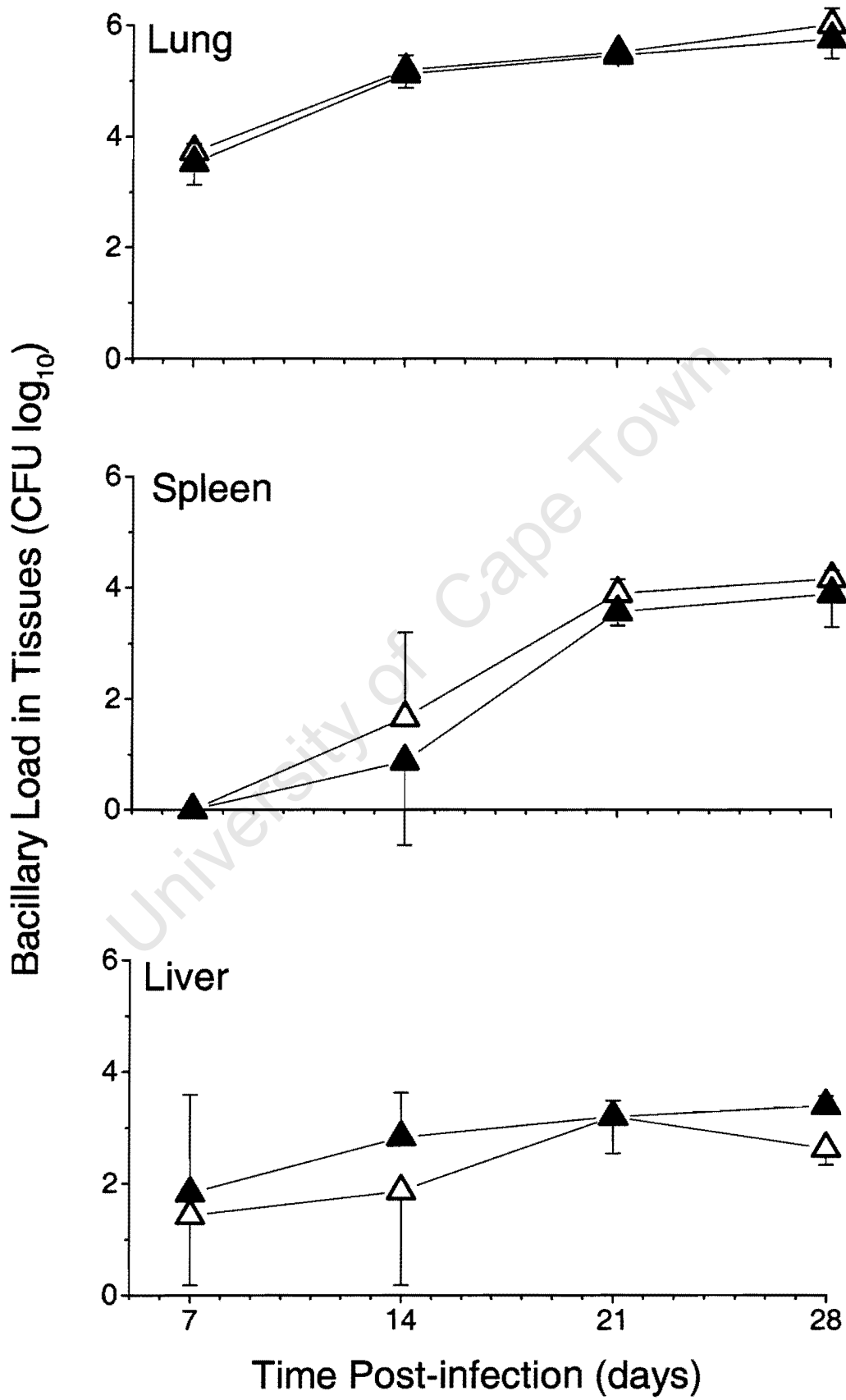


Figure 4-2

Effect of heat killed *M. tuberculosis* on the bacillary load in the tissues of infected mice. Mice were infected by aerosol with a dose of viable bacilli (open triangles) or a similar dose of viable bacilli plus heat killed bacilli (1:1) (closed triangles). Results are from 4 mice per group per time point expressed as mean CFU +/- one SEM.



post infection (Figure 4-3). By day 28 post infection cytokine mRNA levels returned to levels noted in mice infected with viable organisms only. This increase in mRNA levels was associated with larger lung granulomas (Figure 4-4). The mean granuloma size at 21 days post infection was 40% larger in the mice infected with viable plus dead organisms compared to those infected with viable organisms only (Table 4-1 and Figure 4-4).

Table 4-1 Granulomatous response to infection in the lung

Type of infection	Number of animals	Area of granulomas* mm ² (SEM)
Viable bacilli	20	438 (64)
Viable + dead bacilli (1:1)	20	617 (130)

*Evaluated on H&E stained lung sections by morphometry.

Discussion

The results obtained using *M. tuberculosis* infected mice suggested that the TNF- α associated clinical worsening observed in patients upon initiation of antibiotic therapy could be caused by mycobacterial components generated by killing and fragmentation of the organisms. The mycobacterial components may have activated macrophages to produce and release elevated levels of TNF- α . In association with the increased TNF- α levels, enhanced leukocyte activation and increases in symptoms may have occurred.

A wide variety of structurally diverse components derived from the mycobacterial cell wall stimulate TNF- α production in macrophages. The cellular recognition system for the stimulatory mycobacteria cell wall component, LAM has not been defined fully. However, it may be similar to the recognition system for LPS, a stimulatory component of gram negative bacteria. Macrophage responses to LPS and LAM are enhanced by LPS-binding protein (LBP), a molecule that binds LPS and transfers it to CD14. The macrophage CD14 is an extracellular protein that may interact with toll-like receptors to transmit a signal for TNF- α production [Chow JC, Young DW, Golenbock DT, et al., 1999].

Figure 4-3

Effect of heat killed *M. tuberculosis* on cytokine mRNA levels in the lungs of infected mice. Mice were infected by aerosol with viable bacilli (open triangles) or viable plus heat killed bacilli (closed triangles). Results are from 4 mice per group per time point expressed as means +/- one SEM.

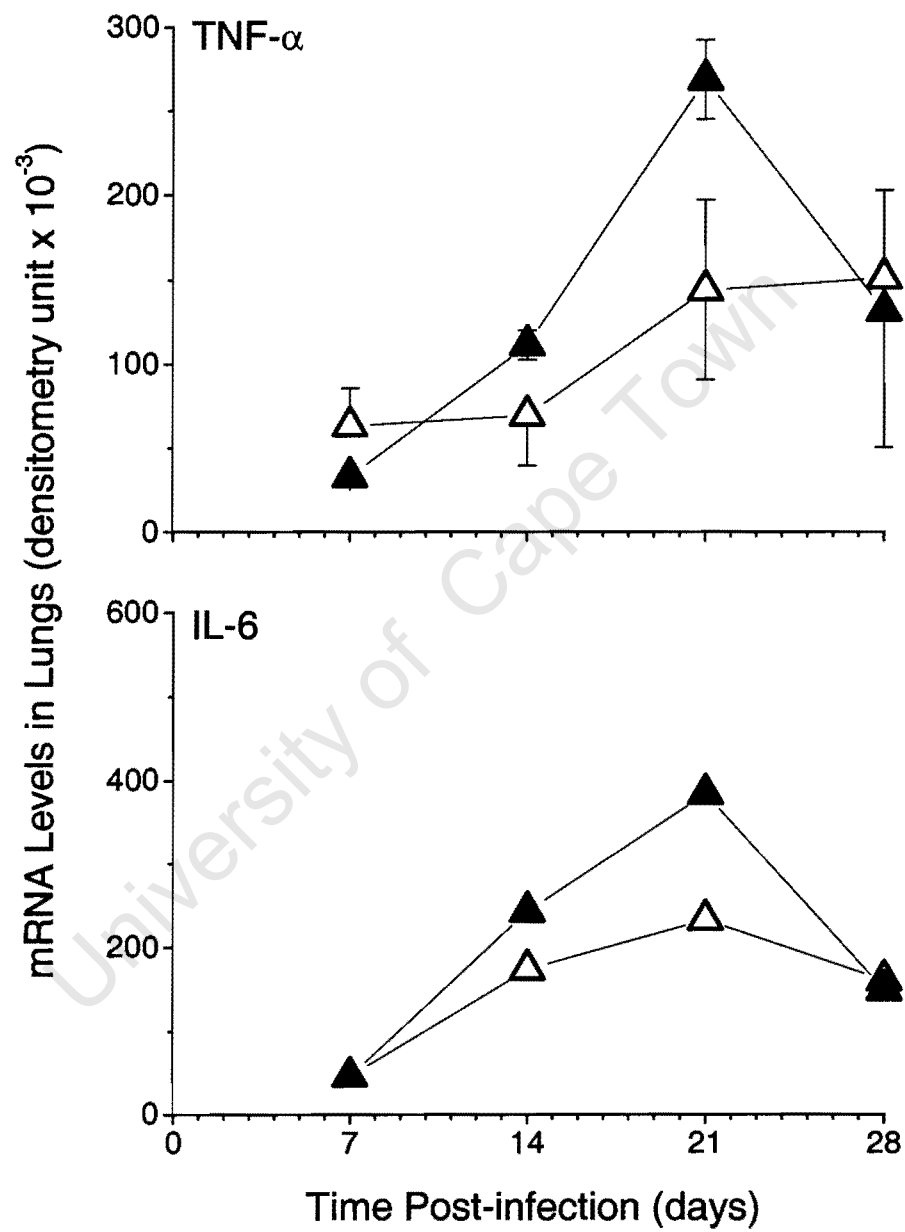
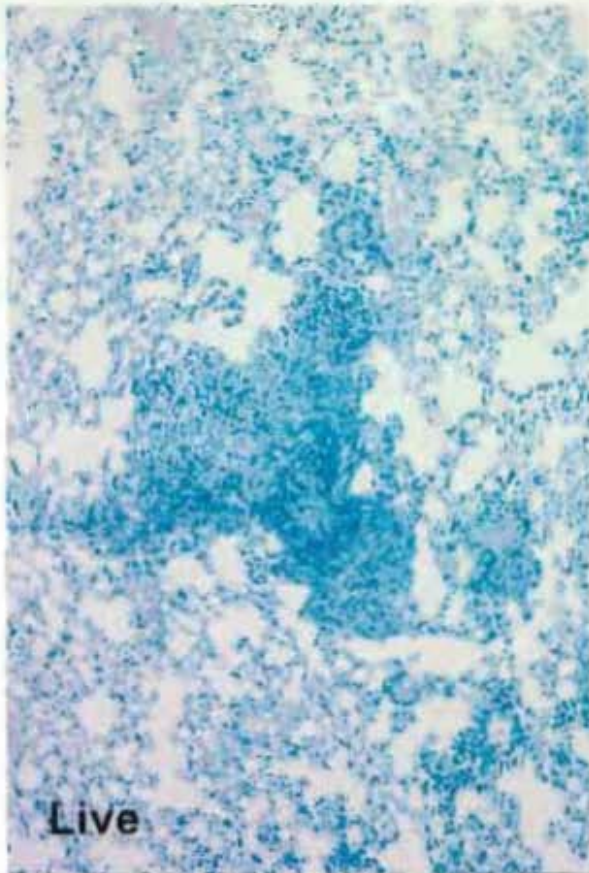
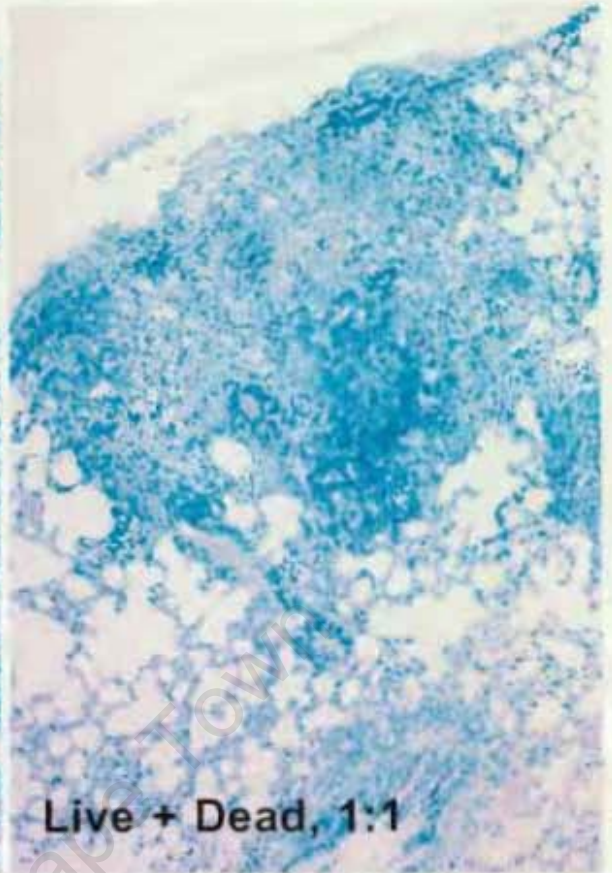


Figure 4-4

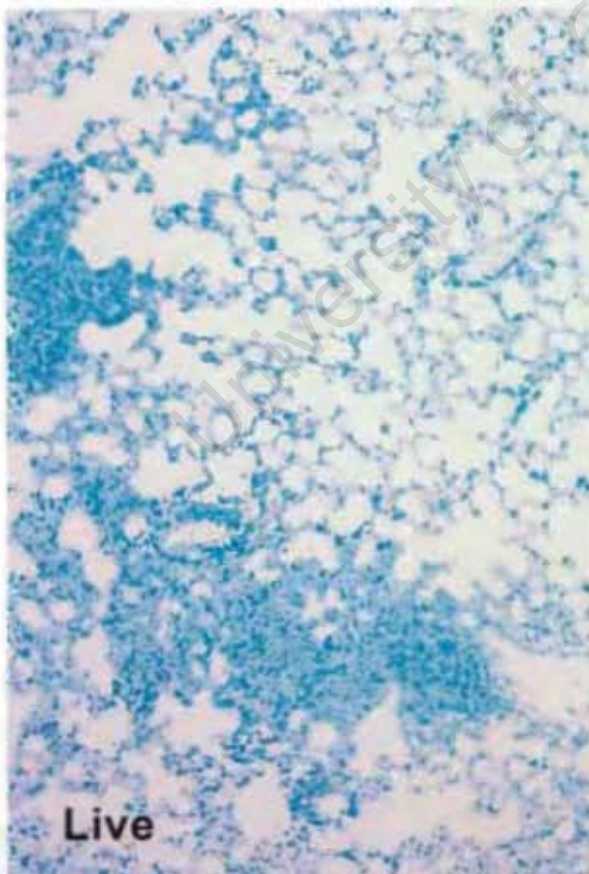
Effect of heat killed *M. tuberculosis* on the granulomatous response in the lungs of infected mice. Mice were infected by aerosol with viable bacilli (Live) or viable plus heat killed bacilli (Live + Dead, 1:1). At 21 days post infection mice were sacrificed and lungs were prepared for histologic evaluation. Ziehl-Neelsen stain, magnification x10.



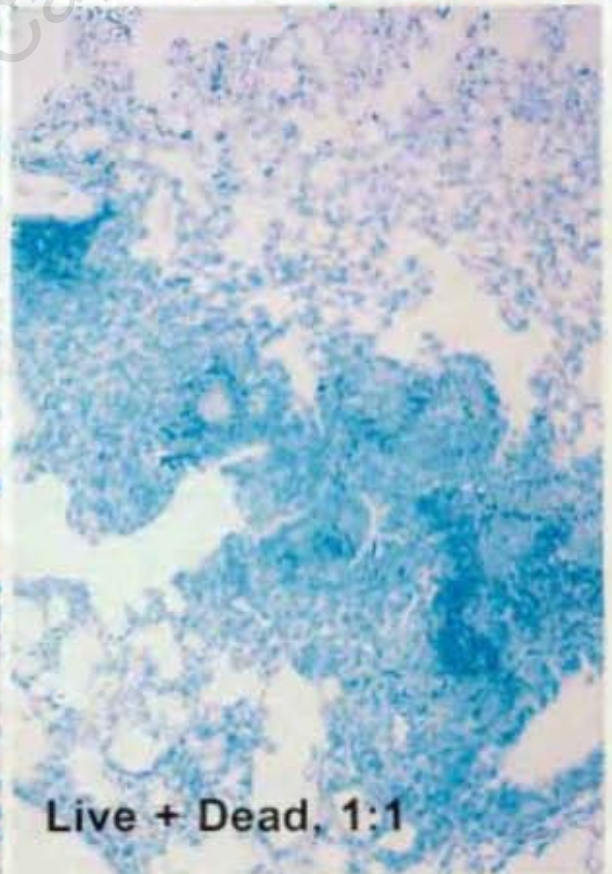
Live



Live + Dead, 1:1



Live



Live + Dead, 1:1

Toll-like receptors comprise a family of innate immune signaling receptors that are related to the *Drosophila* Toll protein, a molecule that is implicated in defense against fungal infection in the fly. The roles of the equivalent 6 mammalian homologues are just beginning to be elucidated. In a recent report it has been shown that upon exposure to *M. tuberculosis*, murine macrophages are stimulated to produce the pro-inflammatory cytokine TNF- α in a toll-like receptor (TLR)-dependent manner [Underhill DM, Ozinsky A, Smith KD, et al., 1999]. Furthermore, TLR2 was identified as the principle mediator of the proinflammatory signal induced by whole *M. tuberculosis* infection *in vitro*. Three structurally diverse fractions of the mycobacterial cell wall, namely LAM, mycolylarabinogalactan-peptidoglycan complex and *M. tuberculosis* total lipids were shown to induce TNF- α production via TLR2. This pathway is distinct from the LPS-signaling that occurs through TLR4. Thus, in the present studies mycobacterial products generated during antibiotic killing of the bacilli probably induce production of TNF- α via the TLR2 signaling pathway.

It is interesting that a similar experiment to the one described in this chapter was inadvertently carried out in patients by Robert Koch in 1890 in Berlin. In his book, *The White Plague*, Dr. Rene Dubos [Dubos R and Dubos J, 1952] describes the phenomena: *“In 1890, Koch released a new bombshell by announcing before the Tenth International Congress of Medicine in Berlin that he had discovered a substance that could protect against tuberculosis and even cure the established disease. Under the pressure of public criticism he (Koch) announced that it (the substance) was merely a glycerin extract of tubercle bacilli, the product now known under the name of “Old Tuberculin”.*

Dr. Dubos further writes: *“The first English physician to arrive in Berlin after the announcement of Koch discovery was A. Conan Doyle. Doyle pointed out that treatment with tuberculin stirs into activity all those tubercle centers which have become dormant. In one case the injection given for the cure of a tubercular joint caused an ulcer of the eye, which had been healed for 20 years, to suddenly break out again, thus demonstrating that the original ulcer came from a tubercular cause and the fever after the injection is in some cases so very high (41°C) that it is hardly safe to use in the case of a debilitated patient”.* In fact, Koch himself describes an unexpected fever, shock like state and death

in some of the patients given “Tuberculin Lymph” as therapy and the resulting condition became known as “Tuberculin Shock”.

In our experiments in the mouse model the upregulation of TNF- α was not very striking and the increase in granuloma size in the lungs was only moderate. In addition these changes were not accompanied by significant measurable clinical toxicity. This is probably because mice are relatively resistant to mycobacterial infection and do not normally manifest the symptoms of tuberculosis observed in man. We therefore proceeded to establish an experimental infection model which enabled us to control the amount of TNF- α produced in the tissues of infected mice (see below).

University of Cape Town

Chapter 5. THE IMMUNOPATHOLOGIC EFFECTS OF TNF- α IN MURINE MYCOBACTERIAL INFECTION IS DOSE DEPENDENT.

Introduction

The importance of TNF- α in the mouse defense against mycobacterial infection has been appreciated for some time. In experimental murine models TNF- α production has been shown to be necessary for the formation and maintenance of the granulomas which seal off foci of infection and thus limit dissemination of the bacteria. When mice infected with *M. tuberculosis* received daily injections of recombinant murine TNF- α , a significant reduction in the number of viable bacteria in the lungs and spleens was observed [Denis M, 1991]. Similarly, treatment of *M. bovis* BCG -infected mice with TNF and TNF-mimetic peptide (consisting of amino acids 70-80 of TNF), resulted in changes in the granulomas and a decrease in the bacterial load [Roach DR, Briscoe H, Baumgart K, et al., 1999]. Conversely, when TNF- α was neutralized by treatment with anti-TNF- α monoclonal antibody, granuloma formation in BCG infected mice was abrogated and the bacilli multiplied in an uncontrolled manner, leading to decreased survival of the animals [Kindler V, Sappino A-P, Grau GE, et al., 1989]. The protective role of TNF- α was further demonstrated in studies using mice in which the genes encoding TNF- α or the receptor for TNF- α TNF-R1 were disrupted. In TNF-R gene disrupted mice, infection with *M. tuberculosis* was not contained and the animals died soon after infection [Flynn JL, Goldstein MM, Chan J, et al., 1995]. In TNF- α gene disrupted mice, *M. tuberculosis* infection led to dysregulated granuloma formation, resulting in large accumulations of cells and mycobacteria in the lungs, as well as extensive necrosis and neutrophil infiltration [Bean AG, Roach DR, Briscoe, H, et al., 1999].

