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by

Ivy M. Dambuza

Investigating transmembrane TNF and transmembrane p55TNFR mediated signaling in host immune function during *Mycobacterium tuberculosis* infection

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By

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Thesis presented for the Degree of Doctor of Philosophy

Division of Immunology

Department of Clinical and Laboratory Sciences

Faculty of Health Sciences

University of Cape Town

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Declaration

I,.....

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Dedication

To Regina & Abram Dambuza,
Mom and Dad, I know what you have done for me. I am eternally grateful…

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All thanks to the Alpha and Omega, Who made everything possible. To my family, thank you for your support and encouragements, your talents and passion inspire me to always want to do better. To Weziwe Seme, thank you for the words of wisdom and for always being there..

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Publications

The following published articles are directly associated with the thesis as well as experimental work performed outside the context of its philosophy:

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Index

Abbreviations

2-ME	Mercaptoethanol
AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cells
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
CFUs	Colony forming units
CO_2	Carbon dioxide
DAB	3,3 diaminobenzidene tetrahydrochloride
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ESAT-6	Early secretory antigenic target
FACS	Fluorescent activated cell sorting
FCS	Fecal calf serum
h	hour
H. capsulatum	*Histoplasma capsulatum*
H_2O	Water
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IL	Interleukin
INH	Isoniazid
L. monocytogenes	*Listeria monocytogenes*
LPS	Lipopolysaccharide
LT	Lymphotoxin
M. bovis BCG	*Mycobacterium bovis* bacillus Calmette-Guérin
M. smegmatis	*Mycobacterium smegmatis*
MFI	Mean fluorescence intensity
MHC-II	Major histocompatibility complex class II
min	Minute
MOI	Multiplicity of infection
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ND	Not detected
OADC	Oleic acid albumin dextrose catalase
PBS	Phosphate buffered saline
PPD	Purified protein derivative
RD 1	Region of deletion
RIF	Rifampicin
RPMI	Roswell Park Memorial Institute Medium
SD	Standard deviation
sec	Second
T. brucei	*Trypanosoma brucei*
Th	T helper
TRAPS	TNF receptor-associated periodic fever syndrome
WHO	World health organization

Abstract

The importance of TNF-TNFR signaling in immunity against *M. tuberculosis* has been established.

The aims of this study were to characterize the functions of membrane-bound TNF (Tm-TNF) and soluble TNF (solTNF) and to investigate the role of membrane-bound p55TNFR signaling as well as the *in vivo* significance of TNFR shedding in host immune responses during infection with *M. tuberculosis* H37Rv. To address this, mice expressing only the membrane-bound TNF or membrane-bound p55TNFR were exposed to a low dose of *M. tuberculosis* H37Rv by aerosol inhalation infection. The results presented in this dissertation illustrate that Tm-TNF mice were able to control acute *M. tuberculosis* infection but succumbed to chronic exposure to *M. tuberculosis* with pneumonia. We demonstrate that Tm-TNF mice displayed heightened pulmonary macrophage activation reflected by enhanced cell surface expression of MHC-II, CD80 and CD86 as well as enlargement of granulomas. Furthermore, our results show that solTNF has a regulatory function that modulates the magnitude of Th1 immune responses during acute and chronic stages of the infection.

The evaluation of the functions of Tm-TNF and solTNF in host immune function in the presence of an established mycobacteria-specific immune response was carried out using a _drug-based' *M. tuberculosis* reactivation model. Here, mice that were challenged with a low dose of *M. tuberculosis* were exposed to INH-RIF treatment for six weeks in drinking water, after which therapy was withdrawn and immune responses during reactivation were analyzed. Our results demonstrate that complete absence of TNF resulted in host susceptibility to recrudescence tuberculosis in the presence of a mycobacteria-specific immune response. TNF deficient mice were unable to suppress bacilli growth and formed diffused granulomas and succumbed early to reappearing tuberculosis compared to WT mice. By contrast, we show that Tm-TNF was sufficient for containment of reappearing mycobacterial growth and sustaining immune pressure in a manner comparable to WT control mice.

Lastly, the analysis of host immune responses in mice expressing a non-sheddable p55TNFR revealed that persistent p55TNFR cell surface expression does not afford better protection to low dose *M. tuberculosis* infection. However, we observed a transient elevation in the frequency of pulmonary $CD11b^{+}$/MHC-II^{+} cells in mice expressing a non-sheddable p55TNFR relative to WT mice as well as reduced cell surface expression of CD44 on $CD4^{+}$ T cells. We also found that pulmonary IL-12p70 and TNF concentrations were elevated whereas IFNγ levels were reduced in mice expressing a non-sheddable p55TNFR relative to WT mice. Furthermore, data presented here describe the *in vivo* functional significance of p75TNFR shedding. We demonstrate using a double mutant mouse strain that in the absence of p75TNFR, mice expressing a non-sheddable p55TNFR display enhanced ability to control *M. tuberculosis* infection.

1 General introduction

i Tuberculosis, epidemiology and immune responses

Tuberculosis

"Since the time people have realized tuberculosis is preventable and since they have learned how to avoid the infection, mortality rates caused by tuberculosis have declined in industrialized countries and signs are starting to appear that it can be eliminated. This is the right time to combat tuberculosis." Robert Kock, 1882.

Tuberculosis is still a major global health issue and continues to claim the lives of both young and adult humans faster than any other single disease causing organism. The tubercle bacilli have evolved ways to circumvent destruction and defiantly persist in the face of hostile innate and adaptive host immune defense mechanisms. In this regard, in some immunocompetent humans, the bacteria induce a lung pathology which ingeniously allows for transmission of bacteria by aerosol from one person to another. Lung pathology is initiated at sites of bacterial implantation with attraction and accumulation of macrophages and lymphocytes which multiply to form compact granulomas that contain the bacteria (Saunders and Cooper, 2000). In some humans, the granuloma structure can result in cavities with central caseous necrosis (Fayyazi et al., 2000). The key feature of granuloma formation is the development of fibrosis within the granuloma and the surrounding lung parenchyma giving rise to the tubercle nodules. Depending on the metabolic status of the bacteria, the disease advances as a necrotizing pneumonic process that can involve bronchioles and result in the spreading of infection to other areas of the lung.

The pathogen identified to be the causative agent of tuberculosis is *Mycobacterium tuberculosis* (*M. tuberculosis*) was discovered in 1882 by Robert Kock. Classical characteristics of *M. tuberculosis* include that it is a gram-positive, slow growing, facultative intracellular pathogen (Manganelli et al., 1999). It shares with other members of the *Mycobacterium* genus a unique waxy cell wall of which the majority of the constituents are mycolic acids which make up more than 50% of the bacteria's dry weight (Brennan and Nikaido, 1995). This allows the bacteria to retain basic dyes in the presence of acid alcohols, hence their acid fastness (Allen, 1992).

Another characteristic feature of *M. tuberculosis* is the ability to remain latent in infected hosts. Of those who are infected, 90% do not develop active disease (WHO report, 2009). These individuals represent a large reservoir of *M. tuberculosis* with the potential for reactivation and driving the epidemic. Furthermore, factors such as the increase of the world's poor, the menace of HIV/AIDS, emigration due to political or economic instabilities, and global interdependency are set to increase tuberculosis cases globally for the foreseeable future. Epidemiologists' estimate that 2 billion of the world's population is infected with *M. tuberculosis,* and 9.27 million new tuberculosis cases occurred in 2007 alone (WHO report, 2009). According to the global distribution pattern, the majority of high tuberculosis incidences (Fig. 1) are restricted to developing countries and this phenomenon is associated with high incidence of human immunodeficiency virus (HIV) confection (Fig. 2).

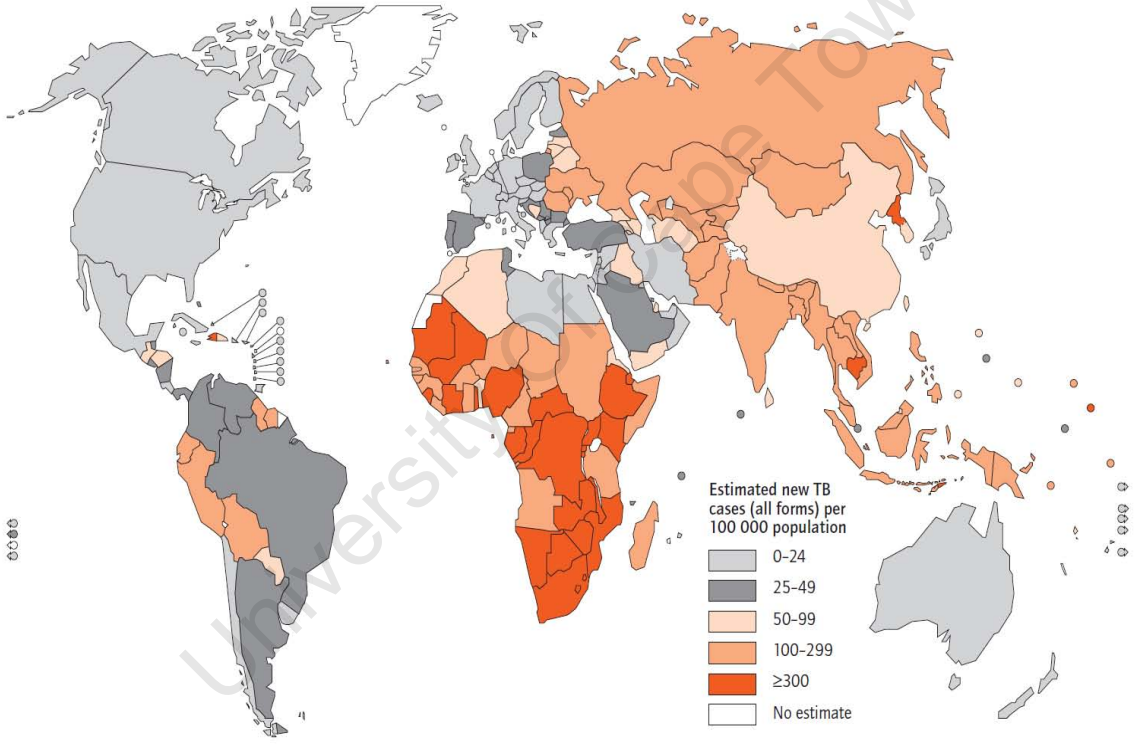


Figure 1*. *The global distribution pattern of estimated tuberculosis incidences in 2007 shows that the developing countries are highly afflicted. (The map was adapted from WHO Report 2009).

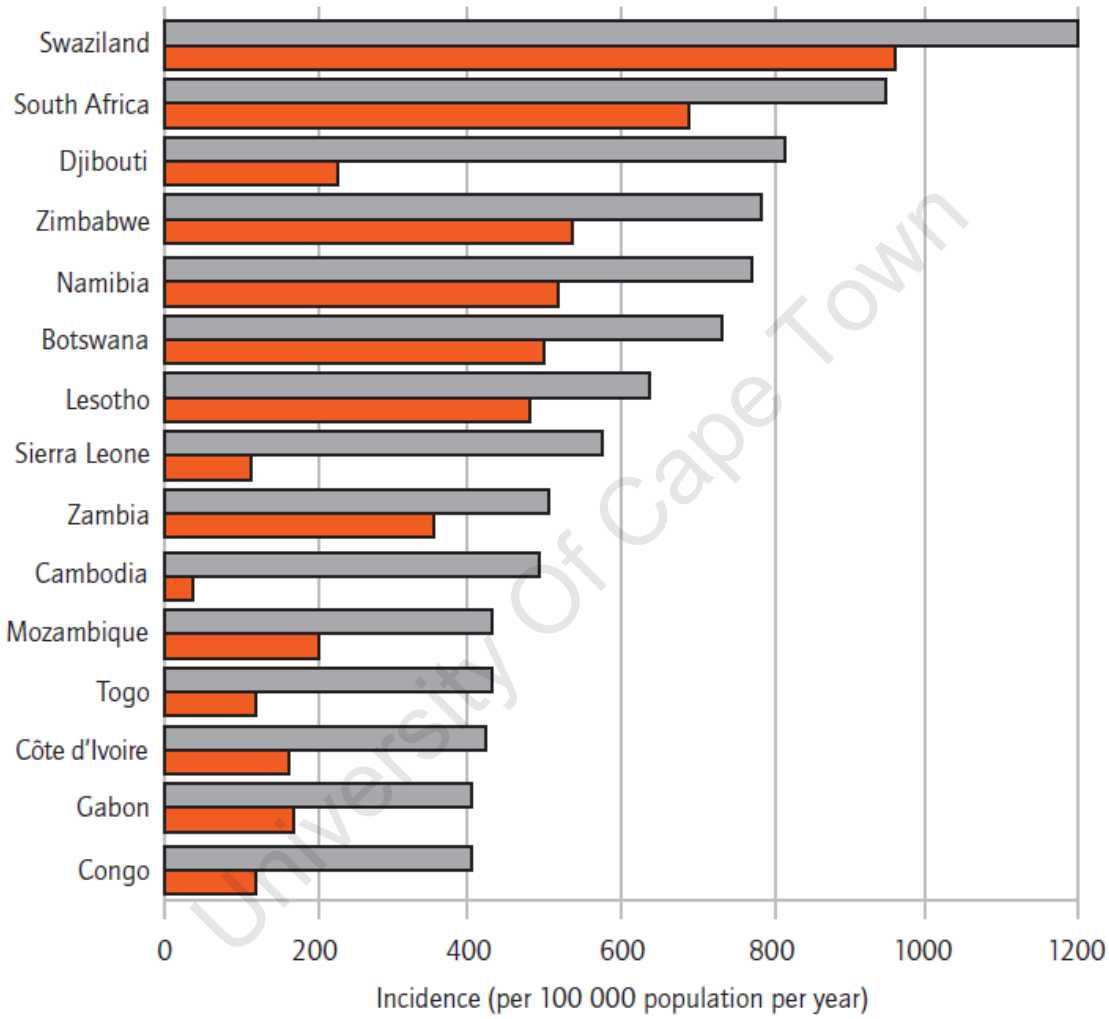


Figure 2. ***Of the 15 countries with the highest estimated tuberculosis incidence rates per capita in 2007, 13 are in Africa***. (Grey bars indicate tuberculosis in all ages and the blue bars indicate the corresponding co-infection with HIV among adults aged 15-49 years). Bar graph was adapted from WHO Report 2009.

Experimental tuberculosis

Most of what is known about the *in vivo* host response to *M. tuberculosis* infection comes from the use of animal models of the disease, mostly mice. This animal host has also proved valuable in investigating aspects of host-pathogen relationships such as the molecular basis of *M. tuberculosis* virulence, a feature that can be investigated only in terms of environmental changes induced in the host. The availability of inbred strains that differ from one another with respect to the degree of resistance to tuberculosis, the availability of genetically altered mutant mouse strains and the fact that mice are much less expensive to purchase and to maintain, make them attractive tuberculosis animal models compared with other host species.

Pattern of infection in mice

The growth of *M. tuberculosis* in mice is well characterised. Mouse strains resistant to challenge with *M. tuberculosis* are able to control lung infection. In resistant C57BL/6 and BALB/c strains (Medina and North, 1996; North et al., 1999; North and Medina, 1998), infection via the respiratory route with 100 or fewer *M. tuberculosis* bacilli results in exponential bacterial replication in the lungs reaching up to 6.5 logs by day 20 post-infection. The onset of mycobacteria specific adaptive immunity results in inhibition of further bacterial growth which give rise to an approximate stationary phase of the infection that persists from day 20 until the mice succumb to infection by day 250. Smith and colleagues (Schell et al., 1974) first described the description of this pattern of lung infection in mice more than 30 years ago. The infection is however not confined to the lungs but is disseminated to other organs via lymph and blood (Chackerian et al., 2002). The bacilli can be recovered from livers and spleens by day 15 post-infection and bacilli growth progresses until day 20 and, when systemic immunity is expressed, further bacterial growth ceases and the stationary phase of the infection ensues (Mogues et al.,

2001). McCune and Tompsett (1956) described the events of mice infected via the intravenous route nearly 50 years ago and revealed that most of the bacilli implants in liver (95%) and spleen (about 4%) and 0.1% implants in the lung after mice are inoculated via the iv route. Interestingly, in the lung, the tubercle bacilli replicate progressively for 20 days compared to 10 days in the liver and spleen before station phase of the infection is established (Medina and North, 1996).

Progression of infection in *M. tuberculosis* susceptible mouse strains is different. Infection of susceptible strains such as DBA/2, C3H, CBA, 129Sv (Medina and North, 1996; Medina and North, 1998; Mitsos et al., 2003) via the respiratory route or the intravenous route result in rapid bacterial growth in the lungs for the first 20 days as in resistant mouse strains but the established chronic phase in these mice is unstable as a result they succumb to infection with a mean survival time of 100 - 150 days. In addition, studies by Gruppo et al., 2002, revealed that susceptibility of these mouse strains was associated with reduced and delayed trafficking of T lymphocytes in the lung which correlated with a poor upregulation of adhesion and integrin molecules necessary for populating the lung.

Immunity to tuberculosis

That immunity to *M. tuberculosis* and other facultative intracellular bacteria is cell mediated was proposed by Mackaness in the 1960s. He saw lymphocytes as the cells that specifically mediate immunity and the macrophages as the cells that indiscriminately express it (Mackaness, 1969). Based on his analyses, before macrophages can express immunity, they need to be activated into assassin mode, and it is the function of antigen-specific lymphocytes that give macrophages the licence to kill the invading bacteria. It was later established in the 1970s that antigen-specific T cells achieved this by secreting humoral factors, known later as lymphokines.

Cellular immunity

There is extensive evidence to support a central role for a type 1 (Th1) immune response in mice and humans for the generation of a protective immune response against *M. tuberculosis* infection. Many components of the immune system appear to be necessary for induction of a protective response, which include T cells, cytokines such as gamma interferon (IFNγ) and tumor necrosis factor (TNF), and macrophage activation. The macrophage activating and Th1 polarizing phenotype is favoured over an antibody-dominated Th2 response by the local release of interleukin-12 (IL-12) from infected cells (Ladel et al., 1997). Studies from mouse models and evidence obtained from individuals with susceptibility to disseminated infection with *M. bovis* BCG and other weakly pathogenic mycobacteria identifies the IL-12-IFNγ loop to be crucial for protection against infection (Altare et al., 1998; Cooper et al., 1993; Cooper et al., 1997; Flynn et al., 1993; Jouanguy et al., 1996; Newport et al., 1996).

CD4$^+$ T cells

Within the infected macrophage, *M. tuberculosis* resides primarily in a phagosome resulting in MHC Class II presentation of mycobacterial antigens to CD4$^+$ T cells. Several studies have shown that the primary effector function of CD4$^+$ T cells in protection against tuberculosis is the production of IFNγ that is essential for activating macrophages (Cooper et al., 1993; Flynn et al., 1993; Orme et al., 1993). Mice deficient in CD4$^+$ T cells had diminished ability to control infection indicating the importance of this subset in protective immunity against *M. tuberculosis* (Caruso et al., 1999; Tascon et al., 1998). Furthermore, adoptive transfer of CD4$^+$ T cells enhanced protection against tuberculosis in mice (Orme et al., 1987). Moreover, in human hosts, HIV$^+$PPD$^+$ individuals revealed that loss of CD4$^+$ T cells greatly enhanced the susceptibility to acute and reactivation tuberculosis (Selwyn et al., 1989). Other possible roles of CD4$^+$ T cells have been suggested, these include mediating apoptosis, which is thought to play a role in *M. tuberculosis* infection, conditioning of antigen-presenting cells, providing help for B cells and CD8$^+$ T cells, and production of other relevant cytokines (Balcewicz-Sablinska et al., 1998; Keane et al., 1997).

CD8$^+$ T cells

CD8$^+$ T cells recognize antigens derived from the cytoplasm of infected cells presented by the MHC Class I molecules. Although *M. tuberculosis* resides predominantly inside vacuoles of infected cells, reports have suggested that the tubercle bacilli may gain access to the cytoplasm via pore formation in the vacuole membrane (Teitelbaum et al., 1999). Several studies in mice supported a role for CD8$^+$ T cells in protection against *M.*

tuberculosis (Flynn et al., 1992; Orme et al., 1987; Rolph et al., 2001; van Pinxteren et al., 2000). Although mycobacteria specific cytotoxic $CD8^+$ T cells have not been isolated from mice or humans, a $CD8^+$ T cell clone isolated from mice vaccinated with mammalian cells expressing mycobacterial 65kDa antigen, was able to transfer protection and was cytotoxic against infected macrophages *in vitro* (Silva et al., 1994).

$CD8^+$ T cells also recognise antigens presented by Class 1b molecules. These are non-polymorphic and include the CD1 molecules (Porcelli and Modlin, 1999) as well H2-M3 (Chiu et al., 1999) and present mycobacterial lipids and glycolipids vastly expanding the repertoire of possible antigen pool.

Effector functions of $CD8^+$ T cells include amongst others, production of IFNγ and TNF (Caruso et al., 1999; Scanga et al., 2000) as well as lysis of infected cells. The lysis of target cells was shown to be mediated by production of perforin and granzymes or via the Fas/FasL pathway (Stenger et al., 1998). Using CD8 deficient mice, CD95 and CD95L deficient mice, Turner and colleagues (2001) showed that CD8 T cell mediated immunity was not required during early stages of the infection, but was important during chronic *M. tuberculosis* infection. Moreover, in the same study, the authors also revealed that perforin gene-disrupted mice were not more susceptible to chronic infection that WT control mice. In humans, $CD8^+$ T cells also produce granulysin, which was shown to be directly toxic to *M. tuberculosis* representing another effector mechanism by which $CD8^+$ T cells contribute to the killing of the tubercle bacilli within cells (Stenger et al., 1998; Stenger et al., 1997).

γδ T cells

Non-protein components of mycobacteria such as isopentenyl pyrophosphate and prenyl pyrophosphate were shown to activate a further set of T cells carrying antigen-specific receptor made up of the γ and δ chains (Constant et al., 1994). Even though the role of γδ T cells in immunity has not been fully described, disruption of T cell receptor (TCR) δ genes proved to have detrimental effects in mice challenged with *M. tuberculosis* (D'Souza et al., 1997). Here, IFNγ production and lysis of target cells also appeared to be contributory effectors in tuberculosis immunity afforded by γδ T cells (Tsukaguchi et al., 1995).

Macrophages as the effectors of Th1 immunity

The macrophage is central to the control of *M. tuberculosis* infection. Tubercle bacilli multiply in resting macrophages, but activation of these infected macrophages with IFNγ resulted in induction of a mycobacteriostatic or mycobactericidal state *in vitro* (Flesch and Kaufmann, 1987). Tumor necrosis factor and IFNγ were shown to collaborate to induce production of nitric oxide (NO) and related reactive nitrogen intermediates (RNIs), which represent one of the antimicrobial defence mechanism launched by activated macrophages (Ding et al., 1988). NO is generated from L-arginine via the enzymatic action of the inducible isoform of nitric oxide synthase (NOS2) (Ding et al., 1988) and its importance in protective immunity was confirmed in studies where mice deficient of NOS2 activity were shown to be susceptible to *M. tuberculosis* infection (Chan et al., 1995; Flynn et al., 1998; MacMicking et al., 1997; Scanga et al., 2001). The relevance of this mechanism in human infection remain controversial, however, there are

reports of NOS2 induction and/or RNI production by human macrophages (Bonecini-Almeida et al., 1998; Nicholson et al., 1996; Rockett et al., 1998). In addition, production of reactive oxygen, which is generated by the transfer of an electron from NADPH to molecular oxygen by the action of NADPH-oxidase is required for antimicrobial defence. Studies with mice incapable of expressing NADPH-oxidase were found to be susceptible to *M. tuberculosis* compared to WT mice (Cooper et al., 2000).

Why does immunity not achieve sterility to the infection?