In addition to its protective effects in the generation of immunity against pathogens, TNF- α has been shown in many systems to induce immunopathology *in vivo*. Tissue necrosis and cachexia or wasting have been associated with elevated TNF- α levels [Beutler B and Cerami A, 1988; Tracey KJ and Cerami A, 1992]. In patients with tuberculosis, increases in this cytokine have been implicated in clinical worsening (see Chapter 3). In a study in mice, we showed that a reduction in granuloma size and necrosis were associated with a decrease in TNF- α levels in the infected lung [Moreira AL,

Tsenova L, Wang J, et al., 1997]. Although these results are suggestive of a pathogenic role for this cytokine it has been difficult to directly demonstrate severe deleterious effects of TNF- α in tuberculosis.

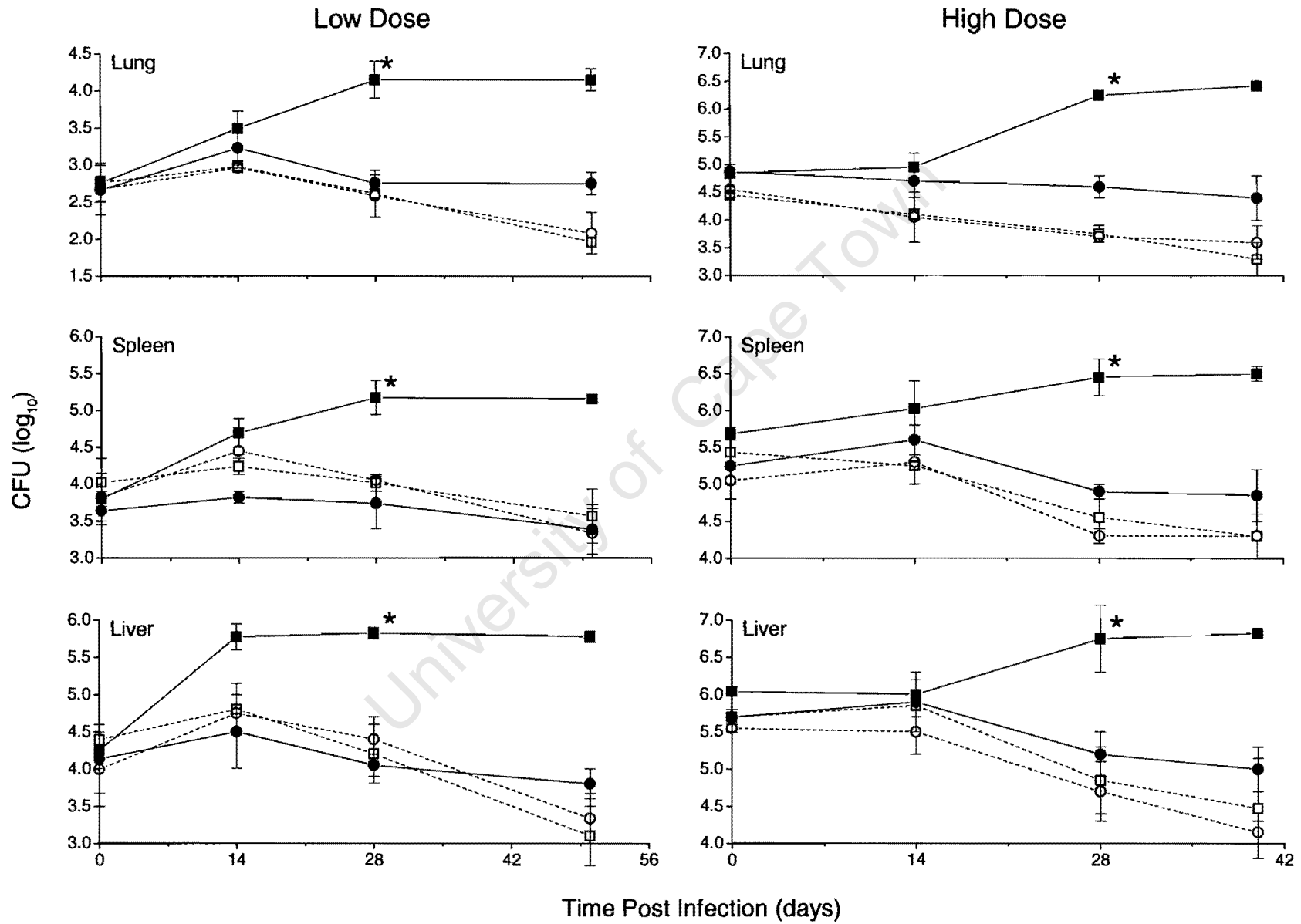
To directly demonstrate the detrimental effects of excess TNF- α in the host murine response to mycobacterial infection, we used a strain of recombinant BCG that secretes murine TNF- α (BCG-TNF) to infect TNF- α gene disrupted (TNF-KO) C57BL/6x129 mice and their equivalent wild type controls. Some mice were given an unusually high inoculum of bacilli to increase the amount of TNF- α at the site of infection. Following intravenous infection, we evaluated the growth of the recombinant BCG in the lungs, livers and spleens, the granulomatous response in the lungs and liver, cytokine mRNA production in the lungs, and survival of the infected animals.

Results

Effect of TNF- α on growth of recombinant BCG in TNF-KO mice: The effect of local production of TNF- α on mycobacterial growth in the organs of mice infected by the intravenous route was evaluated. We compared the bacillary load in the lungs, spleen, and liver of mice following infection with 2×10^5 (low dose) and with 1×10^8 (high dose) BCG-TNF to the bacillary load in mice infected with the control BCG-vector. When the TNF-KO mice were infected with BCG-vector the infection was not controlled and the bacillary load increased in all organs at both doses of infection (Figure 5-1). The increase in CFU count from baseline to the 28 day time point of the study was significant at both doses for all organs tested ($p < 0.05$). On the other hand, when TNF-KO mice were infected with 2×10^5 or 1×10^8 BCG-TNF, bacillary growth was controlled and CFU decreased slightly from baseline to the final time point, similar to the response seen in wild type mice (Figure 5-1). By 28 days post infection there was a significant difference in CFU between the BCG-TNF and BCG-vector in the TNF-KO mice ($p < 0.04$). Thus, in the TNF-KO mice, TNF- α produced at the site of infection appeared to reconstitute the host response, resulting in control of bacterial growth even when the initial infecting inoculum was very high.

Figure 5-1

Effect of TNF- α on the bacillary load in the tissues of infected mice. TNF-KO mice (closed symbols) or wild type mice (open symbols) were infected intravenously with a low dose (2×10^5 organisms/mouse) or a high (1×10^8 organisms/mouse) dose of BCG-vector (squares) or BCG-TNF (circles). * denotes statistically significant ($p < 0.05$) differences between the BCG-vector and the BCG-TNF infected TNF-KO mice. Results are means \pm one SD of two independent experiments for each dose with 4 mice per group per time point.



No TNF- α was detected in the plasma of any of the mice infected at the low dose. However, this cytokine was detected at day 28 and at day 40 in the plasma of TNF-KO mice infected with the high dose of BCG-TNF (10-13 pg/ml). Therefore, the presence of TNF- α at levels high enough to be detected systemically reconstituted the antibacterial response.

Effect of TNF- α on histopathology of lungs and livers following infection of TNF-KO mice with recombinant BCG: The cellular accumulation and organization (granulomatous response) in the lungs and livers of TNF-KO mice following infection with high or low doses of BCG-TNF or BCG-vector was compared. At 28 days post-infection with 2×10^5 organisms of either recombinant, the lungs appeared relatively unaffected with only small scattered cellular aggregates in the parenchyma (Figure 5-2 A and I). These aggregates consisted of lymphocytes and macrophages (Figure 5-2 B and J), some of which stained for iNOS expression (Figure 5-2 D and L). The iNOS-staining macrophages appeared more focal in the BCG-TNF infected lungs (D), compared to the BCG-vector infected lungs (L). By 45 days the two infections differed markedly. In TNF-KO mice infected with low dose BCG-vector, the granulomas had enlarged extensively and occupied most of the lung (Figure 5-2 M). These contained large undifferentiated macrophages, many lymphocytes and polymorphonuclear leukocytes (Figure 5-2 N). iNOS staining was still evident (Figure 5-2 P). In comparison, in mice infected with low dose BCG-TNF, the granulomas had enlarged somewhat by 45 days but were clearly distinct from the majority of the lung which appeared normal (Figure 5-2 E). The granulomas consisted of lymphocytes and macrophages (F), some of which were still iNOS positive (H). Only a few polymorphonuclear leukocytes were seen.

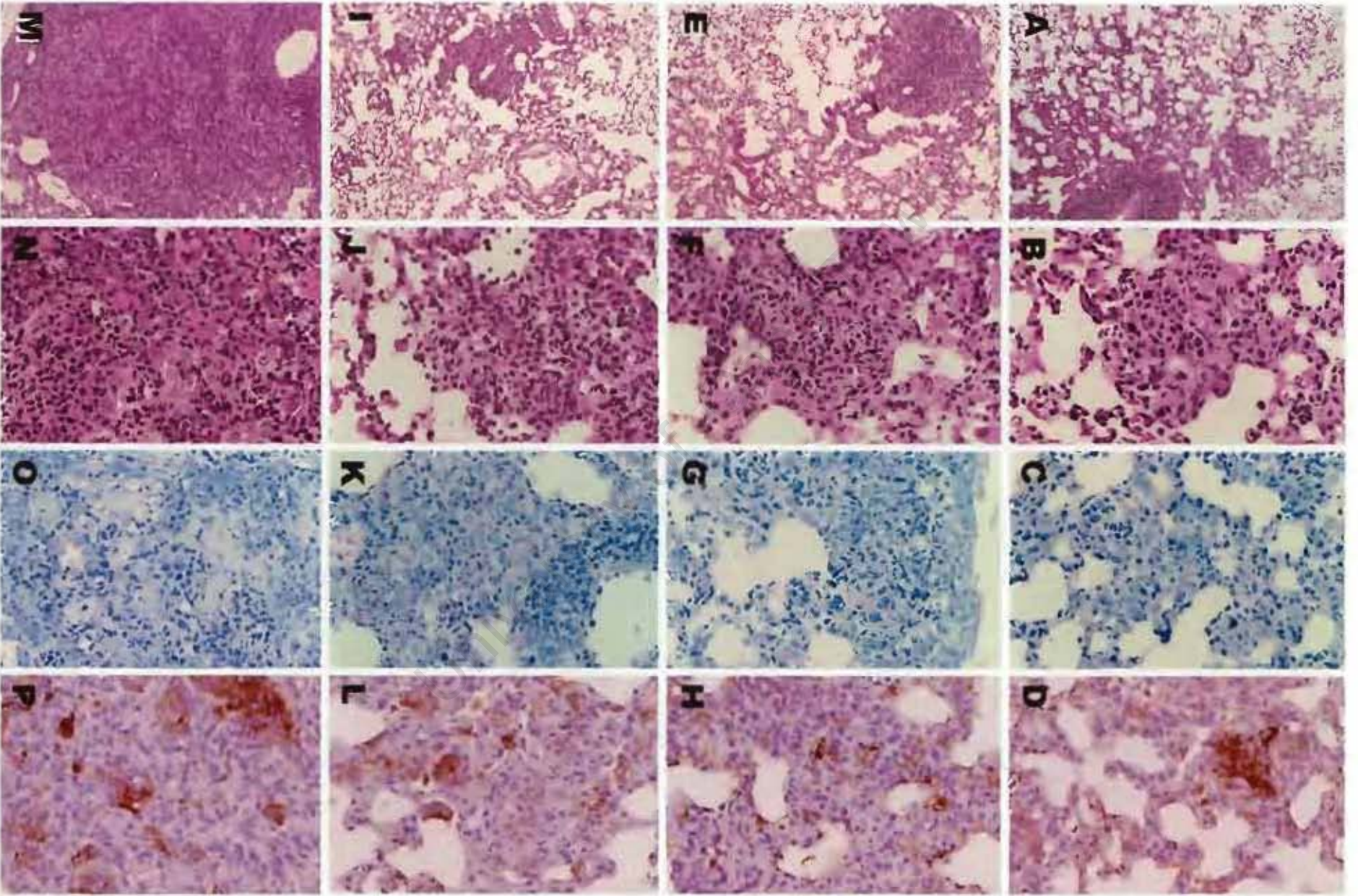
A comparison of the histology of the livers of TNF-KO mice infected with BCG-vector or BCG-TNF revealed differences in the kinetics of the granulomatous response in this organ. Infection with BCG-TNF induced granulomas already present at day 14 (not shown). By day 28 post infection multiple clearly demarcated granulomas with many lymphoid cells were observed (Figure 5-3 Top A and B). By day 45 only very few granulomas were seen suggesting that with the reduction in bacillary load (Figure 5-1) much of the granulomatous response had resolved (Figure 5-3 Top C and D). By comparison infection with BCG-vector resulted in a somewhat delayed but more

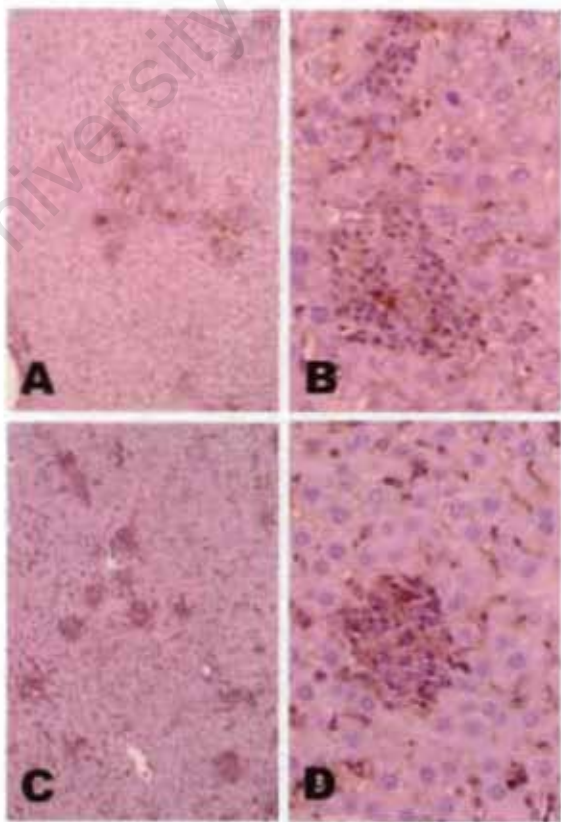
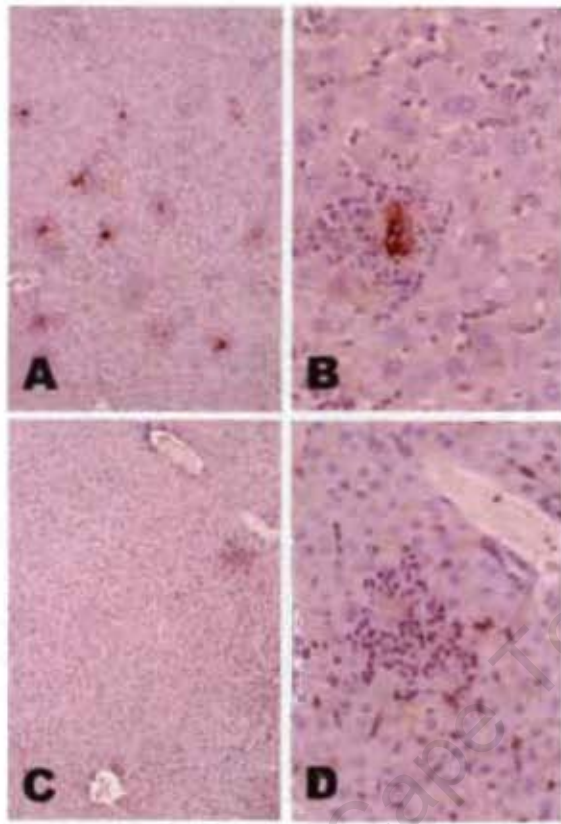
Figure 5-2

Morphology of the lungs of mice infected with low dose recombinant BCG. TNF-KO mice were infected intravenously with a low dose of BCG-TNF (A-H) or BCG-vector (I-P). The lungs were examined at 28 days (A-D and I-L) and at 45 days (E-H and M-P). Sections were stained with H & E (A,B,E,F,I,J,M and N), with Ziehl-Neelsen (C,G,K and O) or for iNOS protein expression (brown staining cells) (D,H,L and P). Magnification x 10 (A,E,I and M); x40 (B-D, F-H, J-L and N-P).

Figure 5-3

Morphology of the livers of mice infected with low dose recombinant BCG. TNF-KO mice were infected intravenously with a low dose of BCG-TNF (Top A-D) or BCG-vector (Bottom A-D). The livers were examined at 28 days (A-B) and at 45 days (C-D). Sections were stained for iNOS protein expression (brown staining cells). Magnification x 10 (A and C); x 40 (B and D).





sustained granulomatous response. At day 14 post infection no granulomas were seen in the liver (not shown). By day 28 multiple granulomas were noted (Figure 5-3 Bottom A and B). These persisted to 45 days and beyond (Figure 5-3 Bottom C and D). iNOS staining was seen in the macrophages in the granulomas in response to both BCG-vector and BCG-TNF although the reaction was more focal in response to the latter infection (Top B).

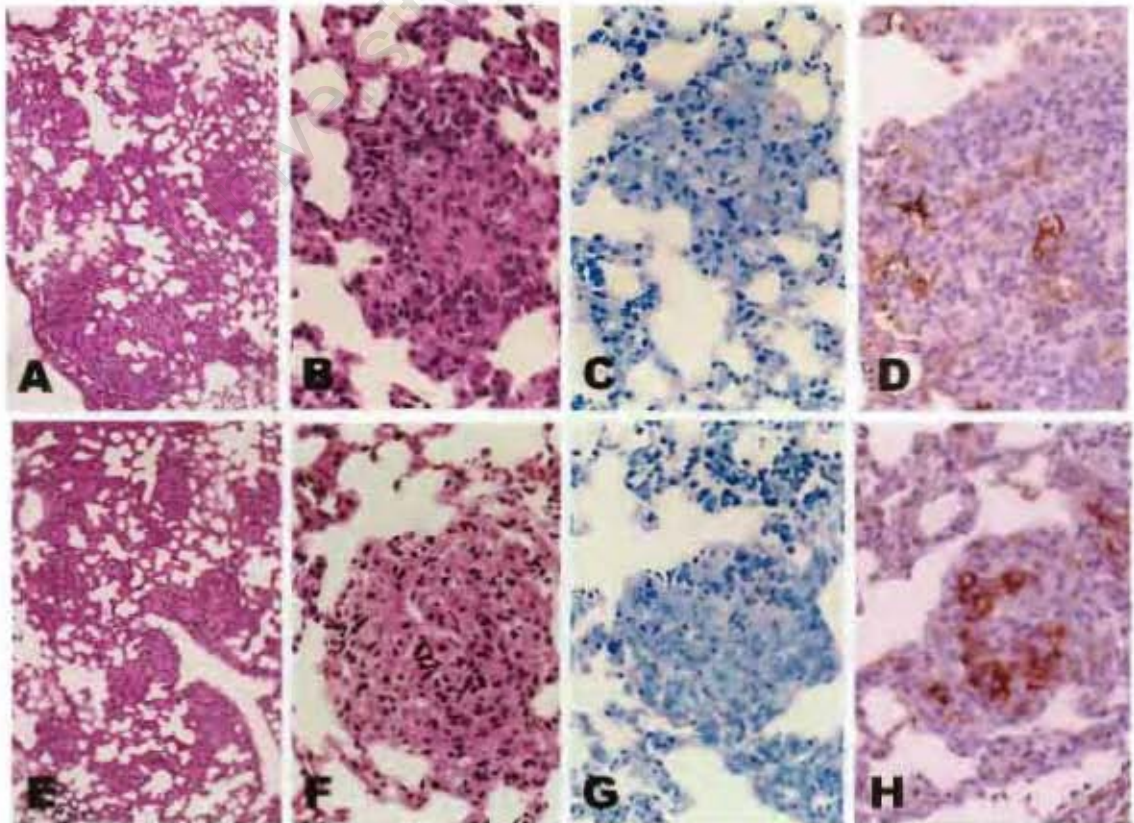
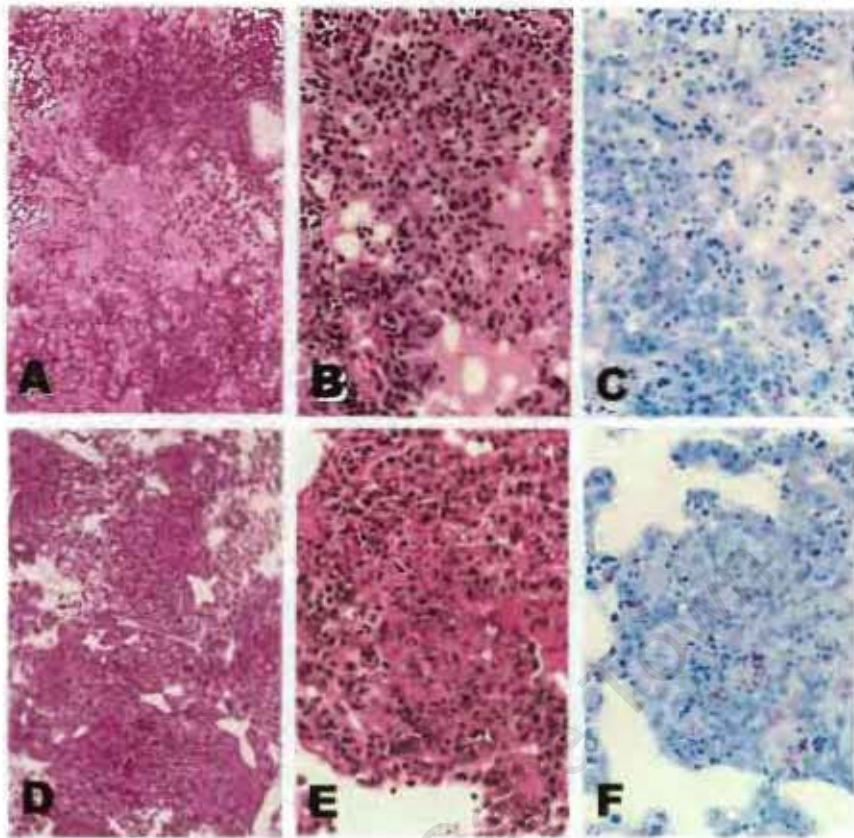
Infection of wild-type mice with this low dose of BCG-TNF or BCG-vector resulted in little or no detected granulomatous response (not shown).