Deficiency in Th1 immunity versus macrophage function:

A puzzling question in tuberculosis research is explaining why immunity to *M. tuberculosis* is not sufficient to resolve lung infection in mice, guinea pigs, rabbits, or susceptible humans to stop the development of disease. Macrophages as the cells that express immunity directed by mycobacteria-specific T cells have been implicated as one the reasons for failure of mice and other hosts to resolve the infection. This might be due to innate defects that prevent macrophages from expressing anti-mycobacterial functions or, alternatively, to an inability of the host to mount a sufficient Th1 macrophage activating response. Also not known is whether macrophages would acquire improved mycobactericidal abilities that would resolve the infection if the number of mycobacteria-specific Th1 cells were to be substantially increased.

Other researchers have suggested that a Th1 immune response is not capable of resolving the infection because of the immunoregulating effects of Th2 immunity (Lin et al., 1996). However, studies conducted in mice incapable of mounting a Th2 response did not

demonstrate an improved ability to resolve *M. tuberculosis* infection in comparison to WT mice (Hernandez-Pando et al., 1996). Likewise, the anti-inflammatory cytokine IL-10, produced by macrophages and T cells during the *M. tuberculosis* infection and known to posses macrophage deactivating properties including downmodulation of IL-12 which in turn decreases IFNγ production by T cells, was found not be responsible for failure of immunity to resolve infection. This was demonstrated in IL-10 gene-deficient mice, which displayed similar *M. tuberculosis* growth kinetics to WT control mice (North, 1998). Another potent immunoregulator, transforming growth factor (TGF)-β, has been implicated in suppression of T cell responses in tuberculosis patients (Hirsch et al., 1997) and was shown to participate in macrophage deactivation by inhibiting IFNγ-induced NOS2 production (Ding et al., 1990). TGF-β can be detected in granulomas of tuberculosis patients and its transcription was enhanced in human monocytes after stimulation with *M. tuberculosis,* PPD (Toossi et al., 1995), or lipoarabinomannan (Dahl et al., 1996). Although the *in vivo* role of TGF-β in resistance or susceptibility in tuberculosis has not been directly tested, neutralization of TGF-β activity resulted in enhanced bacterial control in monocytes infected with *M. tuberculosis* (Toossi et al., 1995).

Breaking the resolve: Immune evasion

The ability of *M. tuberculosis* to evade the immune response should be considered a key factor influencing resolution of infection. The ability of *M. tuberculosis* bacilli to persist within macrophages, whose primary function is to kill invading microbes suggest the evolution of various mechanisms by *M. tuberculosis* to evade RNI toxicity; one of the

anti-mycobacterial mechanisms of activated macrophages (Chan et al., 1995; MacMicking et al., 1997). Studies by Chen and colleagues (1998), showed that *M. tuberculosis ahpC,* which encodes the peroxiredoxin alkyl hydroperoxide reductase subunit C (AhpC), protects *Salmonella typhimurium* and mammalian cells from RNI toxic effects. AhpC was shown to catalytically catabolize peroxynitrite anion ($ONOO^-$) which is a potent oxidant formed by the reaction between NO and superoxide oxide (O^-) produced by activated macrophages (Bryk et al., 2000). In addition, *M. tuberculosis* AhpC peroxiredoxin in concert with mycobacterial dihydrolipoamide dehydrogenase (Lpd), dihydrolipoamide succinyltransferase (SucB), and thioredoxin-like AhpD, were reported to form an antioxidant complex with nicotinamide adenine dinucleotide (reduced)-dependent peroxidase and peroxynitrite reductase activity (Bryk et al., 2002).

Another means by which *M. tuberculosis* evades the immune system is achieved by interfering with the tethering and fusion machinery that is involved in the maturation of phagosomes into phagolysosomes (Desjardirns M, 1995; Viera OV, 2002). In addition, it is reported that once inside the phagosomes, bacilli are able to maintain an intraphagosomal pH environment that is suitable for its survival by way of excluding vacuolar H^+ ATPases (Sturgill-Koszycki S, 1994).

In other studies, *M. tuberculosis* was found to inhibit MHC II antigen processing and presentation. $CD4^+$ T cells' recognition of infected macrophages depend on MCH II presentation of mycobacterial antigens by macrophages (Mazzaccaro et al., 1996). Studies have shown that upregulation of MHC II by IFNγ was inhibited by *M.*

tuberculosis infection through intracellular sequestration of MHC II molecules (Hmama et al., 1998), decreasing the expression of the class II transactivator (CIIT) (Wojciechowski et al., 1999), or by inhibiting IFNγ signalling pathways in the macrophages (Ting et al., 1999). In addition, studies addressing antigen processing in the phagosomes showed that MHC II-mycobacterial peptide complexes are formed in the phagosomes. It was observed that phagosomes containing viable *M. tuberculosis* had fewer MHC II-mycobacteria peptide complexes compared to phagosomes containing heat-killed *M. tuberculosis,* suggesting that live bacilli inhibited the processing of antigens (Ramachandra et al., 2001).

In summary, there is extensive evidence to support a central role for a type 1 (Th1) immune response in mice and humans for the generation of a protective immune response against *M. tuberculosis* infection. Many components of the immune system appear to be necessary for induction of a protective response and these include T cells, cytokines such as IFNγ and TNF, and macrophage activation. A deeper understanding of the underlying molecular mechanisms in the complex network of these immune responses will help in designing of effective vaccines and better treatments for tuberculosis.

ii TNF, signaling and role in disease

Brief History

Tumor necrosis factor (TNF, TNFα) was discovered due its tumor necrotizing activities in mice (Carswell et al., 1975). Independently, a molecule termed ‗cachectin' associated with wasting and hypertriglyceridemic state in rabbits infected with *T. brucei* was investigated (Kawakami and Cerami, 1981; Pekala et al., 1983). In the mid 1980s, the amino acid sequence of human TNF was determined and it was found to be homologous to mouse cachectin thus establishing the identity of TNF and cachectin (Aggarwal et al., 1985; Nedwin et al., 1985). Although initially identified as a product of activated macrophages (Carswell et al., 1975), TNF is now known to be produced by many cell types including T cells (Steffen et al., 1988), B cells (Sung et al., 1988), mast cells (Gordon and Galli, 1990), keratinocytes (Kock et al., 1990), astrocytes and microglial cells (Sawada et al., 1989), smooth muscle cells (Warner and Libby, 1989), intestinal paneth cells (Keshav et al., 1990), and mesengial cells (Baud et al., 1989).

TNF and its receptors

TNF is a member of the TNF superfamily (TNFSF), which is a class of structurally related cytokines that are involved in diverse immunological and developmental pathways (Table 1) (Bodmer et al., 2002; Ware et al., 2003). TNF is initially synthesized as a 26kDa non-glycosylated type II transmembrane molecule (Tm-TNF) that can be released from the cell surface by the metalloprotease TNF-α-converting enzyme (TACE) to generate a 17kDa portion, and subsequent to homotrimerisation, a 51kDa soluble TNF (solTNF) molecule is formed (Black et al., 1997; Kriegler et al., 1988; Mueller et al., 1999; Perez et al., 1990). Both TNF forms are biologically active and the plethora of

TNF functions is mediated by two TNF receptors: p55TNFR (TNFRSF1A, CD120a, TNFR1) and p75TNFR (TNFRSF1B, CD120b, TNFR2) with Tm-TNF preferentially signaling through p75TNFR and solTNF binding strongly to p55TNFR (Grell et al., 1998). In addition, in its homotrimeric form, LTα (a ligand within the TNFSF) also interacts with TNFRs to elicit biological responses similar to that of TNF (Smith et al., 1994) while the heterotrimer, LTαLTβ complex interacts with LTβ-R (Crowe et al., 1994).

Table 1. TNF ligand superfamily (TNFSF). (Adapted from MacEwan 2002)

Ligands	*Alternative names*
TNF	cachectin, DIF, TNFA, TNFSF2
4-1BB ligand	4-1BBL, TNFSF9
APRIL	TALL2, TNFSF13
CD27 ligand	CD27L, CD70, TNFSF7
CD30 ligand	CD30L, TNFSF8
CD40 ligand	CD40L, CD154, GP39, HIGM1, IMD3, TNFSF5, TRAP
Fas ligand	APT1LG1, FasL, TNFSF6
GITR ligand	AITRL, GITRL, TL6, TNFSF18
LIGHT	HVEM ligand, TL1, TNFSF14
LTα	LT, TNFB, TNFSF1
LTβ	TNFC, TNFSF3, p33
OX40 ligand	gp34, OX40L, TNFSF4, TXGP1
RANK ligand	ODF, OPGL, RANKL, TNFSF11, TRANCE
THANK	BAFF, BLYS, TALL1, TNFSF13B
TRAIL	Apo2 ligand, TL2, TNFSF10
TWEAK	Apo3 ligand, DR3L, TNFSF12
VEG1	TL1, TNFSF15

TNFRs are type I transmembrane glycoproteins sharing 28% homology in their extracellular domains but have distinct cytoplasmic domains and need to recruit cytosolic intermediate proteins in order to transduce signals (Fig. 3). Both these receptors are regulated differently and display distinct expression patterns, with p55TNFR widely expressed in many cell types and, p75TNFR expressed mainly in hematopoietic cells (Santee and Owen-Schaub, 1996).

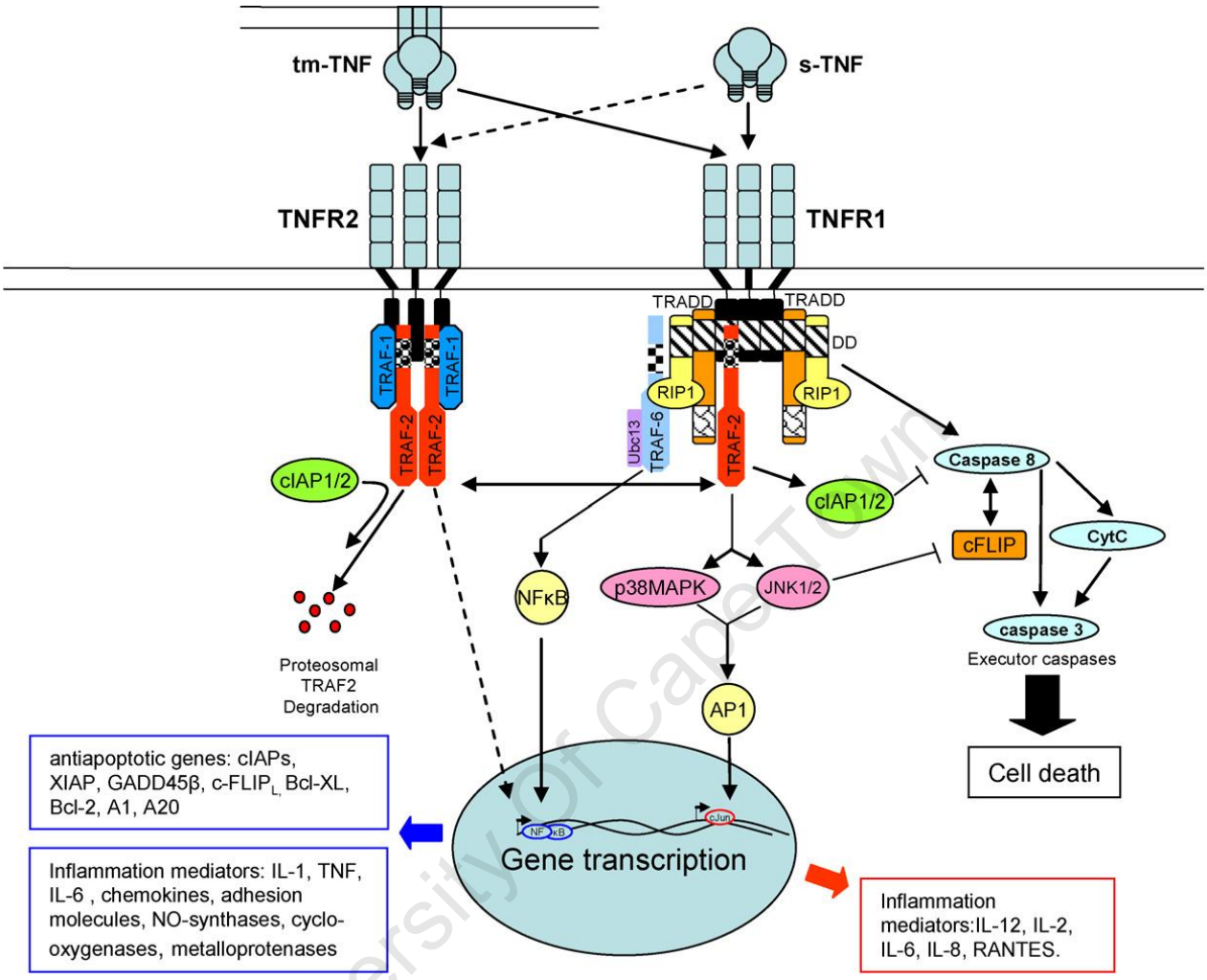


Figure 3. TNF (Tm-TNF and SolTNF) signaling pathway through TNFR1 and TNFR2. TNF interaction with TNFR1 activates the cell survival and proliferation pathways as well as pathways that lead to apoptosis. TNFR2 play an important role in the regulation of apoptosis through TNFR1 although the molecular mechanisms involved in the regulation of cell survival-cell death remain obscure. Abbreviations: cIAP 1/2, cytoplasmic inhibitor of apoptosis 1/2; JNK, cJun N-terminal kinase; p38MAPK, p38 mitogen-activated protein kinase; RIP, receptor interacting protein; ROS, reactive oxygen species; TRADD, TNF receptor-associated death domain; TRAF1/2/6, TNF receptor-associated factor 1/2/6. Figure was adapted from Kruglov et al., 2008.

TNF-TNFR signaling in health and disease

Earlier studies described two opposing functions of TNF, one with seemingly deleterious effect observed when TNF is produced in excess amounts leading to lethal septic shock and the other more beneficial effect resulting in protective immunity against invading intracellular pathogens (Bean et al., 1999; Flynn et al., 1995; Pfeffer et al., 1993; Rothe et al., 1993a; Tracey et al., 1987). With the generation of gene knockout mice, a constellation of TNF-TNFR functions have been described. These include a role in organization of secondary lymphoid tissues. Pasparakis et al., (1997), demonstrated that TNF or p55TNFR gene deficient mice have partial defects in B-cell follicles formation, follicular dendritic cell and germinal centers, but display nearly normal humoral immune responses. However, a more pronounced phenotype was observed in mice lacking LT which displayed a complete absence of lymphoid tissues such as lymph nodes and Peyer's patches (Fu and Chaplin, 1999).

Cancer

With regards to the observed anti-tumor activity of TNF (Carswell et al., 1975), a role in immune surveillance against malignancy has been proposed. However, in the absence of TNF, mice did not develop spontaneous tumors nor did they exhibit accelerated tumor growth when transplanted in the peritoneum (Smyth et al., 1998). Surprisingly, in another study, mice lacking TNF were protected against skin tumors (Moore et al., 1999). It has been suggested that TNF anti-tumor activities in certain mouse models is possible when immunostimulation is limited and the inflammatory response at the sites of tumor is independent of TNF signaling. This hypothesis was tested in a recent study where

transgenic mice developed spontaneous pancreatic tumors (Calzascia et al., 2007); however, this was not observed in studies conducted by Kuprash et al., (2008) in mice lacking p55TNFR. The relevance of these conflicting observations is yet to be established in humans.

Inflammatory disorders

TNF production is tightly regulated by a variety of mechanisms (Biragyn and Nedospasov, 1995; Black et al., 1997; Han et al., 1990; Shakhov et al., 1990; Xanthoulea et al., 2004). Studies have shown that overexpression of TNF leads to pathologic consequences as observed in transgenic mice expressing a human TNF with a modified 3‘-unstralated region of TNF mRNA where these mice developed polyarthritis with synovial inflammation, cartilage damage, and bone destruction (Keffer et al., 1991). In another study, overexpression of TNF in addition to promoting arthritis also induced wasting as well as multiple organ necroses (Probert et al., 1995). Other phenotypes such as Crohn‘s-like inflammatory bowel disease (IBD) (Kontoyiannis et al., 1999) and psoriasis (Schottelius et al., 2004) have been reported to be associated with deregulated TNF levels. Furthermore, the effect of TNF antagonists in disease pathogenesis of rheumatoid arthritis (Lipsky et al., 2000; Setoguchi et al., 2006), Crohn‘s disease (Present et al., 1999) corroborates the functional relevance of TNF and TNFR expression observed in mouse studies. However, considering that TNF is critical for immune regulation and in host defense, a deeper understanding of the therapeutic mechanisms and efficacy of each TNF blocker is required. For instance, a recent publication by Anolik et al., (2008),

demonstrated that anti-TNF therapy in rheumatoid arthritis inhibits memory B cells by affecting lymphoid germinal centers and follicular dendritic cell networks.

TNF-TNFR signaling in host immunity against mycobacterial infections

TNF gene deletion (Bean et al., 1999; Kaneko et al., 1999; Roach et al., 2002), p55TNFR gene deletion (Flynn et al., 1995), and TNF neutralization studies (Eriks and Emerson, 1997; Kindler et al., 1989) demonstrated that TNF has a critical function in protective immunity against mycobacterial infections. In the absence of TNF, mice challenged with either avirulent *M. bovis* (Jacobs et al., 2000b) or virulent *M. tuberculosis* (Bean et al., 1999) were shown to succumb to infection more rapidly compared to the wild type control mice. However, TNF deficient mice displayed survival rates that were comparable to wild type control mice after challenge with a non-pathogenic *M. smegmatis* strain (Roach et al., 2002).

Granuloma structure formation is imperative to host resistance against invading mycobacteria, and their absence usually coincides with mycobacterial dissemination and lethality (Bean et al., 1999; Kindler et al., 1989). TNF has been implicated in inflammatory processes that lead to protective granuloma structure formation. The mRNA expression levels of chemokines that facilitate cellular recruitment and formation of granuloma were observed to be delayed in the livers of TNF deficient mice relative to wild type control mice after challenge with *M. smegmatis* (Roach et al., 2002). However, this early delay in chemokine mRNA expression was not observed in the lungs of TNF deficient mice after *M. tuberculosis* infection (Saunders et al., 2005). Furthermore,

studies by Kindler et al., (1989), demonstrated that treatment of mice with anti-TNF monoclonal antibody interferes with granuloma structure formation if administered after one to two weeks post-infection with *M. bovis* resulting in fewer and smaller granulomas. Additionally, fully developed granulomas disintegrated after treatment of mice with anti-TNF monoclonal antibody. This was further corroborated by studies by Mohan et al., (2001), illustrating the importance of continuous TNF expression in protective granuloma structure maintenance.

An immunoregulatory TNF function has been described in mycobacterial infections. Studies by Roach et al., (2002), demonstrated enhanced Th1 immune responses associated with increased $CD4^+$ T cell numbers and increased IFNγ production in TNF deficient mice compared to wild type control mice after infection with *M. smegmatis*. This is line with Zganiacz et al., (2004), who demonstrated that TNF has a negative regulatory effect of Th1 immune responses. In their studies the authors illustrated that relative to wild type control mice; TNF deficient mice displayed excess IL-12 and IFNγ production, enhanced antigen specific $CD4^+$ and $CD8^+$ T cell responses, and tissue destruction after *M. tuberculosis* challenge.

In summary, the multiple TNF effects described here are an indication of complex TNF biology. The continued generation of more specific transgenic mice, and newer technologies addressing the roles and contributions of cell specific or tissue specific TNF production would allow for more detailed studies in understanding the involvement of TNF in mediating pathology or host defense.

2 Materials and methods

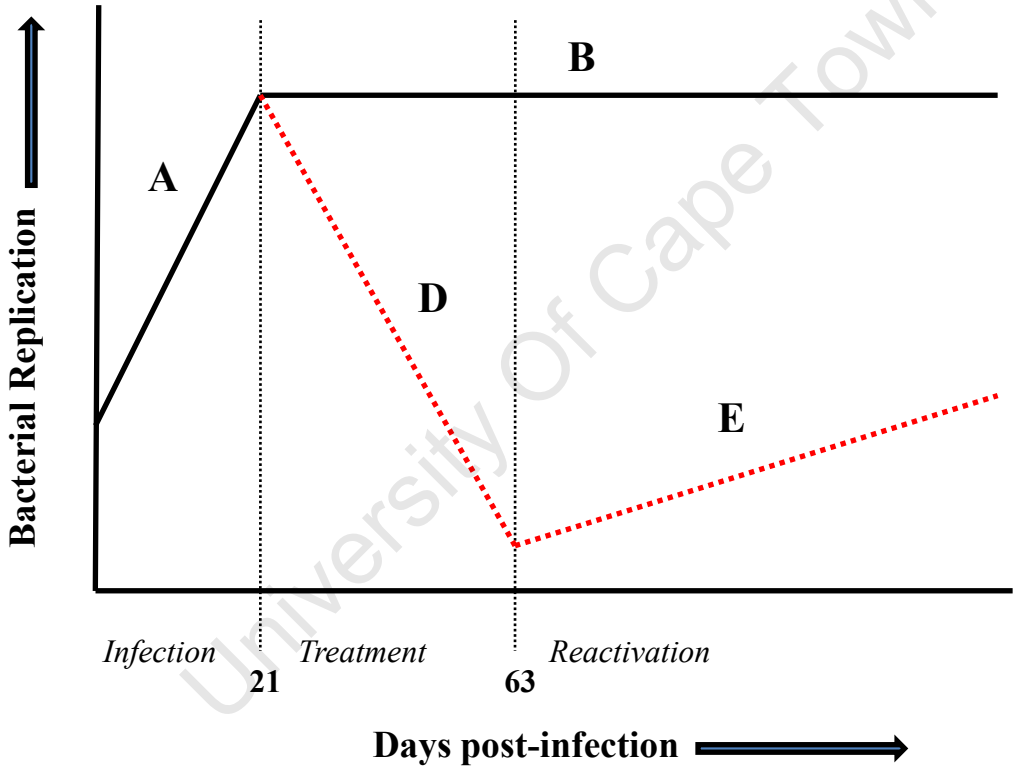
Mice

Wild type (WT) mice were used as controls, $TNF^{-/-}$ mice (deficient in TNF) (Marino et al., 1997), Tm-TNF mice: express only the membrane–bound TNF; the endogenous *TNF* allele was replaced by knocking-in the Δ1-9,K11E *TNF* allele which ensures complete loss of TACE mediated cleavage (Ruuls et al., 2001), $p55^{\Delta NS}$ mice: express only the membrane-bound p55TNFR (Xanthoulea et al., 2004), $p75^{-/-}$ mice (deficient in p75TNFR) (Erickson et al., 1994), and $p55^{\Delta NS}$-$p75^{-/-}$ mice (obtained by mating $p55^{\Delta NS}$ mice and $p75^{-/-}$ mice) were bred, maintained and housed in IVCs under specific pathogen free conditions in the animal unit facility of University of Cape Town (Cape Town, South Africa). For all the experiments, age matched mice on a C57BL/6 background were used. All the experiments and protocols performed were in accordance with the guidelines of Research Ethics Committee of the University Of Cape Town, South Africa.

Bacteria and infections

M. tuberculosis H37Rv was obtained from Trudeau Mycobacterial Culture Collection and grown in Middlebrook 7H9 broth (Becton, Dickinson and Company, Le Pont de Claix, France) supplemented with 10% Middlebrook OADC enrichment medium (Life Technologies, Gaitherburg, MD), 0.5% glycerol and 0.05% Tween 80 at 37°C until log phase. Prior to usage, mycobacterial aliquots were passed 30x through a 29.5 G needle to minimize bacterial clumping. Pulmonary infection with 100 live *M. tuberculosis* H37Rv bacteria was performed using a Glas-Col Inhalation Exposure System, Model A4224. Inoculum size was confirmed 24 h post-infection by determining the bacterial numbers in the lungs of infected mice.