To study the effect of high levels of TNF- α on the cellular inflammatory response in the lungs, TNF-KO mice were infected with high doses (1×10^8) of either BCG-TNF or BCG-vector and compared. High doses of infection induced aggressive and rapid responses. Already at 28 days post-infection, extensive cellular recruitment into the lungs was noted in response to either infection (Figure 5-4 Top A and D). In mice infected with BCG-TNF, the air-space surrounding the cellular aggregates was almost filled with fluid and infiltrated with polymorphonuclear leukocytes (Figure 5-4 Top B). In the BCG-vector infected mice, some lung tissue remained uninvolved (Figure 5-4 Top E). Staining of the lung sections for acid-fast bacilli (AFB) revealed fewer mycobacteria in mice infected with BCG-TNF compared to mice infected with BCG-vector (Figure 5-4 Top C and F respectively), confirming the CFU data (Figure 5-1). When wild-type mice were similarly infected with high doses of BCG-TNF or BCG-vector, multiple small well-organized granulomas were seen in the lungs at 28 days (Figure 5-4 Bottom A and E), with few if any AFB (Figure 5-4 Bottom C and G). iNOS staining macrophages were observed in the granulomas in response to both BCG-TNF and BCG-vector infection (Figure 5-3 Bottom D and H respectively).

Thus, the histologic evidence suggests that the absence of TNF- α in the TNF-KO mice infected with BCG-vector led to uncontrolled cellular recruitment into the infected lungs. On the other hand, the TNF- α secreted by the low dose of recombinant mycobacteria appeared to regulate the granulomatous response in the TNF-KO mice, resulting in smaller and better differentiated granulomas by 45 days of infection. However, high levels of TNF- α (high enough to be detected in the plasma) lead to an

Figure 5-4

Morphology of the lungs of mice infected with high dose recombinant BCG. **Top:** TNF-KO mice were infected with a high dose of BCG-TNF (A-C) or BCG-vector (D-F) and the lungs evaluated at 28 days. Sections were stained with H & E (A,B,D and E) or with Ziehl-Neelsen (C and F). Magnification x 10 (A and D) ; x 40 (B,C,E and F). **Bottom:** Wild type mice were infected with a high dose of BCG-TNF (A-D) or BCG-vector (E-H) and the lungs were evaluated at 28 days. Sections were stained with H & E (A,B,E and F), or with Ziehl-Neelsen (C and G) or for iNOS protein expression (brown staining cells) (D and H): Magnification x 10 (A and E); x 40 (B-D and F-H).



overwhelming inflammatory response that compromised lung function in the host, despite the successful control of the growth of the infecting mycobacteria (Figure 5-1).

The granulomatous response to high dose infection in the livers was also examined. Infection of TNF-KO mice with BCG-TNF gave rise to large very cellular irregular granulomas at 28 days (Figure 5-5 A) which contained few acid fast organisms (Figure 5-5 B). In response to infection with BCG-vector the cellular response in the liver was even more diffuse resulting in cellular aggregates which were not organized into granulomatous structures (Figure 5-5 E) in spite of the aggregates of bacilli clearly seen in the cells (Figure 5-5 F). iNOS induction in the macrophages was noted in response to both BCG-TNF and BCG-vector although again the response was more focal in the presence of TNF- α (Figure 5-5 C and D versus G and H respectively). When wild type mice were similarly infected the response to BCG-TNF (Figure 5-5 I to L) and BCG-vector (Figure 5-5 M to P) was similar. By day 28 post infection, well demarcated small granulomas with differentiated macrophages and few lymphocytes were noted (Figure 5-5 I and M). Small numbers of acid fast bacilli were seen within these granulomas (Figure 5-5 J and N). Macrophages stained for iNOS in response to infection with both BCG-TNF and BCG-vector (Figure 5-5 K and L and O and P). Thus, in the liver too the presence of TNF- α , either of host origin or generated by the recombinant bacilli, was required for the organization and differentiation of the infiltrating leukocytes into mature granulomas. In addition, TNF- α was required for the control of bacillary growth.

Effect of TNF- α on spleen weight in TNF-KO mice infected with recombinant BCG: Spleen weight, which may be used as an indicator of the systemic immune response to infection, was followed in the gene-disrupted mice infected with either recombinant. At each time point the spleens recovered from mice were weighed. When TNF-KO mice were infected with 2×10^5 BCG-vector, there was a dramatic increase in mean spleen weight from 0.065 mg to 0.395mg ($p < 0.03$) (Figure 5-6). In contrast, when TNF-KO mice were infected with 2×10^5 BCG-TNF, the increase in the mean spleen weight was much reduced, from 0.0625 mg to 0.215 mg ($p < 0.01$). The spleens from the latter mice were not much larger than those seen in wild type mice infected with BCG-vector or BCG-TNF (Figure 5-6).

Figure 5-5

Morphology of the livers of mice infected with high dose recombinant BCG. TNF-KO mice (A-H) or wild type mice (I-P) were infected with a high dose of BCG-TNF (A-D and I-L) or with BCG-vector (E-H and M-P) and the livers evaluated at 28 days. Sections were stained with H & E (A,E,I and M), with Ziehl-Neelsen (B,F,J and N) or for iNOS protein expression (brown staining cells) (C,D,G,H,K,L,O and P). Magnification x 10 (C,G,K and O) ; x40 (A,B,D,E,F,H,I,J,L,M,N and P).

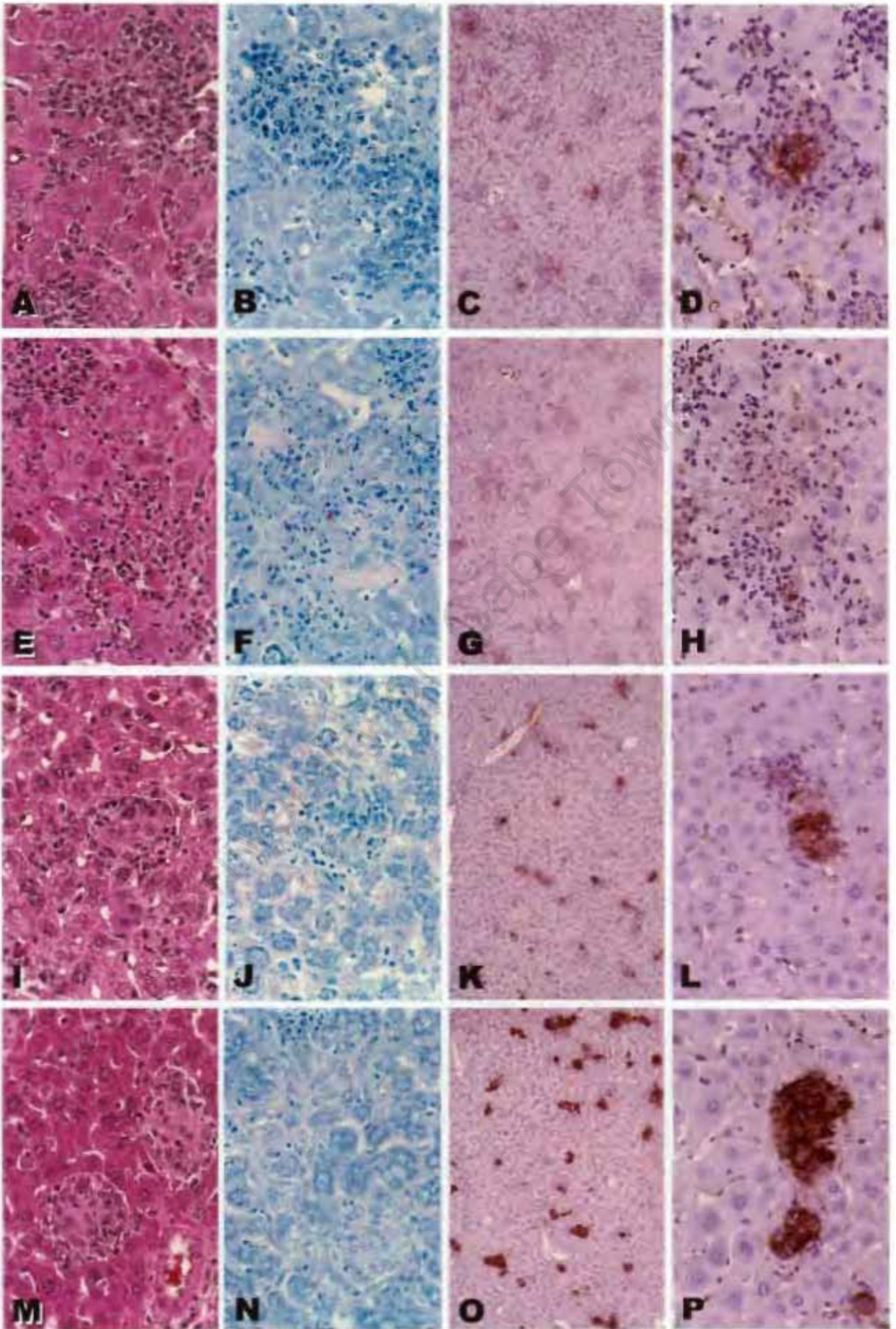
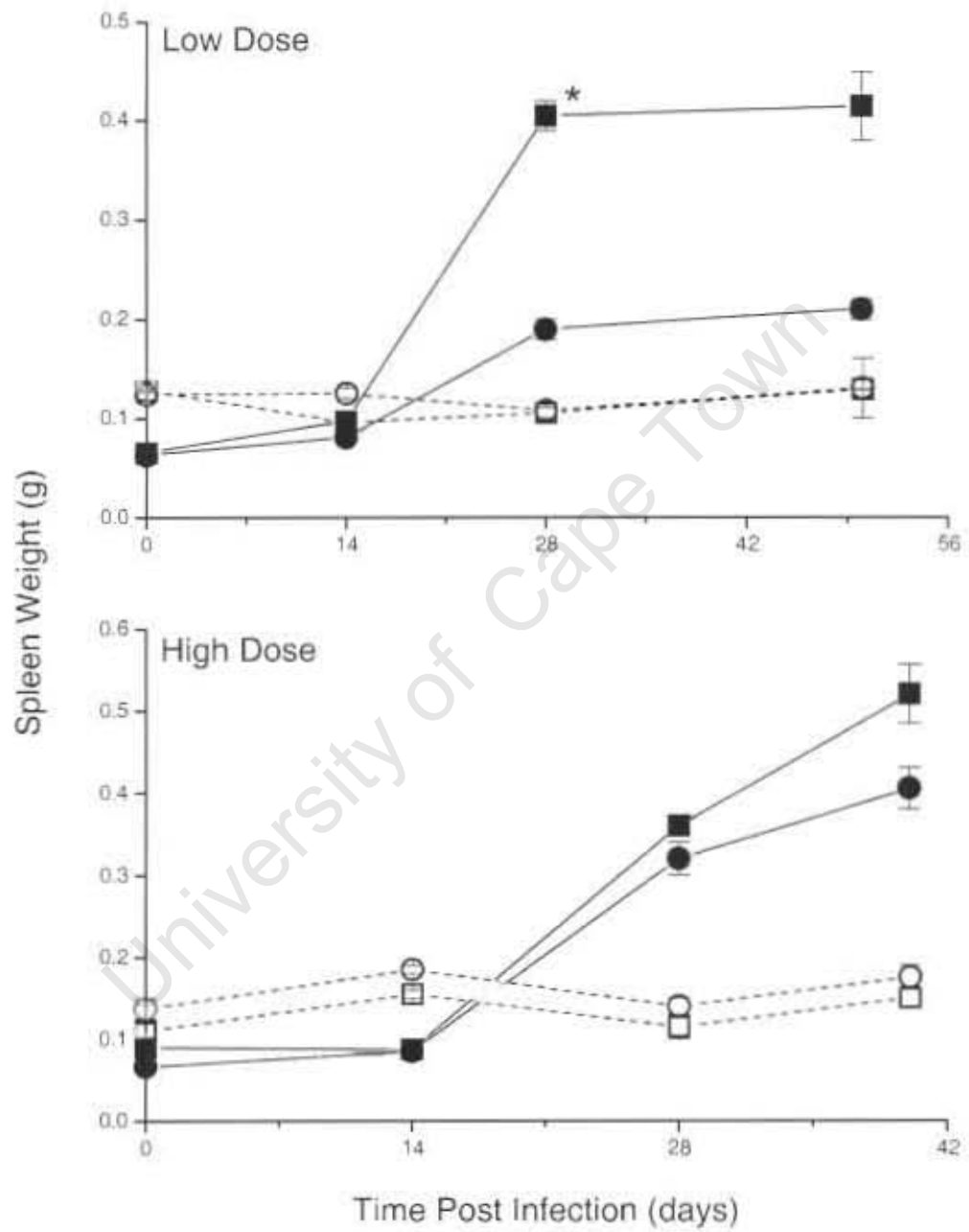


Figure 5-6

Effect of TNF- α on the weight of spleens of TNF-KO mice (closed symbols) or wild type mice (open symbols) infected with BCG-vector (squares) or BCG-TNF (circles).

* denotes a statistically significant ($p < 0.05$) difference between mice infected with BCG-vector versus BCG-TNF. Results are means \pm one SD of two independent experiments for each dose with 4 mice per group per time point.



Following infection with the high doses of BCG-vector the spleens recovered from TNF-KO mice were enlarged (from 0.09 mg to 0.565 mg; $p=0.006$) (Figure 5-6). Interestingly, infection with the high dose of BCG-TNF also resulted in enlarged spleens at day 28, although the size did not increase as much by day 40 (from 0.055 mg to 0.425 mg; $p = 0.002$). The wild type mouse spleens showed no increase in size in response to high doses of either infection (Figure 5-6). Thus, the TNF- α produced by the recombinant BCG reduced the degree of spleen enlargement seen in the absence of TNF- α in the knock-out mice. However, the presence of a large amount of TNF- α appeared to exacerbate the inflammatory process leading to spleen enlargement despite control of bacillary growth (Figure 5-1).

Effect of TNF- α on survival of mice infected with recombinant BCG: The infection of TNF-KO mice with 5×10^6 BCG-vector resulted in early death of the mice (Figure 5-7). By day 56 all animals had succumbed to the infection. Following infection with 5×10^6 BCG-TNF however, the TNF-KO mice survived ($p<0.001$ for BCG-TNF vs BCG-vector). The wild type mice infected at this dose with BCG-vector or BCG-TNF had no deaths over the period of the experiment. Following infection of TNF-KO mice with 1×10^9 BCG-vector, 100% mortality was observed by day 35 (Figure 5-7) ($p<0.001$ for TNF-KO vs wild type mice infected with BCG-vector). Infection with 1×10^9 BCG-TNF resulted in about 70% mortality of the TNF-KO mice by day 39 (Figure 5-7). When wild type mice were infected with this dose of BCG-vector, 100% survived. However, wild type mice infected with this dose of BCG-TNF showed some early mortality. By day 32, 33% of the mice had succumbed ($p=0.06$ for wild type mice infected with BCG-TNF vs BCG-vector). Therefore it appears that the TNF- α from the recombinant mycobacteria restored the ability of the TNF-KO mice to control the infection and survive. However, excess levels of TNF- α produced by unusually large numbers of recombinant BCG-TNF compromised survival of the mice, whether or not the growth of the bacilli was controlled (Figure 5-1).

Survival of TNF-R1 deficient mice infected with recombinant BCG: To determine whether the differences in survival of mice were the result of the absence of signaling by the TNF-R1, TNF-R1 knock out (TNFR-KO) mice were infected

Figure 5-7

Effect of TNF- α on survival of TNF-KO mice infected with recombinant BCG. TNF-KO mice (closed symbols) or wild type mice (open symbols) were infected with BCG-vector (squares) or BCG-TNF (circles). Results expressed as percent survival represent the means of two independent experiments for each dose with 12 mice per group. * $p < 0.001$ for BCG-TNF vs BCG-vector in TNF-KO mice. ** $p < 0.001$ for TNF-KO vs wild type mice infected with BCG-vector.

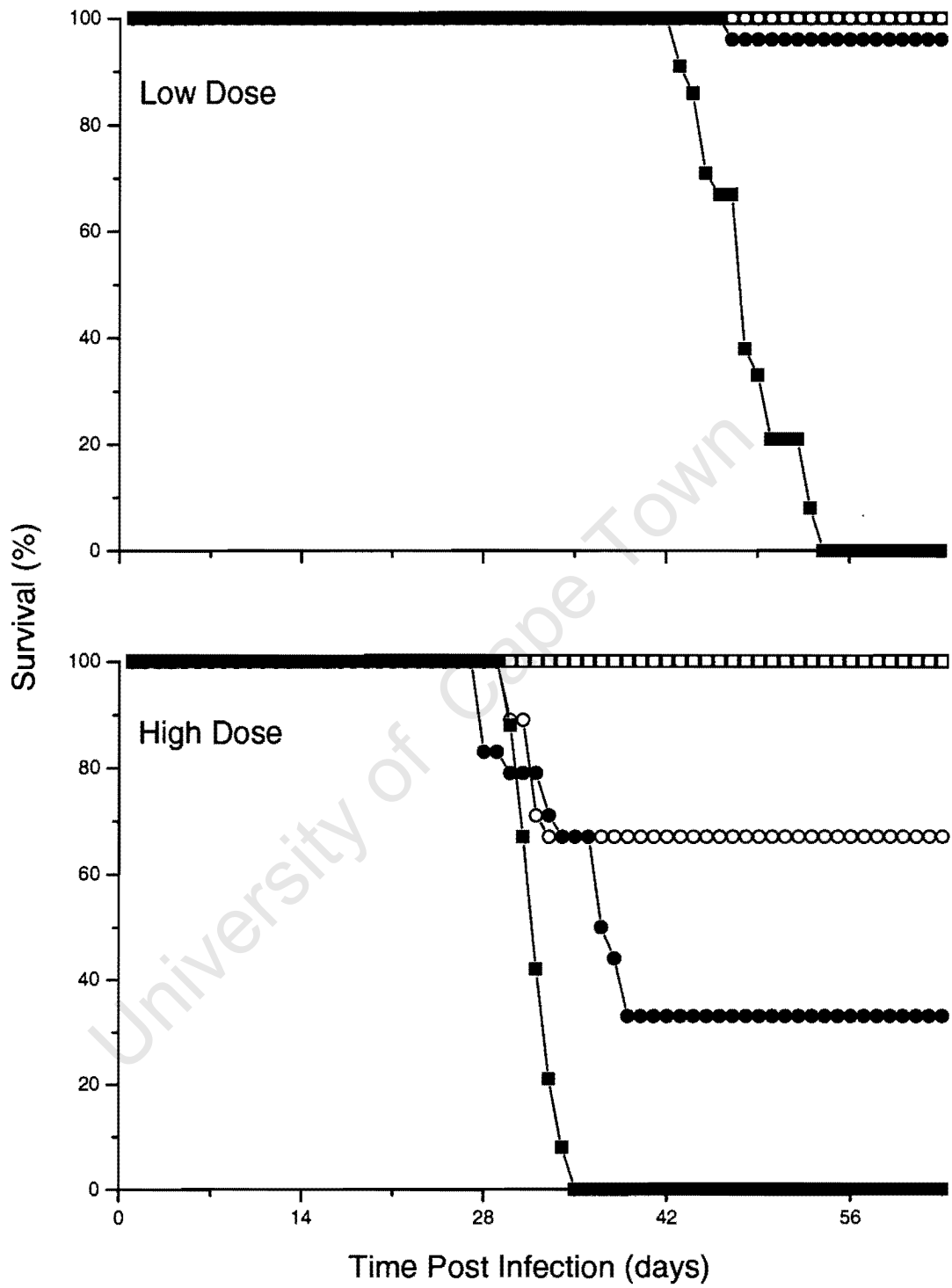
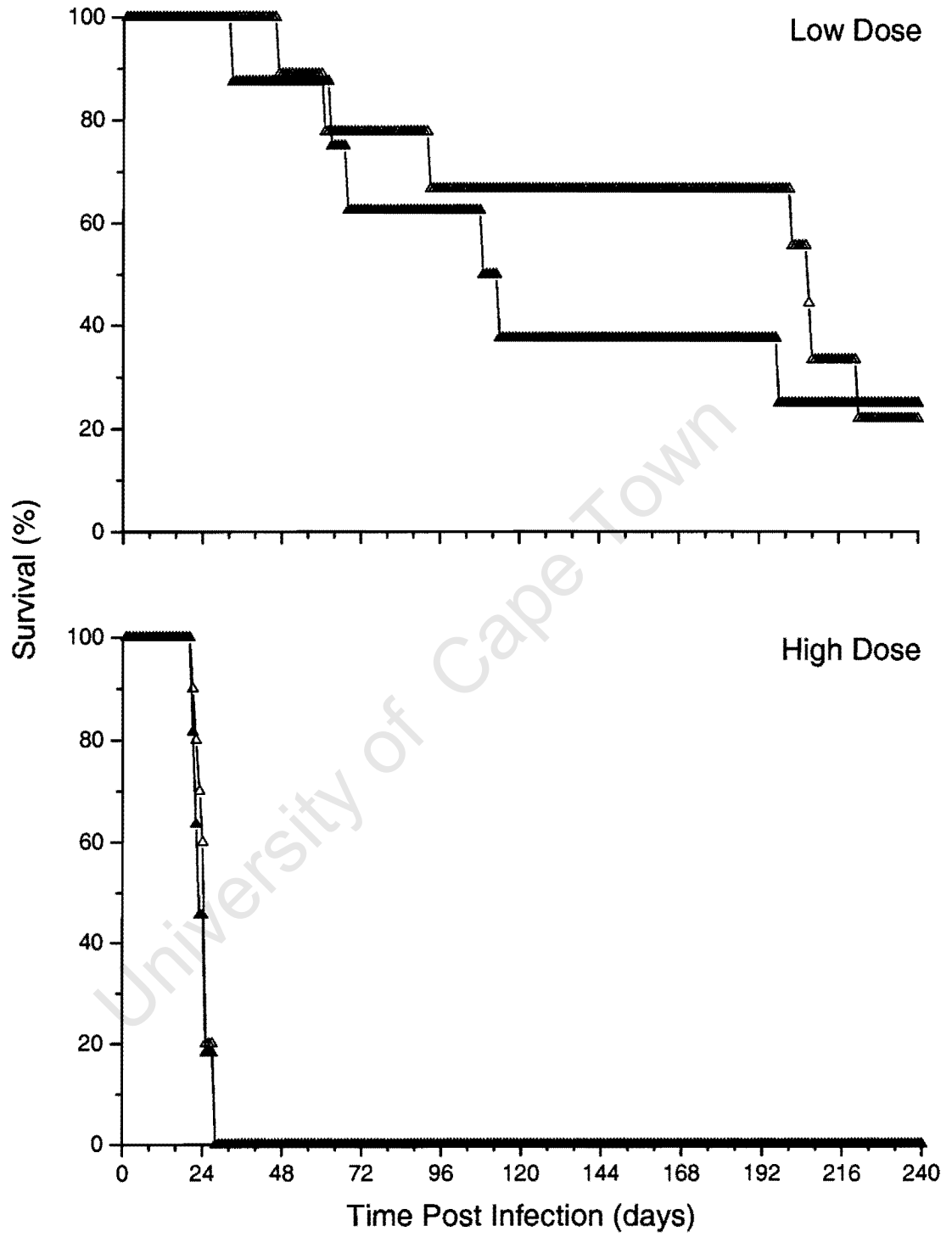