For the *M. tuberculosis* reactivation model (Fig. 1), groups of mice were infected by aerosol inhalation with 100 viable *M. tuberculosis* H37Rv bacilli. The infection was



model; D: Reduction of bacterial numbers to very low levels using a short course regimen of antimycobacterial drugs; E: Reactivation of infection upon cessation of antibiotics.

Colony enumeration assay

Bacterial burdens in the lungs, livers and spleens of infected mice were determined at specific time points after infection with *M. tuberculosis* H37Rv. Organs were weighed and homogenized in 0.04% Tween 80 saline. Tenfold serial dilution of organ homogenates were plated in duplicates on Middlebrook 7H10 (Becton, Dickinson and Company) agar plates containing 10% OADC (Life Technologies, Gaitherburg, MD) and incubated at 37°C for 19-21 days. Colonies on plates were enumerated and bacterial burdens determined.

Microscopic investigation of the lungs

Mice were euthanized by carbon dioxide inhalation at specific time points. Organs were weighed and fixed in 10% formalin and paraffin-embedded (Appendix). Two to 3 µm sections were stained with haematoxylin and eosin (H&E) and a modified Ziehl-Neelson (ZN) method (Appendix). For immunostaining, formalin-fixed paraffin-embedded sections were deparaffinised and rehydrated then stained with rabbit anti-mouse specific inducible nitric oxide synthase (iNOS) as described (Garcia et al., 2000). Sections were then washed in PBS (Appendix) and incubated for 30 min at room temperature with biotinylated secondary antibody then subsequently incubated with avidin-biotin complexes (Vector Laboratories, CA, USA) for 30 min, washed and incubated with DAB substrate (Dako Corporation, CA, USA).

Lung homogenate preparations

Whole lungs were removed from infected mice at specific time points and were homogenized in 1 ml 0.04% Tween 80 saline containing protease inhibitor (Sigma) (Appendix) and the supernatants were collected after low-speed centrifugation, aliquoted and frozen at -80°C.

Cytokine ELISA

Supernatants from organ homogenates or from cultured cells were harvested and assayed for cytokine concentration using commercially available ELISA reagents for TNF, IFNγ, IL-10, IL-12p40, IL-12p70, p55TNFR and p75TNFR (R&D Systems, Germany and BD PharMingen, San Diego), according to the manufactures instructions (See Appendix, brief description).

Lung single cell preparation

Lungs of infected mice were perfused by injecting 5 ml cold PBS (Appendix) containing 20 U/ml heparin (Bodene (PTY) Limited, RSA) in the right ventricle of the heart. Lungs were then removed, sectioned and incubated in PBS (Appendix) containing 50 U/ml collagenase 1 (Worthington Biomedical Corporation, Lakewood, NJ) and 13 µg/ml DNAse 1 (Boeringer-Mannheim, Germany) at 37°C for 90 min with rotation. For single cell suspension, lung tissue was passed through a 70 µm nylon cell strainer (Beckton and Dickinson) washed 2x with PBS (Appendix) and viable cell numbers were determined by counting in the presence of trypan blue.

Flow cytometry and cell surface markers

Isolated lung cells were counted and incubated for 20 min on ice with 25 µl FACS blocking buffer (Appendix), washed 2x with 475 µl ice-cold FACS buffer (Appendix) and centrifuged at 4°C for 5 min at 514 RCF. Cells were then incubated for 20 min on ice with either 25 µl of specific antibody at 2 µg/ml or anti-IgG control antibody at 2 µg/ml in the dark (See Appendix, antibodies used). Following this, cells were washed 2x with 475 µl ice-cold FACS buffer (Appendix) to remove excess antibodies then centrifuged at 4°C for 5 min at 514 RCF, the cell pellet was then resuspended in 200 µl FACS fixation buffer (Appendix). Samples were acquired using FACSCalibur (Beckton and Dickinson) and analysed using FlowJo 7.5 software (Tree star, Ashland, OR, USA).

Preparation of elicited peritoneal macrophages

Mice were injected with 3% thioglycollate (Difco, St. Louis, USA). Five days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with ice-cold RPMI (Sigma, Germany) supplemented with 10% FCS (Gibco, Invitrogen Corporation, Germany). Cells were cultured overnight at 37°C and 5% CO_2 incubator. Adherent monolayer cells were used as peritoneal macrophages and were cultured at $5x10^5$ cells/ml in RPMI supplemented with 10% FCS and stimulated for 20 min with either 100 ng/ml LPS (*E. coli*, serotype O111:B4, Sigma) or *M. tuberculosis* H37Rv (MOI 2:1).

Preparation of primary macrophage cultures

Bone marrow cells were isolated from femurs of 6 to 8 weeks old naive mice and cultivated on 90 mm Sterilin plates (Bibby Sterilin, UK) at $2x10^6$ cells/ml for 7 days in RPMI (Sigma, Germany) supplemented with 2 mM L-glutamine (Gibco, Invitrogen Corporation, Germany), 0.2 μM 2-ME (Sigma, St. Louis, USA), 20% horse serum (Gibco, Invitrogen Corporation, Germany) and 30% L929 cell-conditioned medium at 37°C and 5% CO_2. Adherent cells were harvested by adding PBS (Appendix) containing 0.02% EDTA (Merck Chemicals, Gauteng, RSA) and 4 mg/ml lidocain hydrochloride monohydrate (Sigma-Aldrich, L5647-15G) and incubated for 5 min at 37°C, cells were then washed with PBS (Appendix) and centrifuged at 405 RCF. Following this, cells were resuspended in RPMI (Sigma, Germany) containing 10% FCS (Gibco, Invitrogen Corporation, Germany), 100 U/ml penicillin (Gibco, Invitrogen Corporation, Germany) and 100 ng/ml streptomycin (Gibco, Invitrogen Corporation, Germany) and plated at $5x10^5$ cells/ml in 96 well tissue culture plates (Nunclon, Denmark) and incubated for 24 h at 37°C and 5% CO_2 to allow for cell adherence. Cells were then stimulated with either 100 ng/ml LPS (*E. coli*, serotype O111:B4, Sigma) or live *M. tuberculosis* H37Rv bacteria (MOI 2:1) for 90 min, 150 min, 300 min, 24 h, and 48 h. Supernatants were collected and analysed for cytokine content using ELISA and nitrite concentration using the Griess reagent (3% phosphoric acid, 1% p-aminobenzene-sulphonamide, 1% n-naphthylenediamide) as described (Stuehr and Nathan, 1989).

Bioactive TNF assay

Bioactive TNF was determined using TNF sensitive fibroblast cell line WEHI 164, clone 13 (Walter and Eliza Hall Institute). WEHI cells were cultured on 90 mm tissue culture petridish (Bibby Sterilin, UK) in RPMI (Sigma, Germany) in 10% FCS (Gibco, Invitrogen Corporation, Germany) containing 10 U/ml penicillin (Gibco, Invitrogen Corporation, Germany), 10 µg/ml streptomycin (Gibco, Invitrogen Corporation, Germany) and 0.5x amino acid supplement (Gibco, MEM Amino acids without L-glutamine, Invitrogen Corporation, Germany) until confluent. Adherent cells were harvested using PBS (Appendix) containing Trypsin-EDTA (Gibco, Invitrogen Corporation, Germany), washed in PBS (Appendix) then centrifuged at 405 RCF. Cells were resuspended in RPMI (Sigma, Germany) containing 10% FCS (Gibco, Invitrogen Corporation, Germany), 100 U/ml penicillin (Gibco, Invitrogen Corporation, Germany) and 100 ng/ml streptomycin (Gibco, Invitrogen Corporation, Germany) and plated at $2x10^5$ cells/ml in 96 well tissue culture plates (Nunclon, Denmark) and incubated for 24 h at 37°C and 5% CO_2 to allow for cell adherence. TNF standards (recombinant mouse TNF, BD PharMingen, San Diego) prepared in 2 fold serial dilutions starting at 8 pg/ml to 0.016 pg/ml or samples were added to WEHI cells and incubated for 18 h at 37°C and 5% CO_2. Fifty microliters of 2 mg/ml MTT (Sigma, Germany) solution was added and cells were incubated for further 2 h, after which, the supernatants were aspirated and 50 µl DMSO was added and samples were read at 570 nm using VERSAmax Tunable Microplate Reader (Molecular devices Corporation, California, USA). Data was analysed using SoftMax Pro Version 4.3 software (Molecular devices Corporation, California, USA).

Antigen-specific IFN-γ production

Antigen-specific production of IFNγ was measured from single cell suspension of mediastinal lymph nodes, lungs or BAL prepared from infected mice at specific time points after infection with *M. tuberculosis* H37Rv. Cells were resuspended in RPMI (Sigma, Germany) supplemented with 10% FCS (Gibco, Invitrogen Corporation, Germany), 100 U/ml penicillin (Gibco, Invitrogen Corporation, Germany) and 100 µg/ml streptomycin (Gibco, Invitrogen Corporation, Germany) then cultured at $5x10^5$ cells/well in 96 well round-bottom microplates (Nunc, Naperville, IL), and stimulated with either 10 µg/ml ESAT-6 (Statens, Serum Institut, Denmark), 5 µg/ml anti-CD3/CD28 (BD PharMingen, San Diego) live *M. tuberculosis* H37Rv (MOI 2:1) and incubated at 37°C and 5% CO_2. Supernatants were harvested after 3 days and stored at -80°C for further analysis.

Statistical Analysis

Data were analysed by comparison of WT and gene-deficient/transgenic mice at specific time points and expressed as mean ± SD of 4mice/group (unless stated otherwise). All graphic results were prepared using the GraphPad Prism 4 software and the same software was used for statistical analysis of data (Student's *t* test for comparison of two groups and ANOVA for comparison of three groups or more) and comparative analysis of survival data using designated GraphPad Prism 4 statistical module. p values ≤ 0.05 were considered significant. Each experiment was repeated at least once to ensure reproducibility.

3 Tm-TNF in *M. tuberculosis* infection

The inherent complexity of TNF-mediated protective immunity and TNF-mediated immune pathologies is implicit in the differential bioactivities of the two molecular forms of TNF (solTNF and Tm-TNF) and the differential functioning of its receptors (p55TNFR and p75TNFR). The purpose of this study was to characterize the role(s) of soluble TNF and membrane bound TNF in protective or pathology mediated TNF responses against *M. tuberculosis* lung infection. Because of the progressive nature of *M. tuberculosis* infection this chapter addressed three different phases of tuberculosis disease: acute infection, chronic infection and reactivation drug-induced model.

Section I: Tm-TNF and acute *M. tuberculosis* infection

Summary

The fundamental importance of TNF in immunity against *M. tuberculosis* has been demonstrated previously but the contribution of soluble TNF versus membrane bound TNF in this immunity has not been fully characterized. In this chapter, we demonstrate that Tm-TNF mediate protective immune responses against low dose aerosol inhalation challenge with *M. tuberculosis* H37Rv. We demonstrate that Tm-TNF mice produce normal levels of IL-12p70, IFNγ and IL-10 and mediate a normal inflammatory process comparable to WT control mice. Bactericidal granuloma structure formation is possible in the presence of Tm-TNF molecule and these are composed of activated $CD4^+$ T cells and activated macrophages. In conclusion, membrane expressed TNF is sufficient to protect mice against acute *M. tuberculosis* H37Rv infection.

Results

Tm-TNF mice control acute M. tuberculosis infection.

Previous gene deletion and neutralization studies showed that TNF is required for generation of protective immunity during infection with a non-virulent mycobacterial *M. bovis* BCG strain (Jacobs et al., 2000b; Kindler et al., 1989). However, these studies did not characterize the relative contributions of the two molecular forms of TNF: Tm-TNF and solTNF in this process. A study by Olleros et al., (2002; 2005) revealed that transgenic mice expressing Tm-TNF in the absence of TNF and LTα developed protective immune responses against *M. bovis* BCG as well as *M. tuberculosis* however; the control of bacilli replication observed for *M. bovis* BCG was less efficient for *M. tuberculosis* infection. Dissecting the functions of Tm-TNF versus those of solTNF in these transgenic mice is further complicated by the fact that LTα has also been reported to mediate partial protection against *M. bovis* BCG infection (Bopst et al., 2001). We examined the respective roles of solTNF and Tm-TNF using Tm-TNF mice in which the endogenous *TNF* allele was replaced with an uncleavable form of TNF but retained normal cell surface expression and functional capacity of WT Tm-TNF molecule (Ruuls et al., 2001). In a study where mice were challenged with $1x10^6$ CFUs/mouse via the intranasal route, we found that Tm-TNF mice were rendered susceptible to *M. bovis* BCG infection reflected by significant bacilli burdens in the lungs, spleens and livers compared to WT mice (Dambuza et al., 2008). To determine the effects of virulence on Tm-TNF mediated immunity in a natural *M. tuberculosis* aerosol inhalation challenge model; WT mice, $TNF^{-/-}$ mice and Tm-TNF mice were infected with 100 CFUs/mouse *M. tuberculosis* H37Rv and body weight changes and mortality rates were recorded as

markers of disease progression. WT mice maintained consistent body weights (Fig. 1A) throughout the experimental period and no mortalities were recorded (Fig. 1B). In sharp contrast, $TNF^{-/-}$ mice were highly susceptible to infection, displayed rapid weight loss (Fig. 1A) and succumbed to infection with a mean survival time of 44 days (Fig. 1B) which was significantly different ($p < 0.05$) from WT control mice. Conversely, Tm-TNF mice maintained body weights comparable to WT mice (Fig. 1A) and displayed resistance to infection similar to WT mice (Fig. 1B).

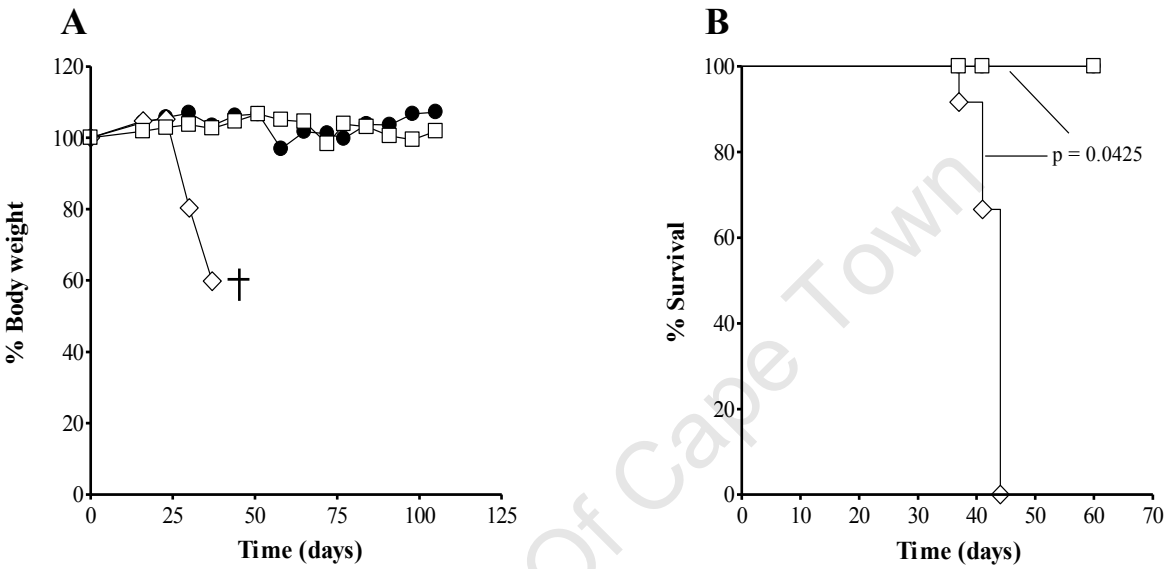


Figure 1. Tm-TNF mice are protected from acute M. tuberculosis infection. WT mice (black circles), $TNF^{-/-}$ mice (white diamonds) and Tm-TNF mice (white squares) were infected with 100 CFUs/mouse *M. tuberculosis* H37Rv by aerosol inhalation. (A) Body weights and mortality rates (B) were assessed over the infection period. Data represent 1 out of 2 experiments performed and are expressed as mean ± SD of 8 mice/group. *p* values were obtained using the Log Rank test for survival curve comparison.

Control of mycobacterial replication was then assessed in the lungs and spleens of mice. As shown in Fig. 2A, bacterial replication in WT mice increased logarithmically and reached 6.5 $\log_{10}$ by day 26 post-infection then decreased to 5 $\log_{10}$ by day 33 post-infection. Bacilli burdens were then maintained at this level until the experiment was terminated 52 days post-infection. In $TNF^{-/-}$ mice however, bacilli growth in the lungs was unrestricted and by day 33 post-infection bacilli burdens reached 8.5 $\log_{10}$ CFUs and were significantly higher ($p < 0.001$) in comparison to WT mice (Fig. 2A). The increased bacilli burdens in the lungs of $TNF^{-/-}$ mice corresponded with the early susceptibility to infection seen in this strain (Fig. 1A & B). Interestingly, Tm-TNF mice displayed a bacilli growth kinetic comparable to that of WT mice (Fig. 2A). Spread of mycobacteria to spleen was determined and a significant difference ($p < 0.01$) in bacterial numbers was observed as early as day 26 post-infection in $TNF^{-/-}$ mice compared to WT mice and by 33 days post-infection, the bacilli numbers declined in both strains but were observed to be higher in $TNF^{-/-}$ mice (Fig. 2B). Conversely, bacilli numbers were comparable between Tm-TNF mice and WT mice at day 26 post-infection and followed a similar mycobacterial growth kinetic determined day 33 and day 52 post-infection, no significant differences were observed (Fig. 2B). Differences in bacterial burdens in the lungs were confirmed histologically by Ziehl-Neelson staining with mycobacteria in WT mice (Fig. 2C) largely localized intracellularly, in contrast, there was a significant amount of extracellular bacilli in $TNF^{-/-}$ mice (Fig. 2D) at day 33 post-infection. Conversely, in Tm-TNF mice (Fig. 2E) the bacterial staining pattern was similar to WT mice with bacilli largely confined intracellularly. Thus, these data indicate that complete loss of TNF

renders mice susceptible to the infection and that Tm-TNF is sufficient for control of the infection.

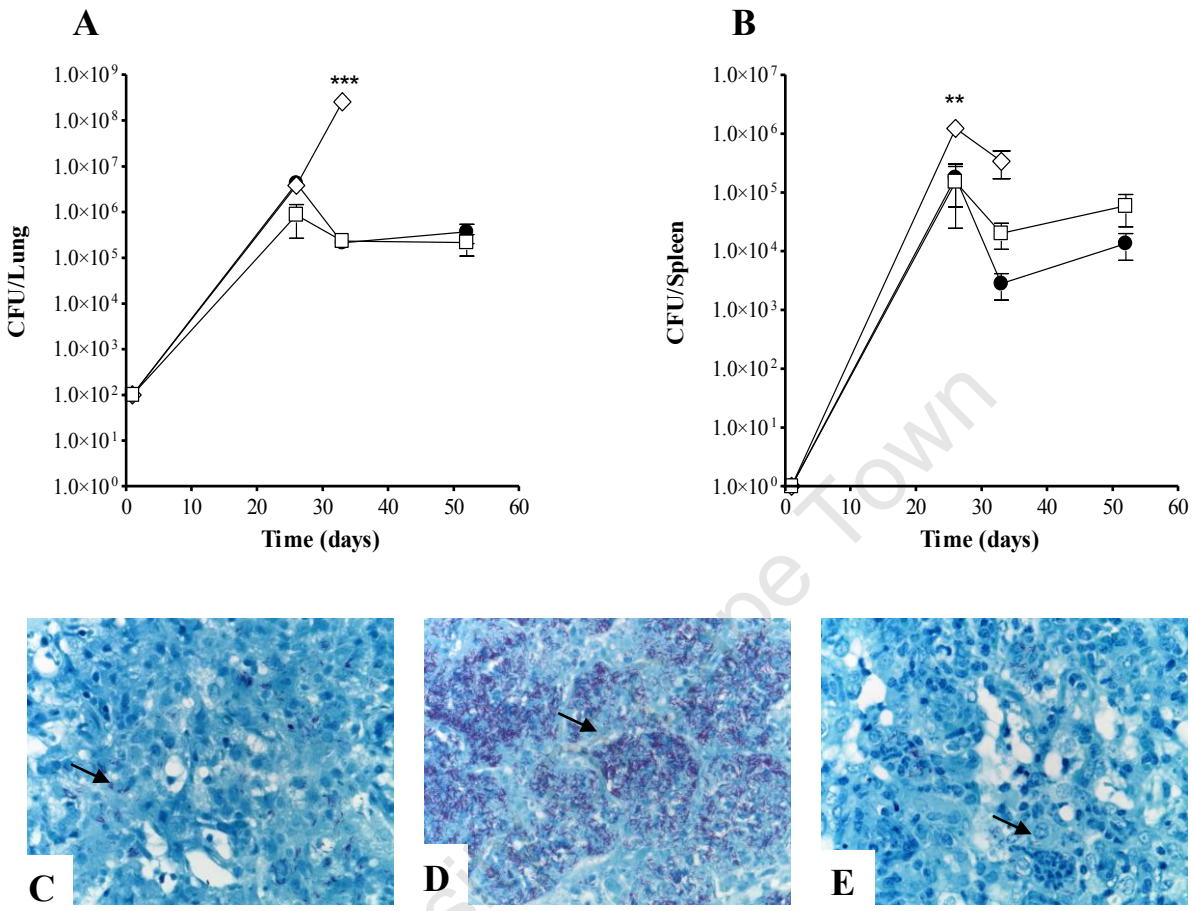


Figure 2. Tm-TNF is adequate for control of the infection. WT mice (black circles), $TNF^{-/-}$ mice (white diamonds) and Tm-TNF mice (white squares) were aerosol-infected with 100 CFUs/mouse *M. tuberculosis* H37Rv and bacilli burdens were assessed in the lungs (A) and spleens (B) at time points indicated. Lung sections from WT mice (C), $TNF^{-/-}$ mice (D) and Tm-TNF mice (E) were analyzed for presence of mycobacteria by Ziehl-Neelson staining at day 33 pots-infection (arrows show acid-fast bacilli). Data are expressed as mean ± SD of 4 animals/group and represent 1 of 3 experiments performed. Significant differences (***$p < 0.001$; **$p < 0.01$) were obtained using ANOVA. Micrographs represent 4 animals/group and shown at x800 magnification.

Tm-TNF mediates controlled inflammatory response and protective granuloma formation during acute M. tuberculosis infection.

Mycobacterial granulomas are focal accumulations of mononuclear cells that form organized structures with centrally located macrophages that differentiate into epitheloid cells surrounded by lymphocytic aggregates, mainly CD4 T cells and this colocation is thought to allow close antigen-specific T cell-macrophage interaction facilitating activation of bactericidal mechanisms (Flynn and Chan, 2001). TNF is essential for the formation and maintenance of the granulomas and many studies have shown that TNF plays a role in the early induction of chemokines and regulation of cell adhesion molecules that facilitate initial leukocyte recruitment and aggregation resulting in granuloma formation (Hickey et al., 1997; Mulligan et al., 1993; Roach et al., 2002). We therefore investigated whether Tm-TNF signaling induced granuloma structure formation.

Initially, we assessed lung weights in infected WT mice, $TNF^{-/-}$ mice and Tm-TNF mice as a surrogate marker of inflammation. WT mice displayed a steady slight increase in lung weights of about 0.1 g over the course of the infection period (Fig. 3.1A). Lung weights were comparable to WT mice determined 26 days post-infection in $TNF^{-/-}$ mice, but a significant increase ($p < 0.05$) was observed at day 33 post-infection in $TNF^{-/-}$ mice (Fig. 3.1A). In contrast, Tm-TNF mice showed similar lung mass comparable to WT mice at time points investigated (Fig. 3.1A). Total lung cell counts were then determined at day 33 post-infection to confirm lung weight data. Results obtained showed that $TNF^{-/-}$ mice had significantly higher ($p < 0.01$) lung cellularity relative to WT control mice, in

contrast, Tm-TNF mice displayed lung cell numbers comparable to WT mice (Fig. 3.1B). Together, these results suggest that Tm-TNF is sufficient to mediate early inflammatory response during *M. tuberculosis* infection and complete absence of TNF results in excessive inflammation which may be a reflection of increased bacilli burdens.