Figure 5-8

Effect of TNF- α on survival of TNFR1-KO mice infected with recombinant BCG. TNFR-KO mice were infected with BCG-vector (open triangles) or BCG-TNF (closed triangles). Results expressed as percent survival are from a single experiment for each dose with 12 mice per group.



intravenously with either low dose or high dose BCG-vector or BCG-TNF. There was no difference in survival between the TNFR-KO mice infected with BCG-vector compared to those infected with BCG-TNF at either dose (Figure 5-8) ($p>0.1$). This result indicated that the reduced survival observed in the TNF-KO mice infected with BCG-vector (Figure 5-7) was indeed due to the lack of TNF- α in the TNF- α signaling pathway.

Cytokine expression in tissues of TNF-KO mice infected with recombinant BCG: The early (14 day) expression of IFN- γ , IL-12 and IL-10 mRNA in the lungs of TNF-KO infected mice was studied. In response to infection of TNF-KO mice with BCG-vector relatively low levels of IFN- γ mRNA were induced compared to the amount of IFN- γ mRNA induced by infection with BCG-TNF (Table 5-1). The levels of IL-12 and IL-10 mRNA expressed in the lungs were also lower in response to infection with BCG-vector compared to BCG-TNF. By later timepoints, as differences in the bacillary load become manifest, cytokine mRNA levels increased in the lungs of mice infected with BCG-vector relative to the lungs of mice infected with BCG-TNF (not shown). These results suggested that TNF- α is required for the efficient generation of the cellular immune response to mycobacterial infection in mice.

Table 5-1. Cytokine mRNA levels in infected lungs (14 days).

Cytokine	Infecting strain	Activity Level *(SD)
IFN- γ	BCG-vector	2,239 (200)
	BCG-TNF	16,014 (6,000)
IL-12	BCG-vector	11,401 (3,120)
	BCG-TNF	21,045 (124)
IL-10	BCG-vector	19,937 (4,397)
	BCG-TNF	57,142 (9,250)

*Results are expressed as mean density units normalized to β -actin from 4 animals per group (standard deviation).

Discussion

The essential protective role of TNF- α in mycobacterial infections is well established in experimental models of tuberculosis [Garcia I, Miyazaki Y, Marchal G, et

al., 1997]. However, this is the first demonstration in the murine model that TNF- α can also be seriously detrimental to the infected host. In this study, we show that both protective effects and deleterious effects are due to the presence of TNF- α at the site of infection. The cytokine induces antibacterial responses or antibacterial responses and pathogenesis depending on the amount of TNF- α .

Disease outcome in the infected mice thus appears to be not merely a function of bacterial load. Outcome is also dependent on the extent of inflammation and cellular accumulation in the infected lung. While the granulomatous response is necessary to control the infection [Kindler V, Sappino A-P, Grau GE, et al., 1989], excessive inflammation including influx of cells and fluid fill the air space, resulting in compromised lung function and death of the animals. This damaging inflammation is similar to that seen in the adult respiratory distress syndrome. In experimental situations where high dose TNF- α is administered systemically to mice, the resulting inflammatory cascade causes leaky capillaries, leukocyte infiltration, neutrophil-mediated endothelial damage and inhibition of pulmonary surfactant [Vassalli P, 1992].

Our studies suggest that the extent of cellular recruitment into the lungs is determined by a number of factors. One of these is the infecting bacillary load. This is clearly demonstrated by the observation that following infection of TNF-KO or TNFR-KO mice with the high dose of BCG-vector, the mice die sooner than following infection with the lower dose (Figure 5-7). The other determinant of outcome is the level of TNF- α . When excessive TNF- α is produced at the site of infection, as in the TNF-KO and wild type mice infected with the high dose of BCG-TNF, the toxic effects of TNF- α override the protective effect of the cytokine and the animals die in spite of control of bacterial growth.

The opposing effects of TNF- α in an infection are also illustrated by a rodent model of malaria infection. In rats, *Plasmodium chaboudi* infection cures spontaneously and TNF- α is not detectable in the blood. *Plasmodium vinckei* infection on the other hand, leads to death of rats after several weeks. In these animals, there is high parasitaemia inducing high TNF- α levels which result in a shock-like condition and focal liver necrosis. Although ultimately the parasitaemia in this infection is cleared, death of the animal from shock still ensues. Similar to our study reported here, this rodent malaria

model shows clearly that TNF- α can be protective against infection, but that when released in high amounts systemically, even though the pathogen is cleared, death of the host results [Clark IA, Cowden WB, Butcher GA, et al., 1987].

TNF- α may contribute to control of mycobacterial infection in a number of ways. It could modify the endothelium, thereby facilitating extravasation of monocytes from the blood to the infected site [Ming WJ, Bersani L and Mantovani A, 1987]. Indeed, our histologic studies of granuloma formation in the infected liver clearly demonstrate the selective accumulation of monocytes and macrophages in tissues exposed to TNF- α (Figure 5-5). TNF- α could activate and foster differentiation of dendritic cells for enhanced antigen presentation [Josien R, Wong BR, Li HL, et al., 1999]. It could activate T-cells or T-cell subsets thereby facilitating the generation of cytokines and/or cytotoxic effector cells [Ueta C, Kawasumi H, Fujiwara H, et al., 1996]. This possibility is confirmed by our results demonstrating higher levels of IFN- γ and IL-12 mRNA induced in the infected lungs in the presence of TNF- α compared to the levels induced by the infection in the absence of this cytokine. Another possible mechanism is via the effects of the death domain of the TNF-R family resulting in apoptosis and elimination of macrophages infected with *M. tuberculosis* [Strasser A and Newton K, 1999]. Finally, it could directly activate macrophages to control the growth and/or kill the intracellular mycobacteria [Denis M, Gregg EO and Ghandririan E, 1990].

One possible mechanism by which activated murine macrophages could kill mycobacteria is via the generation of RNI. It has previously been shown that iNOS induction is associated with killing of mycobacteria [MacMicking JD, North RJ, LaCourse R, et al., 1997]. However, Bean, et al. [Bean AG, Roach DR, Briscoe H, et al., 1999] and our present study show that the presence of the iNOS protein in the macrophages of the infected lungs, is not sufficient to control the growth of the bacilli. Tissue sections of the TNF-KO mice infected with BCG-vector stained for iNOS (Figure 5-2), yet the infection was not controlled (Figure 5-1). Bean, et al. also showed that TNF-KO mice had serum nitrite levels similar to those observed in wild-type mice that were capable of controlling *M. tuberculosis* infection [Bean AG, Roach DR, Briscoe H, et al. 1999]. Thus, it appears that iNOS expression may be necessary but is not sufficient for

the killing of intracellular mycobacteria. These mechanisms are further studies below (see Chapter 6).

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Chapter 6. TNF- α AND MACROPHAGE CONTROL OF MYCOBACTERIAL GROWTH

Introduction.

TNF- α plays a major role in the control of mycobacterial infection both *in vivo* and *in vitro*. This cytokine has been shown to be required for the generation of the granulomatous response in the tissues of infected mice and for the control of tuberculosis infection in mice [Flynn JL, Goldstein MM, Chan J, et al., 1995; Bean AG, Roach DR, Briscoe H, et al., 1999]. Also, it has been shown that the addition of TNF- α to the culture medium of human monocytes infected *in vitro* with *M. tuberculosis* results in a reduction in the number of intracellular organisms [Denis M, Gregg EO and Ghandirian E, 1990]. When cultures of human alveolar macrophages infected with *M. tuberculosis* H37Ra are treated with polyclonal neutralizing antibody to TNF- α , intracellular mycobacterial growth in the macrophages increased [Hirsch CS, Ellner JJ, Russell DG, et al., 1994]. However, how TNF- α contributes to the control of mycobacterial growth is not fully understood.

TNF- α together with IFN- γ activate macrophages rendering them better able to control the growth of and/or kill intracellular organisms. This activation has been associated with the generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). For example, in liquid culture medium under high oxygen tension, the growth of *M. tuberculosis* is inhibited [Gottlieb SF, Rose NR, Maurizi J, et al., 1964]. Also, ROI have been shown to directly kill mycobacteria [Jackett PS, Aber VR, and Lowrie DB, 1978]. In addition, during phagocytosis of mycobacteria by either guinea pig [Jackett PS, Andrew PW, Aber VR, et al., 1981] or mouse macrophages [Gordon AH and Hart PD, 1994], release of ROI occurs, in association with killing of the mycobacteria. However, the results of Akaki, et al. [Akaki T, Sato K, Shimizu T, et al., 1997] suggested that RNI may be more important effectors than ROI in the control of intracellular mycobacteria infection in mouse macrophages. RNI have previously been shown to play a central role in the killing of mycobacteria within mouse cells *in vitro* [Chan J, Tanaka K, Carroll D, et al., 1995]. *In vivo*, when the iNOS gene is disrupted, mice are unable to generate RNI and are highly susceptible to *M. tuberculosis* infection

[MacMicking JD, North RJ, LaCourse R, et al., 1997]. Activation of the RNI pathway in murine macrophages is dependent on exposure of the cells to IFN- γ as well as to TNF- α . Pretreatment of mouse peritoneal macrophages with IFN- γ and TNF- α before infection resulted in potentiation of the antimycobacterial activity of the macrophages and was correlated with the production of nitrites [Sato K, Akaki T and Tomioka H, 1998].

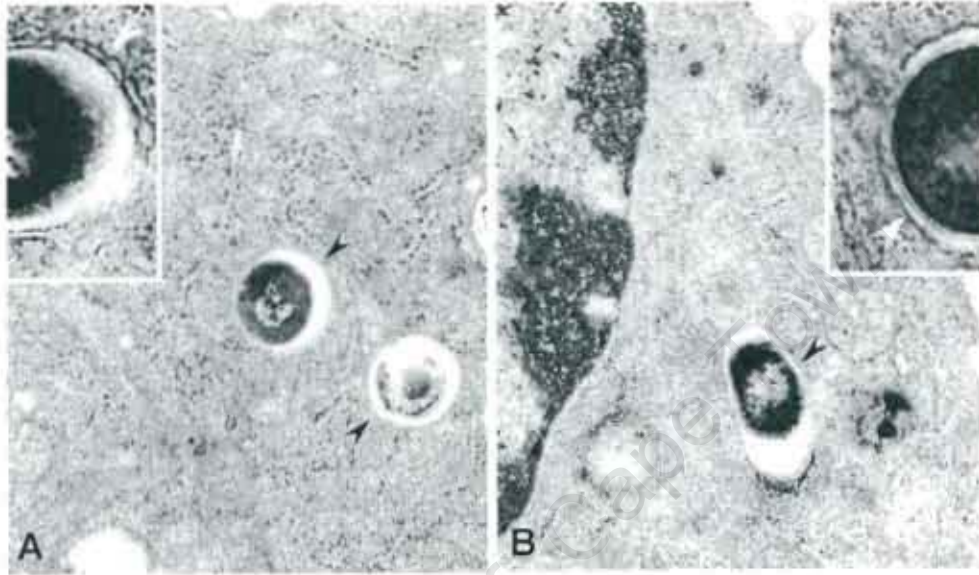
To directly examine the role of TNF- α in these pathways, we have established an *in vitro* infection model using murine macrophages and recombinant BCG secreting murine TNF- α . For our experiments, macrophages were prepared from the peritoneal cavity or from the lungs of either wild-type mice or from mice with specific gene-disruptions (TNF- α , iNOS, or IFN- γ). The macrophages were infected *in vitro* with recombinant BCG secreting murine TNF- α and bacillary growth and survival were compared. In addition, the ability of the cultured macrophages to produce TNF- α , to undergo an oxidative burst, to express iNOS protein and to produce NO were evaluated. The studies reported here suggest that RNI are important for the killing of intracellular BCG in murine macrophages, but RNI are not sufficient for maximal control of the survival and growth of the mycobacteria. The results indicate that in the absence of any iNOS, TNF- α can be shown to contribute to the control of intracellular mycobacterial growth *in vitro*.

Results.

Growth of recombinant BCG in murine macrophages in vitro: To investigate the role of TNF- α in the control of intracellular mycobacterial growth, macrophages from TNF-KO mice were infected *in vitro* with either BCG-vector or a recombinant BCG secreting murine TNF- α (BCG-TNF). Electron microscopic examination of the infected macrophages revealed that both BCG-vector and BCG-TNF were phagocytosed efficiently by the peritoneal and the alveolar macrophages. At 8 hours post infection single organisms were found within phagosomes in the cytoplasm of the cells (Figure 6-1). The double membrane of the phagocytic vacuole was tightly apposed to the mycobacterial cell wall of either recombinant strain (Figure 6-1). No morphologic differences were noted between the phagocytosed BCG-vector (Figure 6-1A) and

Figure 6-1

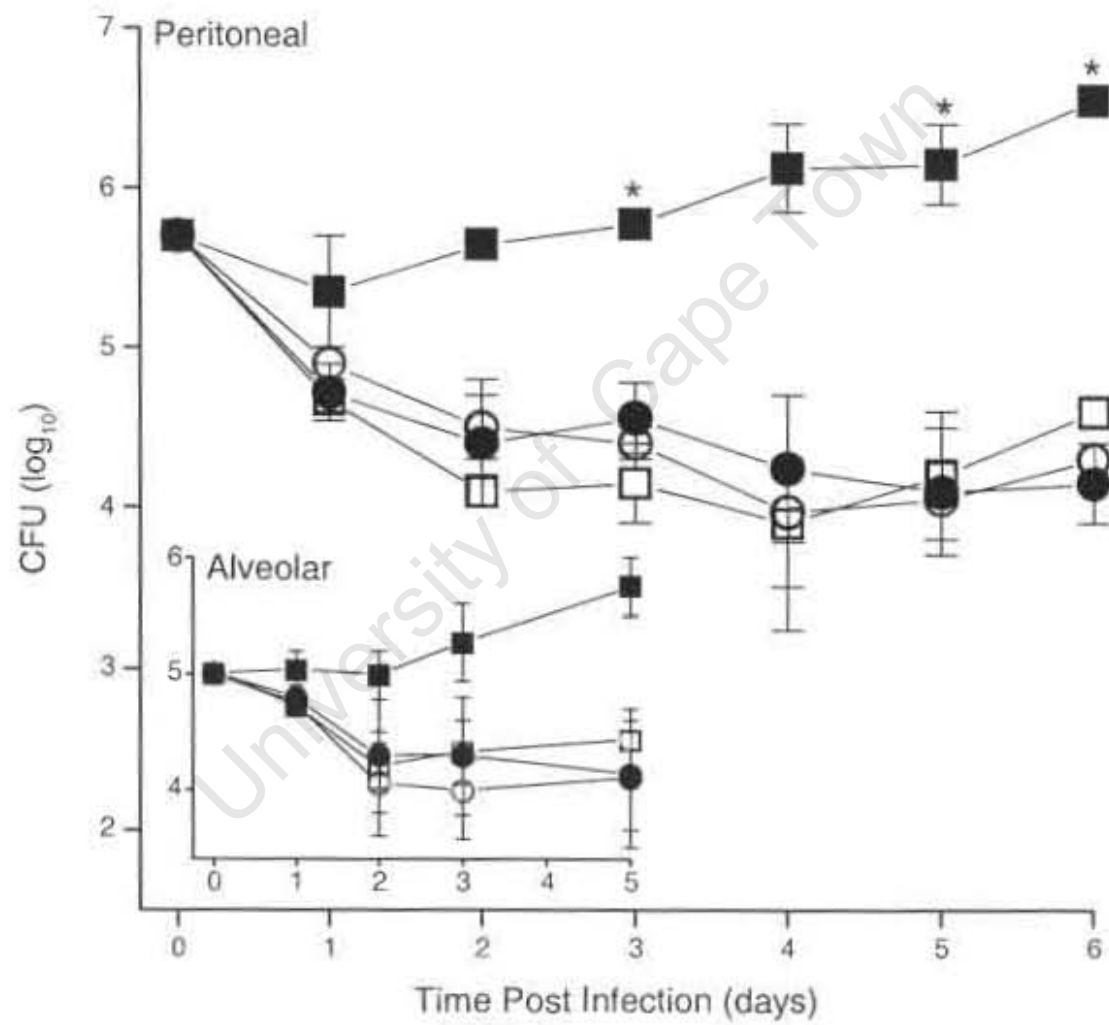
Morphology of macrophages infected with recombinant BCG *in vitro*. **A:** Electron microscopy micrograph of alveolar macrophages from a TNF-KO mouse infected with BCG-vector. **Insert for A:** Enlarged area of peritoneal macrophage infected with BCG-vector. **B:** Electron microscopy micrograph of alveolar macrophage from a TNF-KO mouse infected with BCG-TNF. **Insert for B:** Enlarged area of peritoneal macrophage infected with BCG-TNF. Black arrows show individual bacilli within a phagocytic vacuole in the cytoplasm of the cells. White arrows in inserts show the double membrane of the phagocytic vacuole. Magnification x 2600; inserts magnification x 50 000.



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Figure 6-2

Effect of TNF- α on the survival and growth of recombinant BCG in macrophages *in vitro*. Macrophages from TNF-KO mice (closed symbols) or from wild type mice (open symbols) were infected with BCG-vector (squares) or BCG-TNF (circles). * denote statistically significant ($p < 0.05$) differences in bacillary load between BCG-vector and BCG-TNF infected macrophages. Results are means \pm one SEM of four independent experiments each carried out in duplicate.



BCG-TNF (Figure 6-1B). Similarly, no differences in the morphology of the macrophages were noted following phagocytosis of BCG-vector compared to BCG-TNF.