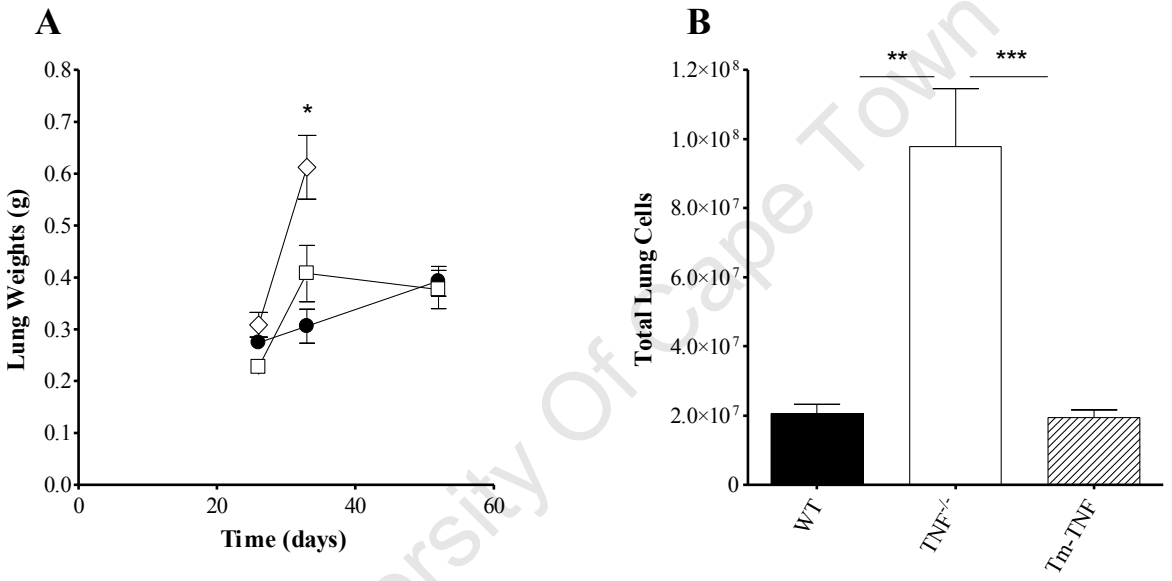


Figure 3.1. Tm-TNF mediates a controlled cell infiltration in the lung. WT mice (black circles), $TNF^{-/-}$ mice (white diamonds) and Tm-TNF mice (white squares) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv and lung weights (A) were determined at specific time points. (B) Lung single cells suspensions were prepared and total cell numbers were quantified 35 days post-infection. Data are expressed as mean ± SD of 4 animals/group and represent 1 of 3 experiments performed. Significant differences (***$p < 0.001$; **$p < 0.01$; * $p < 0.05$) were obtained using ANOVA.

Next, we assessed whether granulomas were formed in presence of Tm-TNF as a correlate of protection. Depicted in Fig. 3.2, results obtained showed that WT mice formed compact well defined granulomas consisting of epitheloid macrophages and interspersed with recruited lymphocytes analyzed at day 26 (Fig. 3.2A) and day 33 (Fig. 3.2D) post-infection and interestingly, granulomas formed in $TNF^{-/-}$ mice (Fig. 3.2B) analyzed 26 days post-infection, however, the lesions increased in size by day 33 post-infection (Fig. 3.2E) becoming more diffuse and involving larger areas of the lung compared to WT mice granuloma. In converse, Tm-TNF mice formed well-structured granulomas comparable to WT mice analyzed 26 days and 33 days post-infection (Fig. 3.2C & F, respectively). These observations are in line with published reports (Fremond et al., 2005; Saunders et al, 2005) and illustrate that Tm-TNF is sufficient to mediate granuloma structure formation that protects mice from acute *M. tuberculosis* H37Rv infection not observed in complete lack of TNF.

We then investigated the expression of cell adhesion and cell homing molecules as determinants for failure to form compact granulomas in $TNF^{-/-}$ mice. Lung single suspensions were generated from infected WT mice, $TNF^{-/-}$ mice and Tm-TNF mice 21 days post-infection and cell surface expression levels of CD11a, CD49a and CD69L on $CD4^+$ T cells (gating strategy, Fig. 3.3.i) were determined by measuring the mean fluorescent intensity using flow cytometry. Results obtained showed that there was a slight but insignificant increase in percentage of $CD4^+$ T cells in $TNF^{-/-}$ mice compared to WT mice but conversely, the percentage of $CD4^+$ T cells present in Tm-TNF mice was comparable to that of WT mice (Fig. 3.3.iiA). Interestingly, no differences were

observed in the level of surface expression of all the leukocyte adhesion and trafficking molecules investigated (Fig. 3.3.iiB, C & D). These results suggest the expression of these molecules is not TNF-dependent in the lung during early inflammation after *M. tuberculosis* infection.

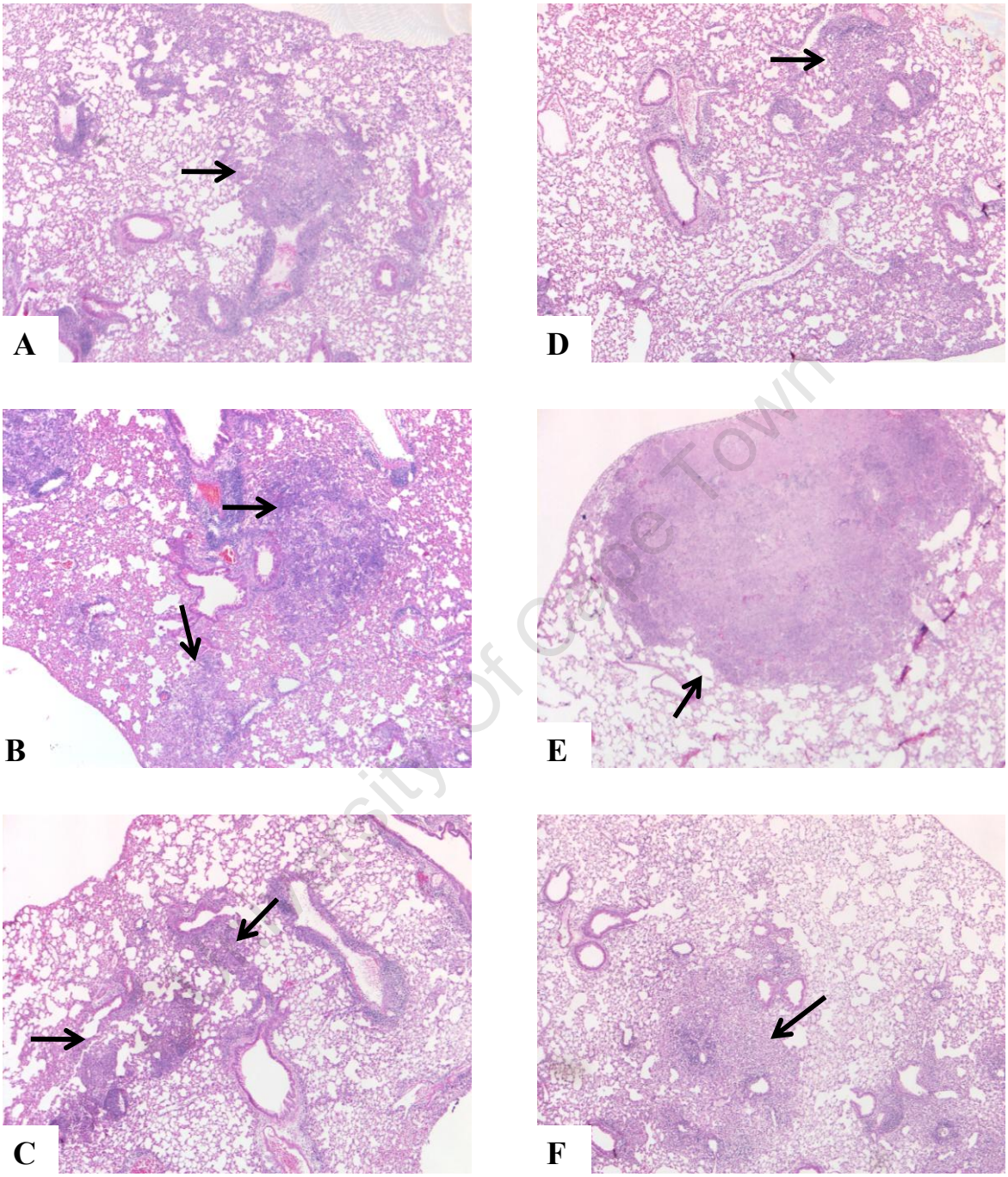


Figure 3.2. Tm-TNF contributes to granuloma formation during acute M. tuberculosis infection. WT mice (A & D), $TNF^{-/-}$ mice (B & E) and Tm-TNF mice (C & F) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv and lung sections were removed 26 days post-infection (left column) and 33 days post-infection (right column) and stained with haematoxylin and eosin (arrows indicate granulomas). Micrographs represent 4 animals/group and shown at x32 magnification.

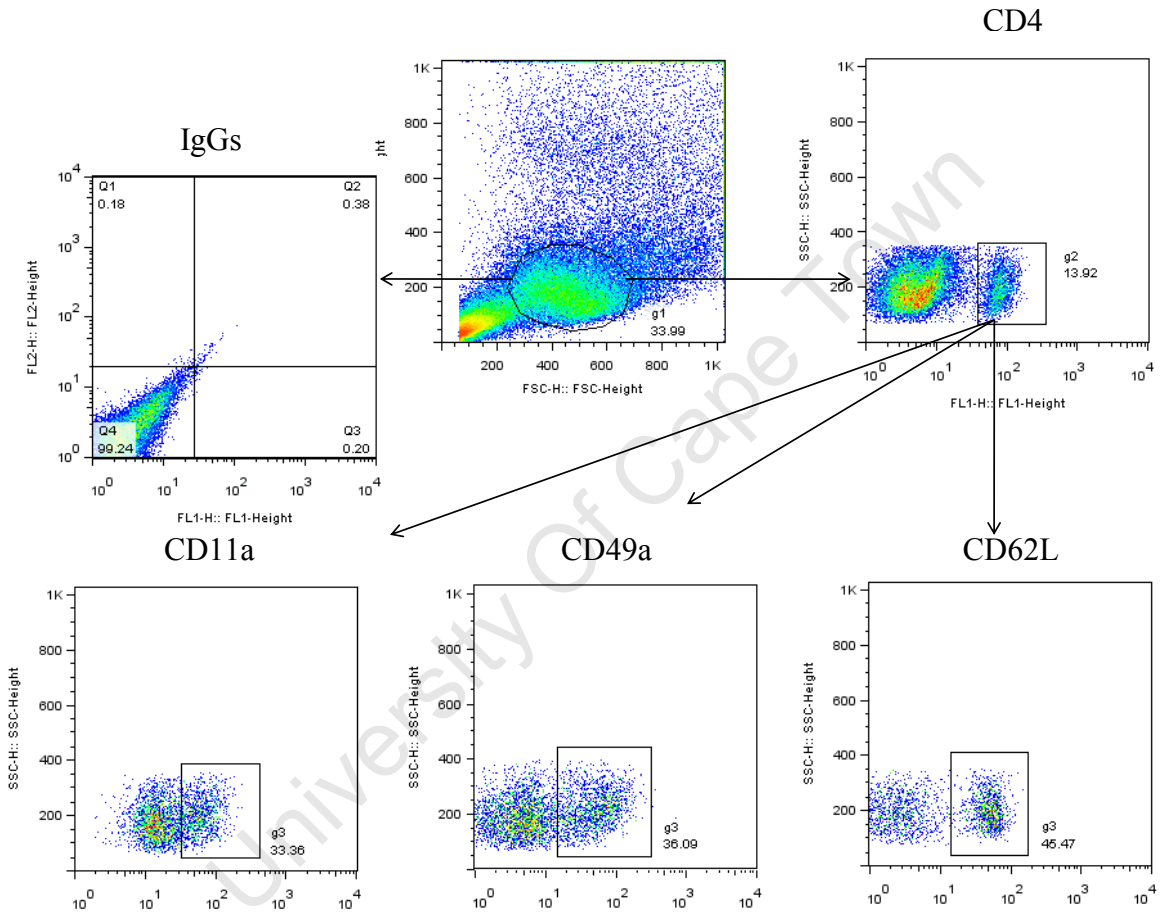


Figure 3.3.i. Gating strategy. Lung single cell suspensions derived from WT mice, $TNF^{-/-}$ mice and Tm-TNF mice were prepared 21 days after aerosol infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Cells were stained with irrelevant antibody (IgG) or anti-CD4 antibody and the MFIs of CD11a, CD49a and CD62L gated on the $CD4^{+}$ T cell subpopulation were determined by flow cytometry.