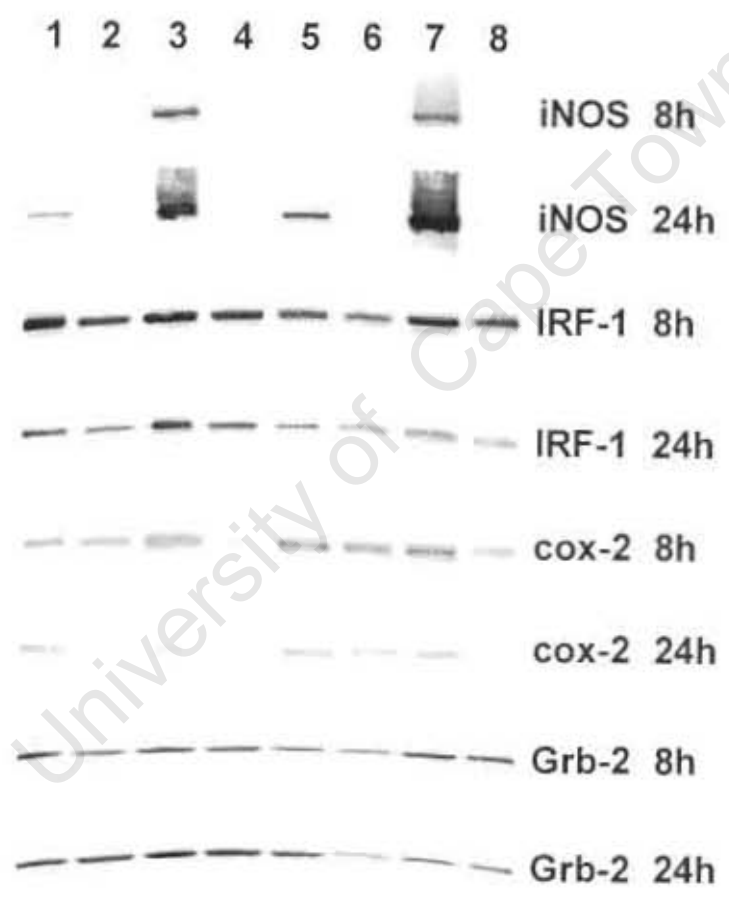
Following infection an initial delay in growth of BCG-vector was noted (Figure 6-2). Thereafter the numbers of BCG-vector, evaluated daily by CFU assay, increased steadily for the next 5 days. The number of bacilli during this period increased about 10 fold; the generation time was 30 h. In contrast when peritoneal macrophages from TNF-KO mice were infected with BCG-TNF the numbers of viable mycobacteria decreased from the time of infection. A statistically significant difference in intracellular bacillary numbers between the two recombinant strains was noted by day 3 post infection ($p=0.005$) (Figure 6-2). The killing of BCG-TNF in TNF-KO macrophages was similar to that observed when wild type peritoneal macrophages were infected (Figure 6-2). Thus the presence of TNF- α , whether of host or pathogen origin, was required for the intracellular control of growth and killing of the organisms.

When alveolar macrophages from TNF-KO mice or wild type mice were infected with either of the BCG strains, the same pattern of growth versus bacillary killing was noted. After a one day delay, alveolar macrophages from TNF-KO mice supported the growth of BCG-vector (Figure 6-2) The generation time in this case was 33.8 hours. However, the TNF-KO alveolar macrophages cells killed BCG-TNF as did the alveolar macrophages from wild type mice infected with either recombinant strain (Figure 6-2). Again, the differences on day 3 were statistically significant. These observations suggest that peritoneal and alveolar macrophages of mice control intracellular growth of BCG similarly and that TNF- α is required for this effect.

iNOS expression in peritoneal macrophage infected with BCG: To examine the role of iNOS expression during infection of murine macrophages *in vitro*, Western blot analysis of cell lysates was carried out at 8, 24 and 48 hours post infection. Uninfected peritoneal macrophages from TNF-KO mice or wild type mice did not express iNOS protein at any time (not shown). These cells did however, express the IFN- γ induced protein IRF-1. Infection of macrophages from TNF-KO mice with BCG-vector resulted in delayed iNOS expression compared to infection with BCG-TNF. Thus, at 8 h post infection iNOS protein was only clearly detected in macrophages infected with BCG-TNF (Figure 6-3 lane 3). By 24 hours low levels of iNOS protein were also detected in

Figure 6-3

Effect of TNF- α on protein expression in peritoneal macrophages infected with recombinant BCG *in vitro*. Cell lysates were analysed by Western blot for the expression of iNOS, IRF-1, cox-2 and Grb-2 at 8 and 24 hours post infection. Lanes 1 and 2: Macrophages from TNF-KO mice infected with BCG-vector. Lanes 3 and 4: Macrophages from TNF-KO mice infected with BCG-TNF. Lanes 5 and 6: Macrophages from wild type mice infected with BCG-vector. Lanes 7 and 8: Macrophages from wild type mice infected with BCG-TNF. The iNOS inhibitor AMG was added to the macrophage cultures represented in lanes 2, 4, 6 and 8.



macrophages infected with BCG-vector (Figure 6-3 lane 1) while higher levels of the protein were induced by infection with BCG-TNF (Figure 6-3 lane 3). Similar patterns of iNOS expression were seen when macrophages from wild type mice infected with BCG-vector (Figure 6-3 lane 5) was compared to BCG-TNF (Figure 6-3 lane 7). By 48 hours post infection iNOS was not detected in any of the cell lysates (not shown). IRF-1 levels were similar in all infected cultures at 8 hours, were reduced at 24 hours and were undetectable at 48 hours. These observations suggest that iNOS induction in the macrophages did not require TNF- α . However, the presence of TNF- α accelerated the expression of the protein.

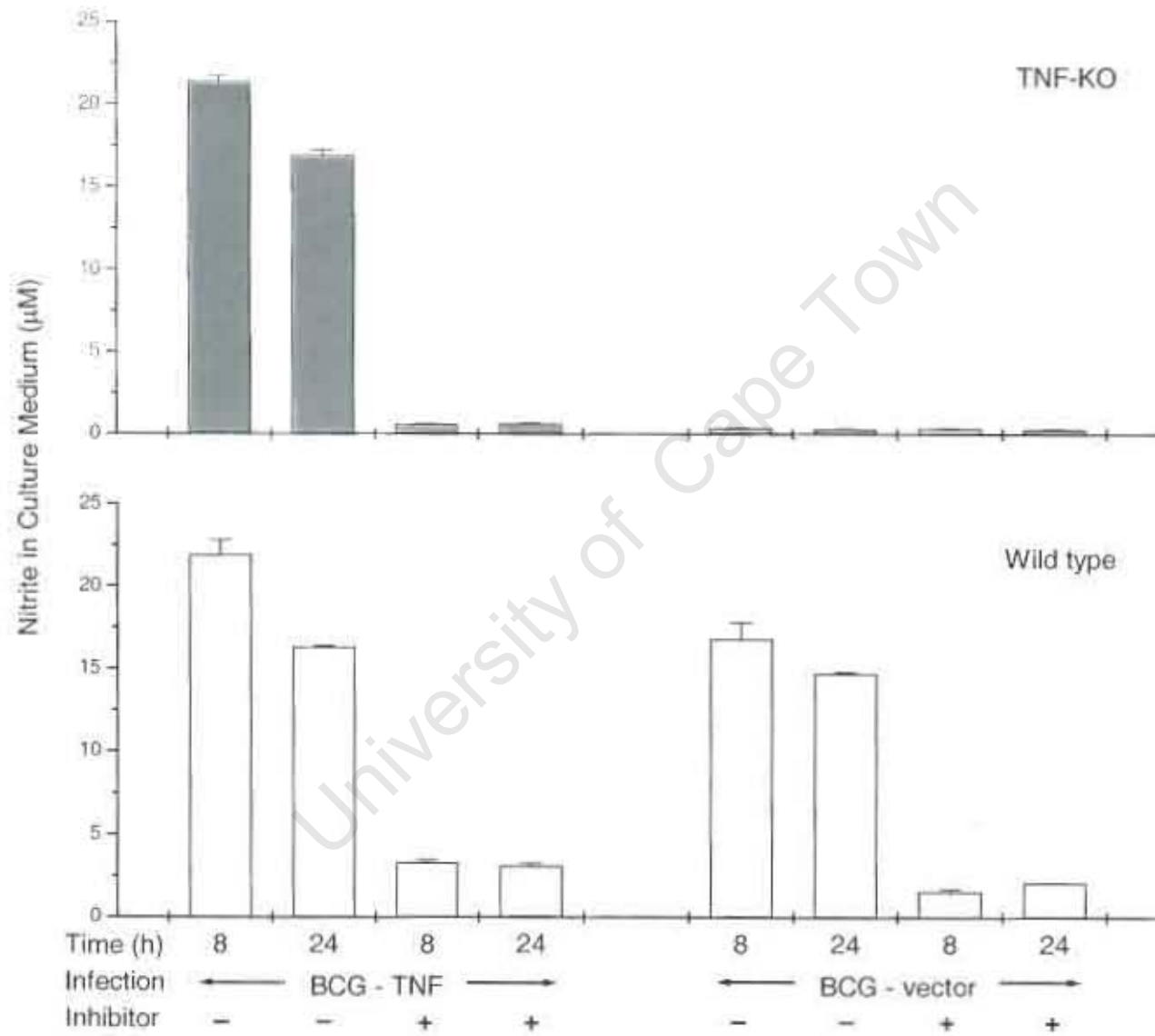
When the iNOS inhibitor AMG was added to the culture supernatants the expression of the protein was fully abrogated in both TNF-KO and wild type macrophages in response to infection with either recombinant BCG strain (Figure 6-3 lanes 2, 4, 6 and 8). AMG in the culture supernatants did not affect the expression of IRF-1.

When exogenous recombinant murine IFN- γ was added to TNF-KO peritoneal macrophages infected with BCG-vector or BCG-TNF, an increase in the level of expression of iNOS was noted in both types of infection (not shown). Levels of expression of IRF-1 were unaffected. Although the addition of IFN- γ induced an increase in iNOS expression the elevated levels of iNOS protein did not change the fate of the intracellular bacilli (not shown).

iNOS activity in peritoneal macrophages infected with BCG: To investigate whether TNF- α affected iNOS activity in macrophages infected with recombinant BCG, nitrite accumulation in the culture medium was evaluated. TNF-KO peritoneal macrophages infected with BCG-vector *in vitro* produced no nitrite (Figure 6-4). However, when the cells were infected with BCG-TNF, nitrite production was observed. The presence of AMG in the culture supernatants fully inhibited this activity. When wild type peritoneal macrophages were infected, both BCG-vector and BCG-TNF induced nitrite production. AMG added to these cultures significantly reduced nitrite production (Figure 6-4). These results suggested that TNF- α is required for iNOS activity although it does not appear to be required for expression of the protein (Figure 6-3). The addition of

Figure 6-4

Effect of AMG on nitrite production by peritoneal macrophages infected with recombinant BCG. Concentrations of nitrite in the culture medium of the cells are expressed as means \pm one SD of two independent experiments each carried out in duplicate.



recombinant IFN- γ to the infected macrophage cultures slightly increased nitrite production (not shown).

Effect of nitrite production on growth of recombinant BCG in macrophages: The effect of inhibition of iNOS activity by AMG on BCG growth and survival was studied next. The presence of AMG in the cultures had no effect on the growth of BCG-vector in the TNF-KO peritoneal macrophages (Figure 6-5 and Table 6-1). However, the drug rendered these macrophages less capable of controlling the BCG-TNF infection. Under these inhibitory conditions the numbers of BCG-TNF were not reduced. Rather the numbers of bacilli increased slowly, resulting in less efficient growth compared to the BCG-vector (generation time of 89.2hrs versus 33.1hrs respectively). The addition of AMG to wild type macrophage mice infected with either BCG-vector or BCG-TNF abolished the killing of the bacilli and rendered these macrophages permissive to mycobacterial growth (Figure 6-5). Thus in the presence of TNF- α , inhibition of nitrite production by AMG only partially restored the growth of the bacilli. In the absence of TNF- α , inhibition of nitrite had no effect on the growth of BCG.

Cytokine production by BCG infected macrophages: The cytokines produced by the peritoneal macrophages during recombinant BCG infection were measured by ELISA. Macrophages from wild type mice infected with BCG-vector or BCG-TNF secreted large amounts of TNF- α into the culture medium. In contrast, when peritoneal macrophages from TNF-KO mice were infected, TNF- α was found in the culture medium only if the cells were infected with BCG-TNF. The addition of AMG to the culture medium reduced the amount of TNF- α present in the medium whether the cytokine was of host or bacterial origin (Table 6-1).

Superoxide production by macrophages infected with BCG: The percentage of macrophages undergoing an oxidative burst was evaluated by superoxide activation of nitrobluetetrazolium (NBT) following infection with BCG. When peritoneal macrophages from wild type mice were infected with either BCG-vector or BCG-TNF, 40 to 50% of the cells were activated (Table 6-1). However, infection of TNF-KO peritoneal macrophages with BCG-TNF resulted in 40% of cells staining while 80% of cells stained following infection with BCG-vector. Again, the presence of AMG in the cultures significantly reduced the percentage of cells producing superoxide (Table 6-1).

and found to be more efficient for the BCG-TNF infection (generation time 90.5hrs) compared to the BCG-vector infection (410 hrs). Similarly, macrophages from IFN-KO mice were also infected *in vitro* with either BCG-vector or BCG-TNF (Table 6-1). Again, no nitrite was detected in the culture medium and TNF- α was detected only when the cells were infected with BCG-TNF. In these macrophages the control of growth of the BCG was more efficient in the presence of TNF- α (generation time 72.2 h) compared to the absence of TNF- α (generation time 33.6 h). These results suggested that in the absence of iNOS activity, TNF- α contributes to the ability of macrophages to control the intracellular growth of BCG.

Discussion

These studies show an unequivocal role for TNF- α in the control of mycobacterial growth in murine macrophages. Although both recombinant strains of BCG were phagocytosed similarly as shown morphologically by electron microscopy, the subsequent fates of the strains were very different. In the absence of any TNF- α , whether of macrophage origin or from recombinant BCG-TNF, the macrophages from TNF-KO mice could not control the growth of BCG. However, when TNF- α was added back by infection with BCG-TNF, the replication of the infecting organisms was controlled and the numbers of viable bacteria was reduced. Similarly, it has been shown that when TNF- α is added to human macrophages infected *in vitro* with *M. tuberculosis*, the cells are more efficient at curbing the growth of the bacilli than when other cytokines or cytokine combinations including IFN- γ are added [Denis M, Gregg EO and Ghandirian E, 1990].

The antimycobacterial effect of TNF- α observed in the present study appeared to be associated with expression of iNOS and with iNOS activity. Interestingly, when AMG was added to these cultures, the antimicrobial activity was less efficient, although not fully inhibited, suggesting the presence of an iNOS independent TNF- α dependent antimycobacterial activity. Furthermore, infection of macrophages prepared from iNOS-KO mice confirmed the observation of an iNOS independent but TNF- α dependent antimycobacterial activity.

The iNOS independent but TNF- α dependent antimycobacterial activity could be mediated by ROI. This pathway has been studied using respiratory burst-deficient gp 91 (phox -/-) mice [Murray HW and Nathan CF, 1999]. These mice are particularly susceptible to mycobacterial infection early in the course of infection. *In vitro*, the oxidative burst has been shown to be important in the control of intracellular infection [Lepay DA, Steinman RM, Nathan CF, et al., 1985]. Since in our studies, AMG inhibited both the RNI and the ROI pathways, it is possible that the role of the oxidative pathway in mycobacterial killing may have been underestimated in previous studies in which AMG was used as a specific inhibitor of RNI [Misko TP, Moore WM, Kasten TP, et al., 1993].

It is well known that both TNF- α and IFN- γ are required for maximal macrophage activation [Oswald IP and James SL, 1996]. In the mouse model, macrophage activation results in iNOS induction [MacMicking J, Xie QW and Nathan C, 1997]. Nitric oxide (NO) then results from the oxidative deamination of L-arginine to L-citrulline by iNOS. In the present studies, iNOS protein was expressed in the macrophages even in the absence of TNF- α . Similarly, in the previous chapter, we observed that iNOS was expressed in the tissues of infected mice in the absence of TNF- α . However, in this *in vitro* study, the enzyme appeared to be inactive, since nitrite was not detected in the culture supernatant. As a result, the mycobacteria were not killed. Nitrites were found in the culture supernatant and the organisms were killed only when TNF- α was present. Thus, complete functional activation of the nitric oxide pathway appears to require TNF- α .

Recent studies have demonstrated the ability of mycobacteria to activate macrophages for cytokine production via the toll-like receptor 2 (TLR2) [Underhill DM, Ozinsky A, Smith KD, et al., 1999; Modlin R, Brightbill HD and Godowski PJ, 1999]. CD14 interacts with toll-like receptors to transmit signals involving the recruitment of interleukin-1 receptor-associated kinase 2 (IRAK2) and tumor necrosis factor receptor-associated factor-6 (TRAF-6) activation of nuclear factor-kappa B (NF- κ B) and subsequent gene transcription. This leads to expression of immunomodulatory genes including cytokines such as TNF- α as well as the induction of enzymes such as iNOS. TLR2 is the principle mediator of macrophage activation in response to mycobacteria. The studies discussed in this chapter may be interpreted to suggest that TLR2 is utilized by BCG to induce iNOS in the TNF-KO macrophages, but both the TLR2 pathway and

TNF- α signaling via the TNF-R signal pathway are required for efficient killing of the intracellular mycobacteria (Figure 1-3). This is probably the receptor utilized by BCG to induce iNOS in the TNF-KO macrophages in these studies. The results suggest that both the TLR2 pathway and the TNF- α signaling pathway are required for killing of mycobacteria.

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Chapter 7. THALIDOMIDE INDUCED ANTIGEN SPECIFIC IMMUNE STIMULATION IN PATIENTS WITH HIV AND TUBERCULOSIS INFECTION.

Introduction

Tuberculosis is the most common coinfection in HIV-1 infected patients in South Africa, causing severe morbidity and accelerated mortality [Connolly C, Davies GR and Wilkinson D, 1998]. This is most likely due to the immune suppression of HIV infection which appears to lead to a greater susceptibility to development of active disease following exposure to *Mycobacterium tuberculosis* as well as an increased rate of reinfection and/or a higher incidence of disease reactivation following anti-tuberculous therapy [Wilkinson D and Davies GR, 1997; Wood R and Hudson C, 1997].

Tuberculosis is a potent inducer of HIV replication, resulting in up to 160-fold increase in viral particles in plasma [Goletti D, Weissman D, Jackson RW, et al., 1996; Shattock RJ, Friedland JS, Griffin GE, 1994]. This is probably because virus replication is stimulated by the immune activation due to concomitant infection [Nakata K, Rom WN, Honda Y, et al., 1997]. It had previously been noted that the immune activation occurring following administration of recombinant IL-2 or immunization was also accompanied by increased viral load [Kovacs JA, Baseler M, Dewar RJ, et al. 1995; Stanley S, Ostrowski MA, Justement JS, et al., 1996]. *In vitro*, antigenic or mitogenic stimulation of blood cells from HIV-infected individuals results in increased viral replication and increased T cell infection [Kinter AL, Poli G, Fox L, et al. 1995; Margolick JB, Volkman DJ, Folks TM, et al., 1987].

Plasma levels of TNF- α are higher in patients with concomitant HIV and tuberculosis infections (HIV/TB) than in patients infected with HIV or *M. tuberculosis* alone [Klausner JD, Makonkawkeyoon S, Akarasewi P, et al., 1996]. TNF- α induces HIV replication via a shared NF κ B-dependent transcription control mechanism. Increases in the levels of this pro-inflammatory cytokine may therefore contribute to enhanced viremia as well as to the wasting observed in HIV/TB coinfection [Osborn L, Kunkel S and Nabel GJ, 1989; Chun TW, Engel D, Mizell SB, et al., 1998; Lawn SD, Shattock RJ, Acheampong JW, et al., 1999]. In Chapter 3 of this thesis, we describe studies in which

initiation of anti-tuberculous therapy in tuberculosis patients resulted in clinical worsening including weight loss, in association with a transient increase in plasma levels of TNF- α .

Thalidomide is an immunomodulatory drug which inhibits monocyte TNF- α production *in vivo* and *in vitro* but does not effect production of TNF- α by T cells [Sampaio EP, Kaplan G, Miranda A, et al., 1993; Sampaio EP, Sarno EN, Galilly R, et al., 1991; Haslett PAJ, Corral LG, Albert M, et al., 1998]. The drug also acts as a costimulator of human T cells *in vitro*, resulting in increased production of Th1 cytokines (IFN- γ and IL-12) [Corral LG, Haslett PAJ, Muller GW, et al., 1999]. In HIV infected patients thalidomide stimulates T cell responses and interleukin 12 (IL-12) production [Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999]. To test whether thalidomide would affect the production of TNF- α and of Th1 type cytokines (IFN- γ and IL-12) in HIV infected patients with tuberculosis, we carried out a double blind, placebo controlled study. We studied the ability of the drug to stimulate antigen-specific T cell responses *in vivo* and *in vitro* as well as the effect of the drug on HIV levels *in vivo* and on HIV replication *in vitro*.

Study Design

Study subjects: Thirty HIV-1 infected anti-retroviral drug-naive seropositive patients, hospitalized with a recent diagnosis of sputum smear positive pulmonary tuberculosis, were enrolled in the study. Exclusion criteria included patients in whom multi-drug resistant *M. tuberculosis* was subsequently identified; patients in whom treatment with immunomodulatory or antiretroviral agents had occurred; patients in whom an additional opportunistic infection had occurred within 4 weeks of the recruitment date. The patients were recruited over a period of eight months from three acute inpatient hospitals in Cape Town. Patients were hospitalized for the duration of the study in a tuberculosis hospital in the Cape Town area.

Female patients of childbearing potential underwent pregnancy tests within 72 hours of joining the study and weekly thereafter. Female patients were required to practice two methods of contraception initiated prior to taking the study drug and to continue contraception for the duration of treatment and for at least 4 weeks thereafter. In

most cases, long acting, intramuscular progesterone and condoms were the methods of choice.

In addition blood was obtained from 8 asymptomatic HIV infected individuals for *in vitro* studies (see below). Patients had the following characteristics: Median age 42 (range 27-52); CD4 count 521/mm³ (380-657); 4 females – 4 males; 6/8 on highly active antiretroviral therapy. None of these patients received treatment with thalidomide. All patients were selected to have T cell proliferative responses to specific known antigens (see below).

Treatment Regimen: HIV/TB patients were enrolled into the study within seven days of the initiation of a standard four drug regimen (rifampicin 10mg/kg; isoniazid 5mg/kg; pyrazinamide 30mg/kg; ethambutol 25mg/kg) of antituberculosis therapy. All patients were also treated with oral pyridoxine 25 mg daily. Patients were randomly assigned in a double-blind manner to receive either thalidomide (200 mg/day), or an identical placebo provided by Celgene Corporation (Warren, NJ). The drug was given orally at night. Pharmacokinetic studies in HIV infected patients have shown that a single oral dose of 200 mg of thalidomide resulted in plasma levels of 1.9 µg/ml [Noormohamed FH, Youle MS, Higgs CJ, et al., 1999]. Randomization was done by a research pharmacist at a ratio of 2:3 (placebo: thalidomide) and the code deciphered at the end of the study. This ratio was chosen to accommodate an anticipated higher dropout rate in the thalidomide treated group due to risk of drug rashes, as reported previously [Haslett PAJ, Tramontana J, Burroughs M, et al., 1997]. The study drug was administered for 42 days followed by a two-week observation period. If a drug rash developed, all drugs including antituberculosis drugs were stopped, and patients were treated symptomatically with anti-histamines and anti-pyretics until the rash disappeared. Subsequently, medications were reintroduced sequentially starting with thalidomide, then PZA, RIF, ethambutol and finally INH, at half-dose for 2 days then at full dose if tolerated. Rechallenge was not attempted if there was any mucosal or ocular involvement. If no adverse events were noted then standard anti-tuberculous therapy was continued throughout and after the completion of the study.

Antiretroviral therapy is limited in Africa; these patients did not receive antiretroviral therapy and are thus representative of the overwhelming majority of HIV infected individuals in the third world.

Clinical Evaluation: Initial baseline assessment of each patient was carried out including full history and physical examination. A careful assessment was made for peripheral neuropathy. A Karnofsky score was assigned for each patient. Patients were weighed, their body mass index calculated, their right mid arm circumference measured and their triceps skin-fold thickness determined using a Lange Skinfold Caliper. We did not record caloric intake but all patients received a standard hospital diet that was similar for all. In many cases this was improved compared to their home diet.

These assessments were repeated at weekly intervals for the duration of the study. Signs of drug toxicity were regularly (at least weekly) sought throughout the study with particular attention paid to signs of peripheral neuropathy, skin rash or excessive sedation.

Clinical laboratory measurements: Full blood count, serum chemistry and urinalysis were performed on blood samples obtained from all patients at study day 0 and every 3 weeks thereafter (days 0, 21 and 42). A final blood sample was also collected at 2 weeks post treatment (day 56).

Bacteriology: Sputum was collected on day 0 and sent for *M. tuberculosis* smear, culture (Bactec, Becton Dickenson, San Jose, CA) and sensitivity to INH and rifampicin. Sputum was collected and re-examined weekly if still available.

Virologic measurements: EDTA-anticoagulated plasma samples were collected on days 0, 21, 42 and 56, and frozen at -70°C until assayed. HIV-1 titres were measured by the Nucleic Acid Sequence Based Amplification (NASBA, Organon Teknika City, The Netherlands). Viral titres in equivalents/ml were expressed as \log_{10} RNA units/ml.

Results

Clinical outcome and adverse events: Thirty HIV-1/TB infected patients were randomized to receive thalidomide (n=18), or placebo (n=12). There were no significant differences in clinical characteristics and immunologic markers between the two groups at baseline (Table 7-1). Three patients were excluded early in the study. One (assigned to

thalidomide) was infected with multi-drug resistant *M. tuberculosis* as noted when mycobacterial sensitivities became available. An excluded second patient (assigned to thalidomide) had a complicating infection (bacterial empyema) at the time of enrollment. The third excluded patient (assigned to placebo), developed evidence of early HIV-1 associated dementia within a week of study commencement and withdrew consent. Of the 27 remaining patients, 11/11 completed the study in the placebo group and 14/16 completed the study in the thalidomide group. Two patients (patient numbers 2 and 21) (assigned to thalidomide) died. Post mortems were performed, confirming the causes of death as hepatitis (probably anti-tuberculous drug induced) and *Pneumocystis carinii* pneumonia respectively, considered unrelated to study drug (Table 7-2).

Table 7-1. Patient characteristics at baseline.

Patient Characteristic	Treatment Group	
	Placebo (n=12)	Thalidomide (n=18)
Age (years)	32.4 (8.6)*	34.1 (11.6)
Gender (M:F)	4:8	9:7
BMI (kg/m ²)	18.8 (2.9)	18.7 (3.4)
CD4 (cells/mm ³)	94 (46)	97.4 (23)
Viral load (RNA U/ml)	Log ₁₀ 5.25 (0.18)	Log ₁₀ 5.57 (0.22)
Karnofsky score	50 (10.8)	49 (11.9)

All parameters are calculated as means. *Standard deviations are given in parentheses.

In the 14 patients assigned to thalidomide and who completed the study, the dose of 200 mg/day of thalidomide appeared to be well tolerated, with no dropouts related directly to therapy (Table 7-2). Two patients complained of increased tingling of their feet but there were no objective clinical changes to suggest progressive peripheral neuropathy. Doubling of the dose of pyridoxine (to 50 mg) and addition of amitriptyline (25 mg) at night lead to resolution of all symptoms. Three patients developed maculopapular rash,

Table 7-2. Patient adverse events.

Patient No.	Gender	Age	Treatment Group	Event
2	M	44	Thalidomide	Died of proven <i>Pneumocystis carinii</i> pneumonia on day 32.
5	M	30	Thalidomide	Proven deep vein thrombosis on day 49. Heparinised, full recovery. Completed study.
8	F	47	Thalidomide	Thalidomide rash on day 9. Successful rechallenge. Completed study.
11	M	28	Placebo	INH rash on day 7. Successful rechallenge.
19	F	20	Thalidomide	Thalidomide rash on day 10. Successful rechallenge. Completed study.
21	F	39	Thalidomide	Died of TB drug-related proven hepatitis on day 22.
27	F	22	Thalidomide	Laparotomy performed to confirm peritoneal tuberculosis on day 30. Thereafter excluded from further analysis.
24	M	54	Thalidomide	Thalidomide rash on day 14. Successful rechallenge. Completed study.

with fever and erythema, typical of the rash seen previously in thalidomide sensitive patients [Haslett PAJ, Tramontana J, Burroughs M, et al., 1997] (Table 7-2). No mucocutaneous or ocular involvement was present in any of these cases and thalidomide was successfully recommenced in all. One patient (assigned to thalidomide) developed ascites confirmed to be due to TB peritonitis at laparotomy on day 30; subsequent data points for this patient were not included for analysis to avoid impact of the surgery on the values. One patient experienced a deep vein thrombosis (Table 7-2). It has previously been shown that this complication is associated with tuberculosis infection [Robson SC, White NW, Aronson I, et al., 1996]. In the 11 patients assigned to placebo one was found

to have an INH drug sensitivity but was successfully rechallenged and completed a full course of anti-tuberculous drugs (Table 7-2).

The patients included in the study were severely immune-suppressed individuals who required hospitalization for tuberculosis. Despite the severity of disease, patient responses to antibiotic treatment of tuberculosis with or without thalidomide were similarly good in both study groups. After the commencement of anti-tuberculous therapy Karnofsky scores (used as a subjective measure of patient well being) showed a significant improvement from a mean (SD) of 49.4 (11.97) (thalidomide) and a mean (SD) of 50.83 (10.84) (placebo) to a mean (SD) of 91.53 (16.75) in the thalidomide group and a mean (SD) of 92.7 (12.72) in the placebo group.

The mean baseline weight in both groups was similar (50.3 kg in the thalidomide and 51.3 kg in the placebo group). Patients treated with anti-tuberculous drugs and either thalidomide or placebo experienced significant ($p=0.004$ or less for both groups) weight gain from day 21 (Figure 7-1). A similar steady increase in weight was observed in both groups.

The mean body mass index for all patients studied at baseline (range: 15 – 30; mean: 18.6) was below the normal values expected for this adult population (normal range: 20-25). In both study groups there was a similar significant increase ($p=0.02$ or less for both groups) in body mass index from day 21 after hospitalization and commencement of anti-tuberculous therapy (Figure 7-1). No significant difference in increase in body mass index was seen between the two groups.

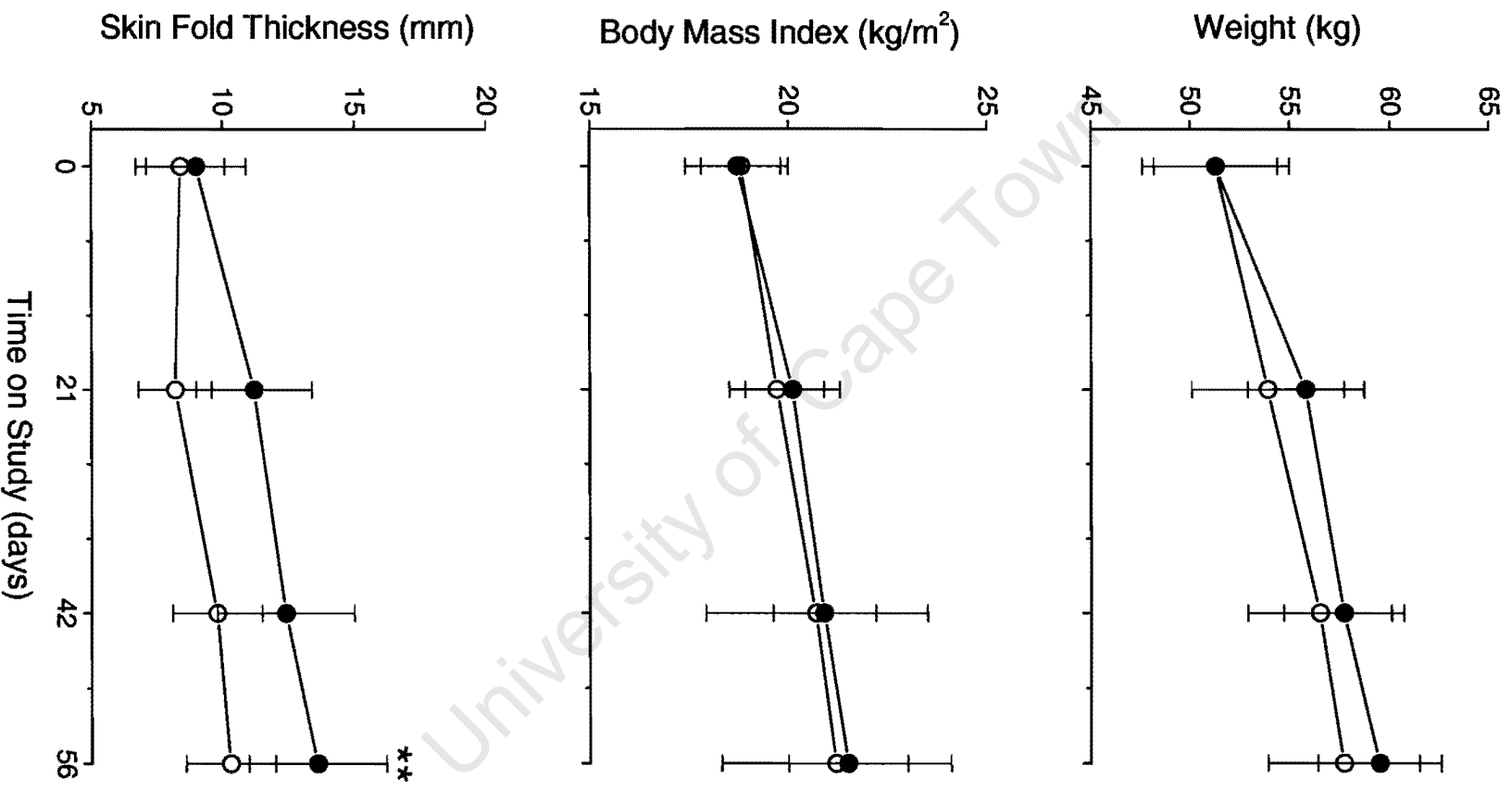
An increase in triceps skin thickness and mid arm circumference was measured in both patient groups. The skinfold thickness increased in both groups but in the thalidomide treated patients the increase from baseline was statistically significant ($p<0.05$) (Figure 7-1). Although there was a clear trend toward a larger increase in skin fold thickness in response to thalidomide the difference between the thalidomide and placebo treated patients was not statistically significant.

Sputum collection and culture of M. tuberculosis: Sputum collected weekly was evaluated for smear positivity and growth of *M. tuberculosis*. The median time to negative culture or the time to inability to produce sputum was found to be 7 days (range

Figure 7-1

Effect of thalidomide treatment (marked by the horizontal arrow) on patient weight, body mass index and triceps skin fold thickness. Patients receiving thalidomide (closed circles); patients receiving placebo (open circles). Results are expressed as means \pm SEM. In thalidomide treated patients **denotes significant difference at day 56 compared to baseline.

← Thalidomide →



3-14 days) in the thalidomide treated patients and 14 days (range 7-42 days) in the placebo treated patients. The accelerated clearance of acid fast bacilli from the sputum of the thalidomide treated patients compared to the placebo treated patients was not statistically significant.

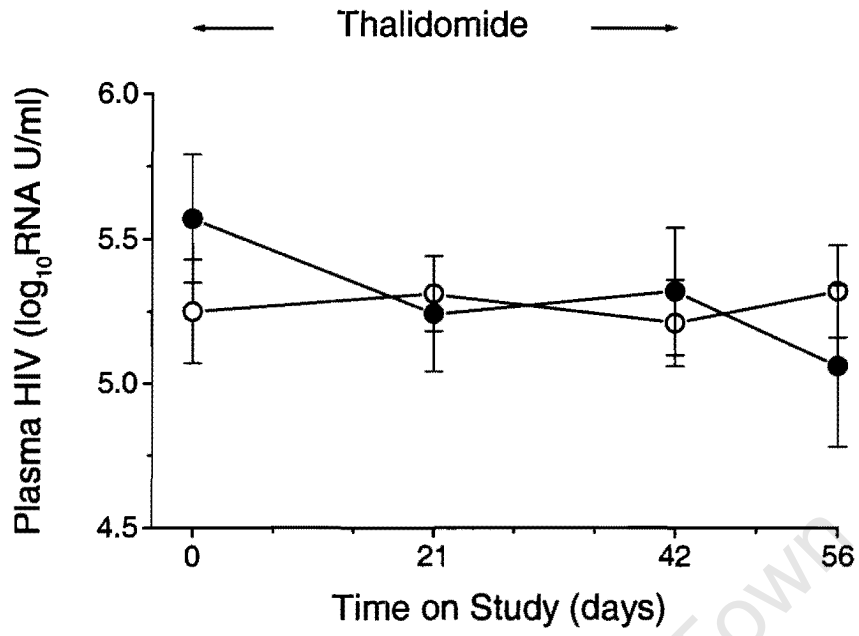
Viral load: The effect of anti-tuberculous therapy and either thalidomide or placebo treatment on plasma HIV-1 levels was evaluated. At baseline, the mean plasma HIV-1 titre in the two treatment groups was 5.25 (± 0.18) log₁₀ RNA equivalents/ml (placebo) and 5.57 (± 0.22) log₁₀ RNA equivalents/ml (thalidomide). No change in plasma HIV-1 levels was observed during the study in the placebo treated patients (Figure 7-2). By comparison, a trend ($p=0.1$) towards a reduction in viral load compared to baseline was noted in the thalidomide treated patients. However, the differences in viral load between the two treatment groups was not significant.

Total lymphocyte counts and T cell subsets: Total lymphocyte counts were similar at baseline in the two treatment groups (973 ± 177 and 832 ± 236 for thalidomide and placebo respectively) and were not significantly different over the study period (1131 ± 171 and 1223 ± 245 respectively). The CD4⁺ T cell counts increased in both groups. The increase from baseline in the thalidomide treated patients occurred earlier (day 21) and was significantly different ($p=0.05$) than the placebo treated patients (Figure 7-3). Similarly, the CD8⁺ T cell counts increased in both groups. The increase compared to baseline in the thalidomide treated patients at day 21 was more pronounced and significantly higher ($p=0.007$) than that of the placebo treated patients (Figure 7-3). Also, a significant ($p=0.05$) increase in sCD8 was observed on day 21 in the thalidomide treated patients compared to the placebo treated patients in whom sCD8 levels remained unchanged (Figure 7-3).

The effect of thalidomide on plasma cytokine and cytokine receptor levels: At baseline, TNF- α levels were higher than normal in all patients (mean normal = 6 pg/ml). During the study there was a transient increase in TNF- α levels observed on day 21 in the thalidomide ($p=0.05$) and the placebo (not statistically significant) treatment groups (Table 7-3). By the end of the study the levels of TNF- α had decreased to just below baseline levels in both groups. Nevertheless levels at 56 days remained elevated compared to normal (uninfected controls). At baseline, IFN- γ levels in plasma were also

Figure 7-2

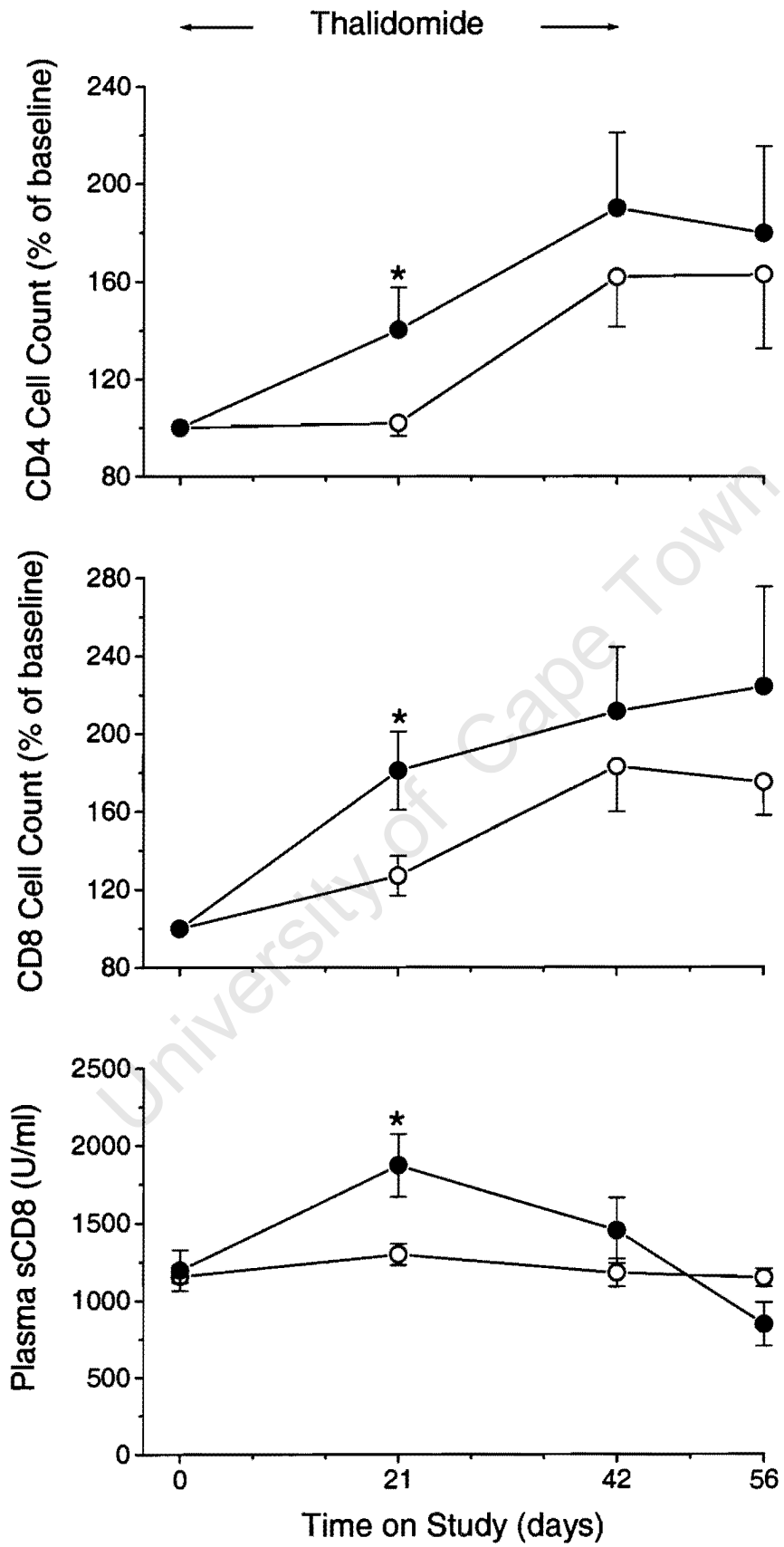
Effect of thalidomide treatment on plasma HIV-1 viral load. Results are expressed as mean \log_{10} RNA U/ml \pm SEM for thalidomide treated group (closed circles) and the placebo treated group (open circles).



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Figure 7-3

Effect of thalidomide treatment on T cells. Mean CD4⁺ T cell counts at baseline were 103 ± 23 and 108 ± 46 and mean CD8⁺ T cell counts at baseline were 590 ± 125 and 434 ± 130 for thalidomide and placebo respectively. Results are expressed as mean percent of baseline ± SEM. Mean plasma soluble CD8 is expressed in U/ml ± SEM. Thalidomide treated patients (closed circles); placebo treated patients (open circles). *denotes significant difference noted between the two treatment groups.



above normal (mean normal = 0.2 IU/ml). An early increase in plasma IFN- γ levels was noted in the thalidomide treated patients but not in the placebo treated group. Thereafter, in both treatment groups there was a reduction in plasma IFN- γ levels by day 56 which was statistically significant ($p=0.01$) only in the patients treated with placebo (Table 7-3).

However at day 56 plasma IFN- γ levels were still higher than normal. IL-6 plasma levels which were elevated at baseline in all patients were also reduced during treatment. The reduction in plasma IL-6 levels was statistically significant ($p=0.0005$) in the placebo but not the thalidomide treated patients (Table 7-3) (mean normal = 8.5 pg/ml).

Table 7-3. Plasma levels of TNF- α IFN- γ and IL-6.

Treatment Group	Cytokine	Cytokine Levels in Plasma**			
		Day 0 (baseline)	Day 21	Day 42	Day 56
		Percent of Baseline (SEM)			
Thalidomide	TNF- α	95.6 (17.2) pg/ml	162*(33)	132 (28)	92 (22)
Placebo		94.1 (23.5) pg/ml	125 (26)	86 (14)	84 (13)
Thalidomide	IFN- γ	1.2 (0.3) IU/ml	130 (26)	58 (13)	60 (13)
Placebo		2.3 (0.4) IU/ml	91 (21)	52 (10)	48*(12)
Thalidomide	IL-6	86.9 (38.7) pg/ml	82 (31)	71 (36)	56 (19)
Placebo		62.2 (25.6) pg/ml	54 (28)	32 (30)	20*(14)

** Results are expressed as means.

* Denotes $P<0.05$ (day of evaluation compared to baseline).

IL-12 plasma levels were elevated in both treatment groups at baseline (Figure 7-4) (mean normal = 81 pg/ml). Thereafter, a significant ($p=0.022$) difference in the plasma IL-12 levels between the two treatment groups was noted. Plasma IL-12 levels increased significantly ($p<0.001$) in response to thalidomide treatment (Figure 7-4). In contrast, the IL-12 level in the placebo treated patients remained constant throughout the study.

In keeping with the thalidomide induced immune activation reflected by CD8⁺ T cell increases and indicated by the enhanced IL-12 and IFN- γ production, non specific markers of immune activation were also affected. The sIL-2R levels, which were elevated

in all patients at baseline (mean normal 1250 pg/ml), significantly increased ($p = 0.03$ at days 21 and 42) in the thalidomide treated patients as compared to the placebo group. The sIL-2R levels remained elevated during thalidomide treatment and were only reduced after discontinuation of the drug. In the placebo treated patients plasma levels of sIL-2R were reduced to normal levels by day 21 (Figure 7-4).

At baseline, TNF- α R levels in plasma were elevated similarly in both groups of patients (Figure 7-4) (mean normal 1.2ng/ml). Here too, there was a significant delay (day 21 $p = 0.008$ and day 42 $p = 0.002$) in the decrease in plasma levels of TNF- α R relative to normal in the thalidomide treated patients compared to the placebo treated patients. However, the levels of the soluble receptor were reduced significantly in both treatment groups by the end of the study (Figure 7-4). Taken together, the changes in cytokine and cytokine receptor levels suggest that thalidomide treatment of HIV/TB patients induced an activation of the host immune cells.

The effect of thalidomide on delayed type hypersensitivity responses and antigen specific lymphocyte proliferation: The skin test response in situ to PPD was compared between the two treatment groups. At baseline, intradermal PPD-induced induration was observed in 3 of 14 thalidomide (21%) and 3 of 11 placebo (27%) patients. At 4 weeks of treatment 10 of 14 thalidomide (71%) and 6 of 11 placebo (54%) patients had positive responses. Although these results suggested that thalidomide treatment may have enhanced the antigen specific T cell response in these patients, statistical significance was not attained ($p=0.2$).

To further evaluate the T cell response to PPD *ex vivo* and to monitor any effect that thalidomide may have had on this response, lymphocyte proliferation assays were carried out before and during treatment. In response to thalidomide treatment, a significant increase in mean stimulation index was observed compared to baseline ($p = 0.0006$ and $p = 0.0001$ at days 21 and 42 respectively) (Figure 7-5). Following discontinuation of thalidomide therapy, lymphoproliferative responses returned to baseline. In contrast, in the placebo treated patients, lymphocyte proliferation did not change throughout the study period (Figure 7-5). Thus, in patients treated with thalidomide there was an activation of antigen specific T cell responses.

Figure 7-4

Effect of thalidomide treatment on plasma cytokine and cytokine receptor levels. Results are expressed as mean plasma IL-12 (pg/ml) \pm SEM, mean plasma sIL-2 receptor (pg/ml) \pm SEM and mean plasma TNF- α receptor (ng/ml) \pm SEM for thalidomide treated group (closed circles) and placebo treated group (open circles). *denotes significant differences between the groups at the specified time points; **denotes significant differences from baseline within each group.

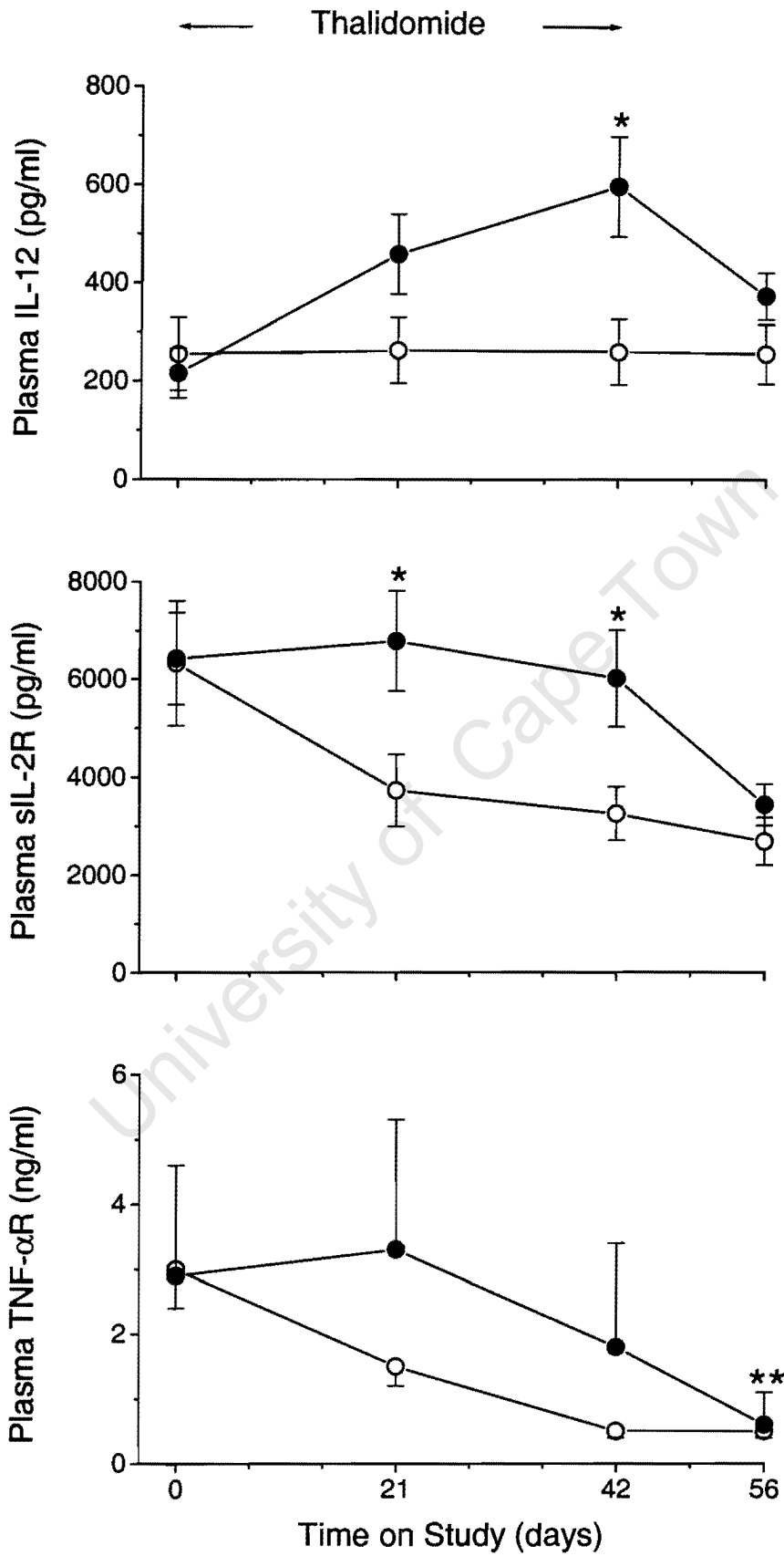
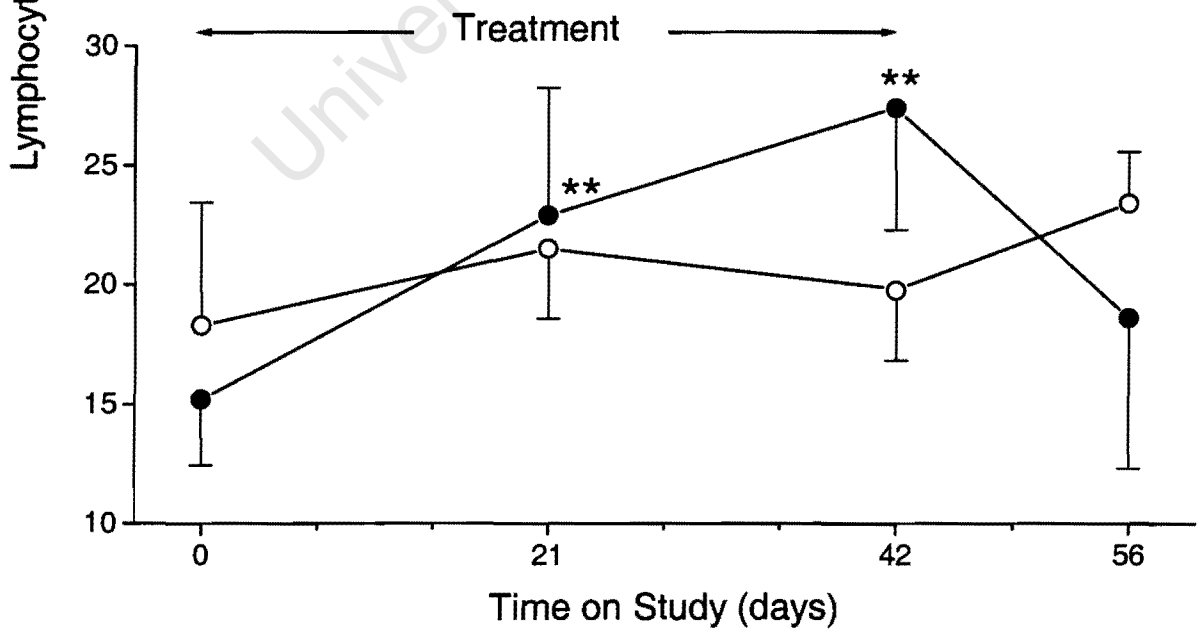
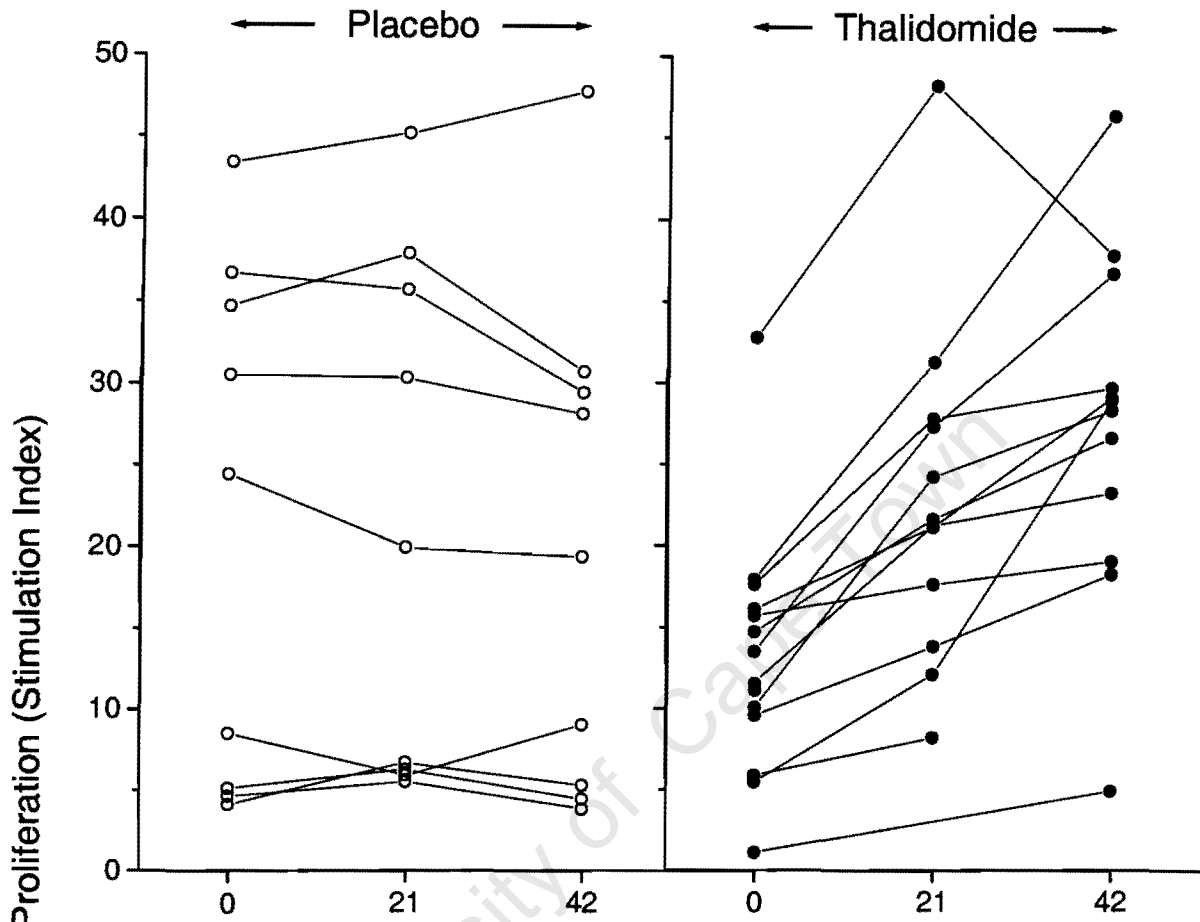


Figure 7-5

Effect of thalidomide on lymphocyte proliferative response to 20 μ g/ml of PPD. Top panels show individual stimulation indices at days 0, 21, and 42 for placebo treated patients (open circles) and thalidomide treated patients (closed circles). Lower panel indicates mean stimulation indices \pm SEM for each group over the whole study period. **denotes a significant difference from baseline at the specified time points, for the thalidomide treated patients.



In vitro thalidomide stimulation of CD4⁺ T cell proliferative responses to antigen: To determine whether thalidomide could directly stimulate T cells obtained from HIV-1 infected individuals, the effect of increasing doses of thalidomide on the *in vitro* proliferative response of CD4⁺ T cells to different recall antigens was evaluated. When antigen pulsed monocytes were incubated with purified CD4⁺ T cells, we observed a thalidomide concentration dependent increase in CD4⁺ T cell proliferation in response to CMV, PPD, and HIV-1 p24 antigen (Figure 7-6A).

To identify the cellular target of the thalidomide-induced increase in antigen-specific proliferation of T cells, the assays were carried out with antigen pulsed DCs which were either live or fixed with glutaraldehyde. Glutaraldehyde fixation renders the DCs deficient in costimulatory function while preserving their capacity to present antigens to T cells [Chain BM, Kay PM and Feldmann M, 1986]. When live antigen pulsed DCs were incubated together with purified CD4⁺ T cells in the presence of thalidomide, a variable enhancing effect of the drug on CD4⁺ proliferative responses was observed (Figure 7-6B). However, when the antigen pulsed DCs were fixed, thalidomide induced a consistent, concentration dependent increase in CD4⁺ proliferative responses (Figure 7-6C). These results show that thalidomide can stimulate an increase in antigen specific CD4⁺ T cell responses *in vitro*, which is most readily apparent in the absence of additional co-stimulatory activity. The drug appears to have a co-stimulatory effect directly on the CD4⁺ T cell.

Effect of CD8⁺ T cells on augmented HIV replication occurring in response to thalidomide costimulation of CD4⁺ T cells: It has been reported that thalidomide treatment of patients with advanced HIV disease resulted in an increase in plasma levels of HIV [Jacobson JM, Greenspan JS, Spritzler J, et al., 1997]. However, in our present clinical study we observed that despite clear evidence of immune stimulation by thalidomide, no stimulatory effect of the drug on HIV replication was observed. To address this question *in vitro*, we examined the effect of thalidomide on PBMC and CD4⁺ T cells obtained from HIV infected individuals. Figure 7-7 shows the results of a representative experiment. When PBMC were stimulated with immobilized anti-CD3 antibody, very low levels of HIV p24 antigen were detectable in the culture supernatant, with no effect of thalidomide (Figure 7-7A). When CD8⁺ T cells were isolated and

Figure 7-6

Effect of thalidomide on antigen specific proliferation of purified CD4⁺ T cells from HIV infected individuals. Purified CD4⁺ T cells were incubated in the presence of varying concentrations of thalidomide with M-APC (A), live DCs (B) or glutaraldehyde fixed DCs (C) which had been pulsed with the following antigens: CMV (closed circles), PPD (closed triangles), tetanus toxoid (closed diamonds) HIV p24 antigen (closed squares), control protein for CMV (open circles), control protein for HIV p24 antigen (open squares) or no antigen (open triangles). Results are for individual patients (means of triplicate samples).

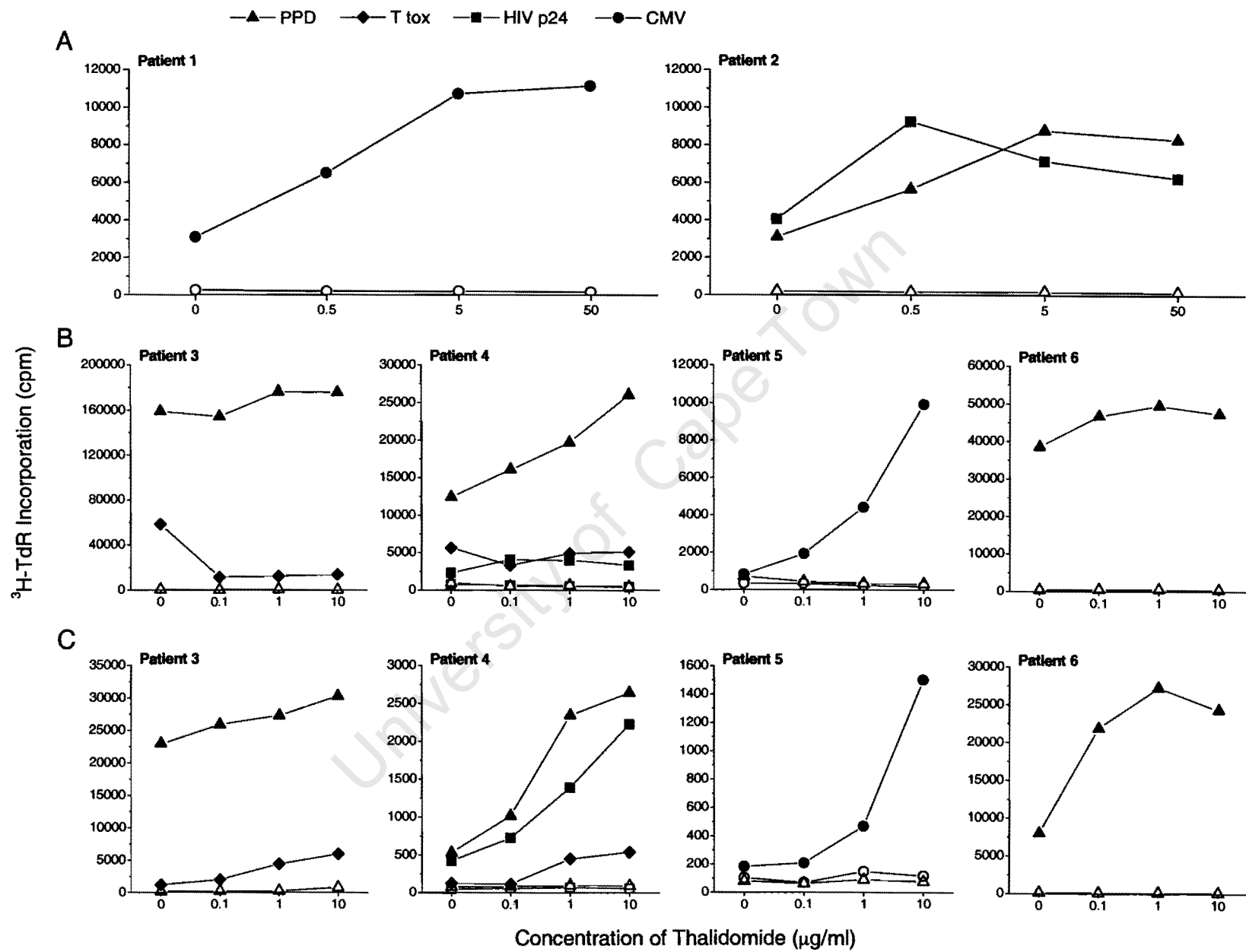
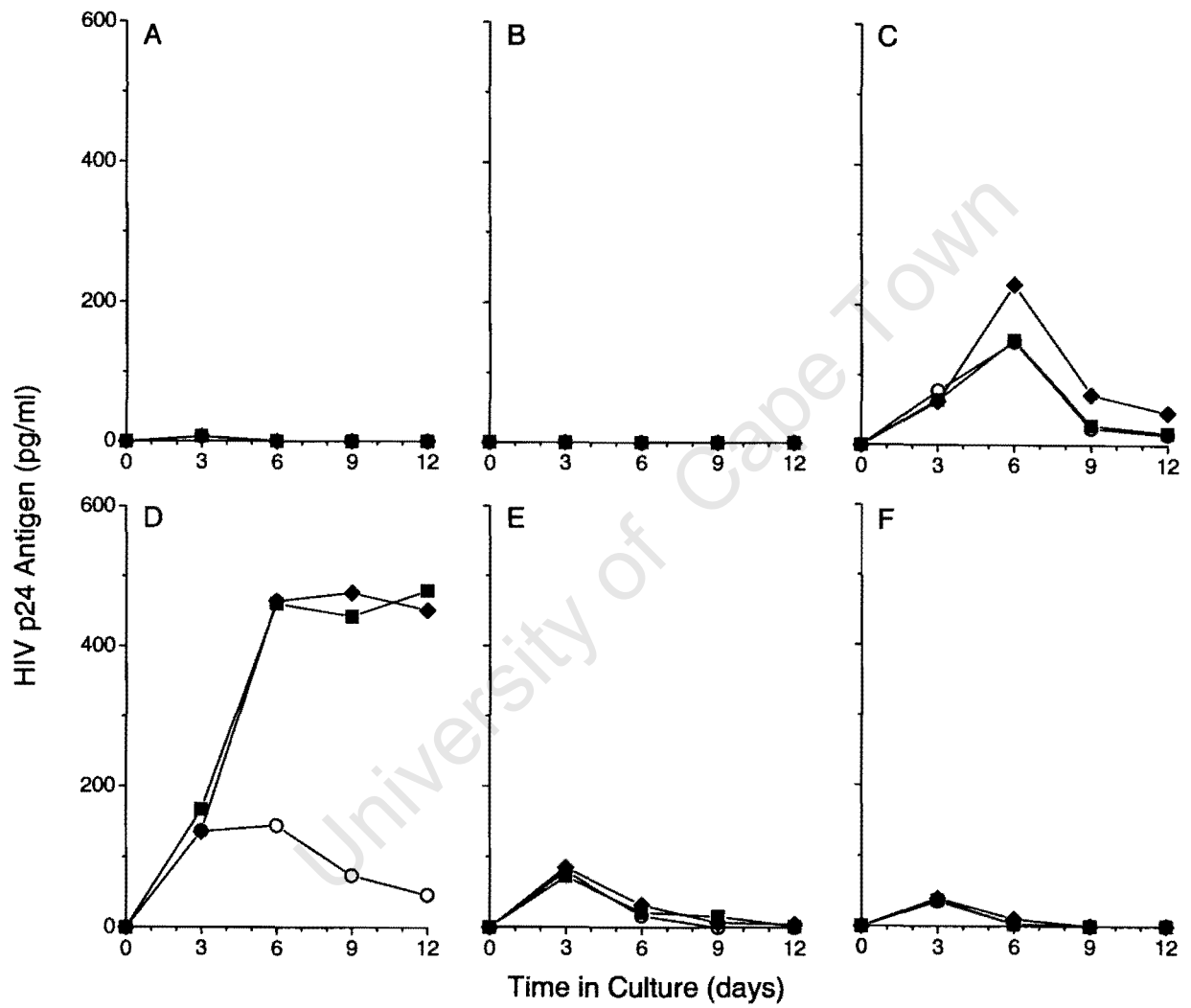


Figure 7-7

Effect of thalidomide on HIV replication in PBMC and T cells stimulated with immobilized anti-CD3 antibody. Cells (7.5×10^6) were cultured with anti-CD3 in the presence of DMSO (open circles), thalidomide 1 $\mu\text{g}/\text{ml}$ (closed squares) or thalidomide 10 $\mu\text{g}/\text{ml}$ (closed diamonds). Supernatants were harvested at the time points indicated and assayed for HIV p24 antigen. Cells used were: Bulk PBMC (A); Purified CD8⁺ T cells (B); PMBC depleted of CD8⁺ T cells (C); Purified CD4⁺ T cells (D); Purified CD4 T cells with autologous CD8⁺ T cells added to achieve a CD4:CD8 ratio of 10:1 (E); Purified CD4⁺ T cells with autologous CD8⁺ T cells added to achieve a CD4:CD8 ratio of 1:1 (F). Representative data from one of 3 independent experiments are shown.



similarly stimulated as expected, HIV was not detected (Figure 7-7B). When the CD8⁺ T cell-depleted PBMC were stimulated with anti-CD3, HIV p24 antigen was readily detected, and p24 production was modestly increased by thalidomide at 10 µg/ml (Figure 7-7C). However, when purified CD4⁺ T cells were similarly stimulated, thalidomide treatment at either 1 or 10 µg/ml caused a marked increase in HIV p24 antigen secretion (Figure 7-7D). When these cultures were reconstituted with CD8⁺ T cells, at CD4:CD8 ratios of 10:1 or 1:1, the enhancing effect of thalidomide on HIV replication was abrogated (Figures 7-7E and 7-7F, respectively). These results suggest that while thalidomide has the potential to stimulate increased HIV replication in activated CD4⁺ T cells, this effect is held in check in the presence of CD8⁺ T cells.

Discussion

In this study thalidomide stimulated antigen specific T cell immunity in advanced HIV/TB coinfecting patients as indicated by enhanced T cell proliferation in response to mycobacterial antigens (PPD). The immunostimulatory effect of thalidomide was also indicated by increases in plasma levels of some of the Th-1 type cytokines and cytokine receptors, including IFN-γ, IL-12, IL-2R and TNF-αR. In addition, increases in the numbers of CD4⁺ and CD8⁺ T cells and plasma sCD8 levels were noted. In *in vitro* studies of CD4⁺ T cells obtained from HIV-1 infected donors, thalidomide induced increased cell proliferation which was also antigen specific.

Because all the HIV infected patients also had documented tuberculosis, and were therefore exposed to *M. tuberculosis* antigens, we had a unique opportunity to evaluate the potential of thalidomide to stimulate antigen-specific T cell responses. Optimal T cell activation resulting in T cell proliferation and cytokine production requires two signals: i) primary T cell receptor stimulus delivered by antigen loaded APCs and ii) a secondary co-stimulatory signal [Mueller DL, Jenkins MK and Schwartz RH, 1989]. We have previously shown that thalidomide has an *in vitro* co-stimulatory effect on purified primary human T cells which are receiving simultaneous stimulation via the T cell receptor using a monoclonal antibody to CD3 [Haslett PAJ, Corral LG, Albert M, et al., 1998]. Exposure of the cells to thalidomide *in vitro* resulted in increased IL-2 mediated T cell proliferation and production of IFN-γ, a Th1 type cytokine. Here we show that the

immune stimulatory effects of the drug in HIV-infected patients *in vivo*, is antigen (PPD) specific (Figure 7-5). This is consistent with our observation that more patients converted to PPD skin test positivity (>5 mm of induration) in the thalidomide treated group compared with the placebo treated group. Our *in vitro* experiments reported here indicate that the drug can act directly on CD4⁺ T cells from HIV infected individuals to co-stimulate antigen specific T cell responses (Figure 7-6).

Studies *in vitro* have shown that thalidomide treatment inhibits IL-12 production by LPS-stimulated monocytes (T cell independent) but that the drug stimulates the production of IL-12 in T cell dependent systems [Moller DR, Wysocka M, Greenlea BM, et al., 1997; Corral LG, Haslett PAJ, Muller GW, et al., 1999; Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999]. Thus, the effect of the drug on production of IL-12 varies according to the stimulus and the target cell type. IL-12 is a central regulatory cytokine in the cellular immune response. The cytokine stimulates Th1 type T cell activation [Trinchieri G, 1998] and T cell activation in turn stimulates the production of monocyte IL-12, completing a positive feedback loop. T cell dependent stimulation of IL-12 production requires the interaction of CD40 on antigen presenting cells (monocytes and dendritic cells) with CD40L, expressed on the surface of activated T cells [Kennedy MK, Picha KS, Fanslow WC, et al., 1996]. In the present study, T cell activation markers were elevated before increases in plasma IL-12 levels were noted, suggesting that IL-12 production occurred as a consequence of thalidomide-induced T cell activation. In previous studies, we observed that thalidomide costimulation induced increases in CD40L expression on T cells isolated from HIV-infected and uninfected donors [Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999; Corral LG, Haslett PAJ, Muller GW, et al., 1999]. These observations, taken together, suggest that in the patients studied here thalidomide treatment may be activating T cells to upregulate CD40L which in turn stimulates increased antigen presenting cell (dendritic cells and monocyte) IL-12 production (T-cell dependent). However, since CD40L expression on peripheral blood T cells was not measured in this study, a conclusive understanding of the mechanism of *in vivo* augmentation of IL-12 production must await further patient studies.

The thalidomide-induced increase in endogenous IL-12 production may be important in controlling both tuberculosis and HIV infection. *In vivo*, an intact IL-12

signaling pathway is required to generate protective immunity to tuberculosis. Indeed, disseminated TB has been reported in a patient with a genetic deficiency in the IL-12 receptor [de Jong R, Altare F, Haagen IA, et al., 1998]. In addition, HIV infection is accompanied by deficiencies in the production of IL-12 [Chehimi J, Starr SE, Frank I, et al., 1994]. Interestingly, *in vitro*, IL-12 production is restored in cells from HIV patients by IFN- γ together with CD40 ligand and *in vitro* IL-12 restores HIV specific cellular immunity and enhances HIV specific CTL responses [Chougnat C, Thomas E, Landay AL, et al., 1998; Clerici M, Lucey DR, Berzofsky JA, et al., 1993; Wilson CC, Olson WC, Tuting T, et al., 1999]. Finally, local mucosal delivery of rIL-12, together with a vaccine, increases CD8⁺ CTL responses in a murine vaccine model [Belyakov IM, Ahlers JD, Brandwein BY, et al., 1998]. The use of recombinant IL-12 therapy in HIV infection has been advocated as an immunostimulatory intervention but is limited by the toxic effects following the administration of the recombinant cytokine [Ryffel B, 1996]. Thus, an intervention such as thalidomide which stimulates endogenous IL-12 production may be a less toxic therapeutic approach. Indeed, there is an anecdotal report of thalidomide treatment-induced clinical improvement of drug resistant *M. avium* infection in a patient with advanced HIV disease [Gori A, Franzetti F, Marchetti G, et al., 1998].

In association with the observed thalidomide induced immune stimulation in these patients we would have expected an increase in viral load. Thalidomide treatment of HIV infected patients has been reported in the past to induce an increase in HIV levels [Jacobson JM, Greenspan JS, Spritzler J, et al., 1997]. However, this was not observed here. In HIV infection, CD8⁺ T cells have been reported to reduce replication of virus in CD4⁺ cells by a variety of mechanisms, including CTL activity and chemokine production [Kinter AL, Bende SM, Hardy EC, et al., 1995]. We suggest that the increased number and/or activation of CD8⁺ T cells noted in our thalidomide treated patients may have downregulated any viral production by thalidomide stimulated CD4⁺ T cells by an as yet undefined mechanism. Our *in vitro* studies (Figure 7-7) indicate that this may indeed be the case.

The increase in CD8⁺ T cells in the thalidomide treated patients may be important also in combating the concomitant TB infection since CD8⁺ T cells have been shown to

contribute to the immune response to tuberculosis infection [Stenger S and Modlin RL, 1999].

In these studies, as well as in one of our previous studies, we note that thalidomide treatment of HIV infected patients did not reduce plasma TNF- α levels [Haslett PAJ, Klausner JD, Makonkawkeyoon S, et al., 1999]. These results contrast with those of some previous studies, in which thalidomide treatment induced a reduction in plasma TNF- α levels in patients with leprosy or with tuberculosis [Sampaio EP, Kaplan G, Miranda A, et al., 1993; Tramontana JM, Utaipat U, Molloy A, et al., 1995]. In another study of HIV/TB patients, a decrease in plasma TNF- α levels was observed following 21 days of thalidomide treatment only in those individuals with the highest levels of TNF- α [Klausner JD, Makonkawkeyoon S, Akarasewi P, et al., 1996]. Our present observations may be explained by the differential effects of thalidomide on monocyte and T cell TNF- α production [Haslett PAJ, Corral LG, Albert M, et al., 1998; Corral LG, Haslett PAJ, Muller GW, et al., 1999]. As already mentioned, thalidomide has been shown to inhibit TNF- α production by LPS stimulated monocytes. However, it is important to note that the drug fails to inhibit TNF- α production by activated T cells [Haslett PAJ, Corral LG, Albert M, et al., 1998]. These findings suggest that in the present study, at least some of the TNF- α detected in the plasma of the HIV/TB patients may be T cell derived and therefore not inhibited by thalidomide. The transient increase in TNF- α levels seen at day 21 of treatment may be attributed to the antituberculosis therapy (see Chapter 3 of this thesis). In the thalidomide treated group, the drug may have augmented this increase, since thalidomide activates T cells.

In conclusion, our study has provided *in vivo* data showing that thalidomide treatment can enhance antigen-specific immunity. This finding suggests that thalidomide may have a hitherto unappreciated potential as an immunologic adjuvant. However, because the effect wanes after removal of the drug, long term treatment may be required. For example, to boost an anti-tuberculous effect the drug may need to be used for the duration of TB therapy.

Chapter 8. CONCLUDING REMARKS

The studies described in this thesis combine laboratory based research on the mechanisms of immunity to *M. tuberculosis* infection with studies of patient responses to experimental immunomodulatory interventions. In the first patient based study we observed that upon initiation of anti-tuberculous therapy, the clinical status of the patients underwent transient deterioration concomitant with clearance of the infection. The clinical deterioration was associated with increases in the levels of TNF- α measured in the plasma of the patients. We hypothesized that the production of TNF- α was stimulated by mycobacterial products released during killing of the organisms by antibiotics. We further hypothesized that TNF- α , whilst an important component of protective immunity, was also contributing to the increased morbidity seen in these patients.

To test this hypothesis we used murine infection models. *In vivo* studies in mice demonstrated that killing of the organisms by antibiotics can induce increased expression of TNF- α in the infected tissues (the lung). Also we showed that the presence of dead organisms led to increased production of TNF- α , resulting in pathological worsening expressed as larger lung granulomas. In the next set of studies, by directly regulating the amount of TNF- α in the tissues we demonstrated that the cytokine had both a protective role and a pathologic role. TNF- α induced clearance of the infection. However at high concentrations of the cytokine, in spite of clearance of the organisms, lung pathology was exacerbated and survival of the mice was compromised.

We further investigated the role of TNF- α in the protection against infection using *in vitro* murine infection models. We showed that this cytokine induces control of mycobacterial growth and killing in macrophages *in vitro*. Killing of the intracellular organisms appeared to be mediated via at least 2 mechanisms: 1) an iNOS dependent pathway and 2) a TNF- α dependent pathway which appears to be iNOS independent. These studies suggested that stimulation of host immunity must be carried out in a rational and controlled way since activation of the immune response (TNF- α production) can be both protective and detrimental.

In our next patient study we demonstrated that it is possible to modify the immune response in patients safely. Using thalidomide as the immune modulatory intervention, antimycobacterial immunity was enhanced in patients with active tuberculosis and HIV

infection as evaluated by increases in T cell proliferative responses *in vitro* to PPD. This immune stimulation was observed in the absence of thalidomide associated toxicities and in the absence of increases in viral titre.

Taken together the studies presented in this thesis demonstrate that an understanding of host immunity in tuberculosis is critical for rational design of immunomodulatory interventions that may help the host immune system to control the infection more efficiently. Such insights are also useful for the design and evaluation of new vaccines which would be used to stimulate the particular immune responses appropriate for protecting individuals from *M. tuberculosis* infection and/or from developing active disease.

This combination of *in vitro* studies, animal studies and patient based studies illustrates the power of translational research. Observations from small patient studies can be carried back to the laboratory, studied in detail with appropriate controls in model systems, and then the insights gained can be applied to design of new patient interventions. In a country like South Africa, where so many suffer from tuberculosis, this multi-faceted approach to research will be increasingly important.

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List of Figures and Tables

	Page
Figure 1-1	South Africa as a health resort for tuberculosis..... 6
Figure 1-2	Cells and cytokines involved in the immune response to mycobacterial infection..... 9
Figure 1-3	Signalling pathways for TNF- α receptor and toll-like receptor..... 19
Figure 2-1	Photograph of the colony forming unit assay..... 29
Figure 3-1	Effect of anti-tuberculosis therapy on patient responses..... 44
Figure 3-2	Effect of anti-tuberculosis therapy on cytokine and cytokine receptor levels in plasma..... 45
Figure 4-1	Effect of anti-tuberculous therapy on murine mycobacterial infection and cytokine response in the lungs..... 50
Figure 4-2	Effect of heat killed <i>M. tuberculosis</i> on the bacillary load in the tissues of infected mice..... 51
Figure 4-3	Effect of heat killed <i>M. tuberculosis</i> on cytokine mRNA levels in the lungs of infected mice..... 53
Figure 4-4	Effect of heat killed <i>M. tuberculosis</i> on the granulomatous response in the lungs of infected mice..... 54
Figure 5-1	Effect of TNF- α on the bacillary load in the tissues of infected mice..... 59
Figure 5-2	Morphology of the lungs of mice infected with low dose recombinant BCG..... 61
Figure 5-3	Morphology of the livers of mice infected with low dose recombinant BCG..... 62
Figure 5-4	Morphology of the lungs of mice infected with high dose recombinant BCG..... 64
Figure 5-5	Morphology of the livers of mice infected with high dose recombinant BCG..... 66
Figure 5-6	Effect of TNF- α on the weight of spleens of mice..... 67
Figure 5-7	Effect of TNF- α on survival of TNF-KO mice infected with recombinant BCG..... 69

	Page
Figure 5-8	Effect of TNF- α on survival of TNFR1-KO mice infected with recombinant BCG..... 70
Figure 6-1	Morphology of macrophages infected with recombinant BCG <i>in vitro</i> 77
Figure 6-2	Effect of TNF- α on the survival and growth of recombinant BCG in macrophages <i>in vitro</i> 78
Figure 6-3	Effect of TNF- α on protein expression in peritoneal macrophages infected with recombinant BCG <i>in vitro</i> 80
Figure 6-4	Effect of AMG on nitrite production by peritoneal macrophages infected with recombinant BCG <i>in vitro</i> 82
Figure 6-5	Effect of AMG on survival and growth of recombinant BCG in peritoneal macrophages <i>in vitro</i> 84
Figure 7-1	Effect of thalidomide treatment on patient weight, body mass index and triceps skin fold thickness..... 96
Figure 7-2	Effect of thalidomide treatment on plasma HIV-1 viral load..... 98
Figure 7-3	Effect of thalidomide treatment on T cells..... 99
Figure 7-4	Effect of thalidomide treatment on plasma cytokine and cytokine receptor levels..... 102
Figure 7-5	Effect of thalidomide on lymphocyte proliferative response..... 103
Figure 7-6	Effect of thalidomide on T cell function <i>in vitro</i> 105
Figure 7-7	Effect of thalidomide on HIV replication in PBMC and T cells..... 106
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Table 3-1	Patient demographics, clinical status and response to treatment..... 42
Table 4-1	Granulomatous response to infection in the lung..... 52
Table 5-1	Cytokine mRNA levels in infected lungs..... 71
Table 6-1	The role of TNF- α , nitrites and ROI in mycobacterial growth and survival..... 85
Table 7-1	Patient characteristics at baseline..... 93
Table 7-2	Patient adverse events..... 94
Table 7-3	Plasma levels of TNF- α , IFN- γ and IL-6..... 100

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