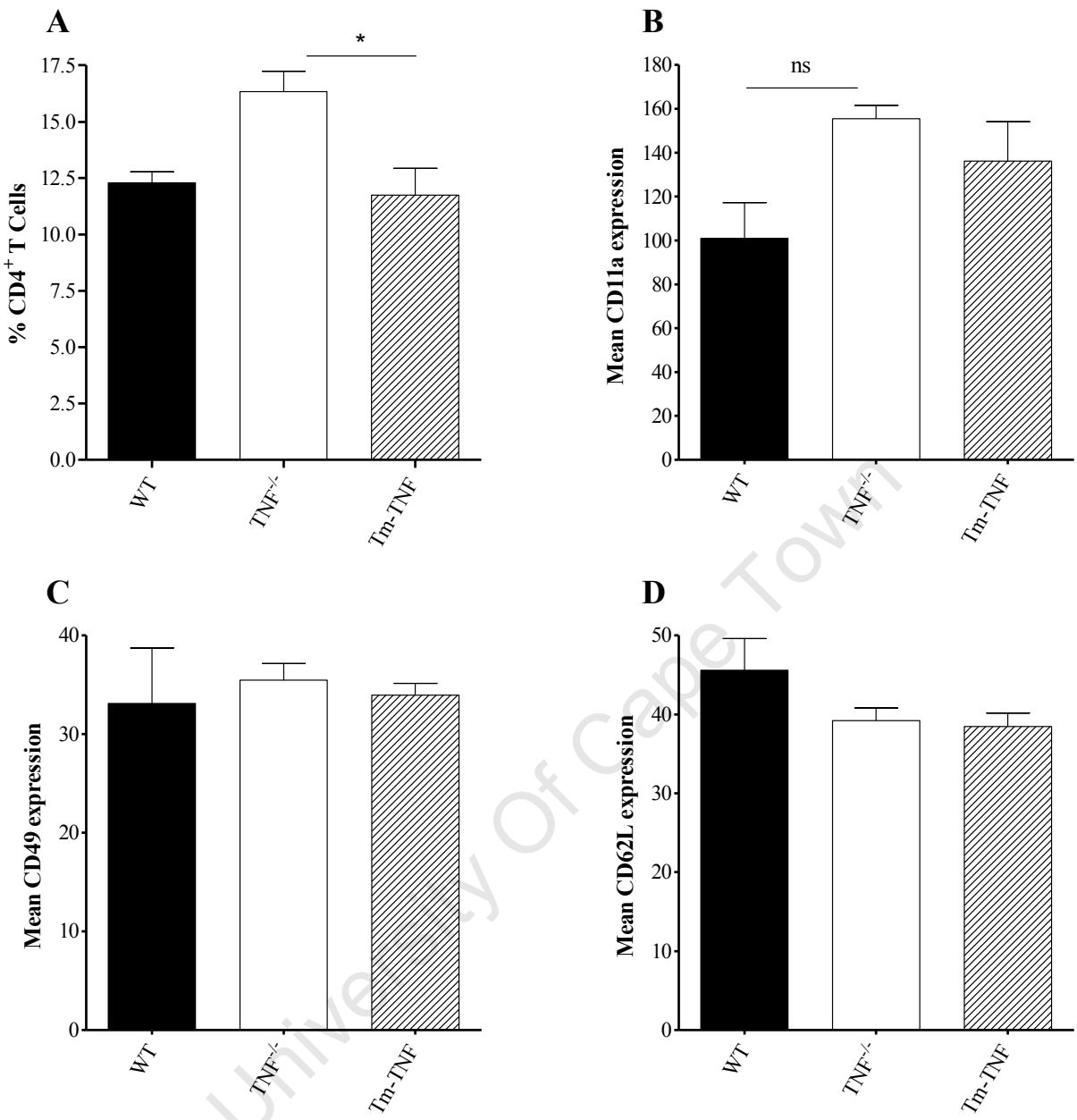


Figure 3.3.ii. Tm-TNF mediates a controlled frequency of pulmonary CD4$^+$ T cells but does not influence cellular adhesion and action markers. WT mice (black bars), TNF$^{-/-}$ (white bars) mice and Tm-TNF (striped bars) mice were challenged by aerosol inhalation infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lung single cell suspensions derived from respective mice groups at 21 days post-infection were stained for CD4, CD11a, CD49a or CD62L expression then analyzed by flow cytometry. The frequency of CD4$^+$ T cells (A) and the mean surface expression levels (MIFs) of CD11a$^+$ (B), CD49a$^+$ (C) and CD62L$^+$ (D) gated on CD4$^+$ T cell subpopulation were determined. The data is representative of 1 of 2 experiments performed and the results are expressed as mean ± SD of 4 animals/group. Significant differences (* $p < 0.05$; ns: $p > 0.05$) were obtained using ANOVA.

Tm-TNF is sufficient to facilitate influx of activated pulmonary CD11b$^+$ cells and CD4$^+$ T cells during acute M. tuberculosis infection.

Next we used flow cytometry to characterize the influx of cells into the lung. Studies have shown that one of the earliest events upon antigen uptake and activation of antigen presenting cells is the upregulation of cell surface expression of co-stimulatory (CD80 and CD86) and activation markers such as MHC-II and CD40 and their expression levels were observed to increase after *M. tuberculosis* infection (Gonzalez-Juarrero et al., 2003). Here, we compared the frequency of lung CD11b$^+$ cells (in murine experimental pulmonary tuberculosis, expressed on macrophages, monocytes, dendritic cells, granulocytes, B and T cells) in WT mice, TNF$^{-/-}$ mice and Tm-TNF mice at day 35 post-infection. Data obtained revealed that compared to WT mice, TNF$^{-/-}$ mice had significantly increased ($p < 0.001$) percentage of CD11b$^+$ cells whereas Tm-TNF mice displayed a comparable frequency of CD11b$^+$ cells to WT mice (Fig. 4.1.iiA). We then assessed the mean surface expression of MHC-II, CD80, and CD86 gated on CD11b$^+$ cells (gating strategy, Fig. 4.1.i). TNF$^{-/-}$ mice displayed significant increase in surface expression of MHC-II (Fig. 4.1.iiB, $p < 0.01$) and CD80 (Fig. 4.1.iiD, $p < 0.001$) compared to WT mice, CD86 expression (Fig. 4.1.iiC) although higher in TNF$^{-/-}$ mice relative to WT mice did not reach statistical significance. These data correlates with the increased bacilli burdens in the lungs of TNF$^{-/-}$ mice (Fig. 2A). Interestingly, in Tm-TNF mice, MHC-II (Fig. 4.1.iiB), CD86 (Fig. 4.1.iiC), and CD80 (Fig. 4.1.iiD) surface expression was equivalent to WT mice. These data suggest that during acute *M. tuberculosis* infection, Tm-TNF contributes to the recruitment of CD11b$^+$ cells to the lung and the activation status of these cells is associated with effective control of

bacterial growth. In addition the excess influx of these cells and their hyperactivation reflect the increased bacilli burdens in the lungs of gene deficient TNF mice.

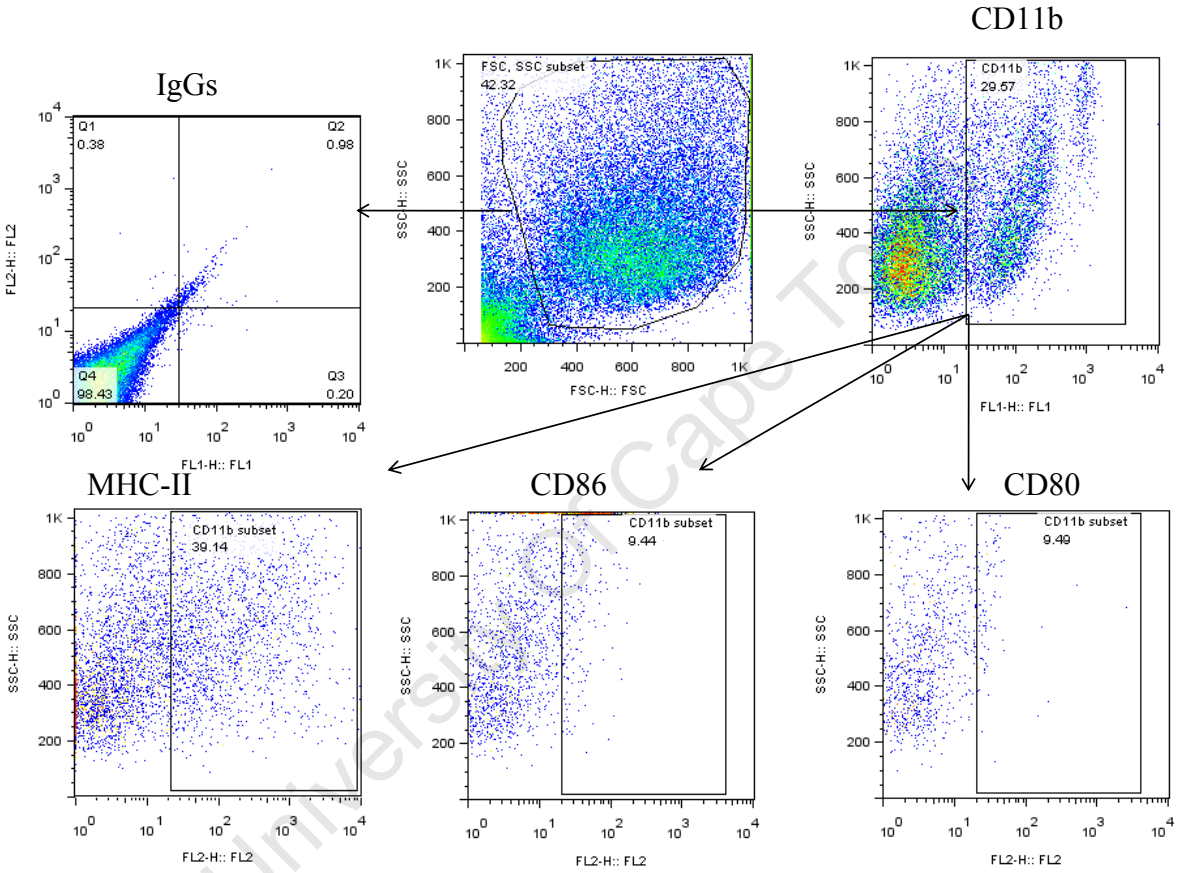


Figure 4.1.i. Gating strategy. Lung single cell suspensions derived from WT mice, $TNF^{-/-}$ mice and Tm-TNF mice were prepared 35 days after aerosol infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Cells were stained with irrelevant antibody (IgG) or anti-CD11b and the MFIs of MHC-II, CD86 and CD80 gated on $CD11b^{+}$ cell subpopulation were determined by flow cytometry.

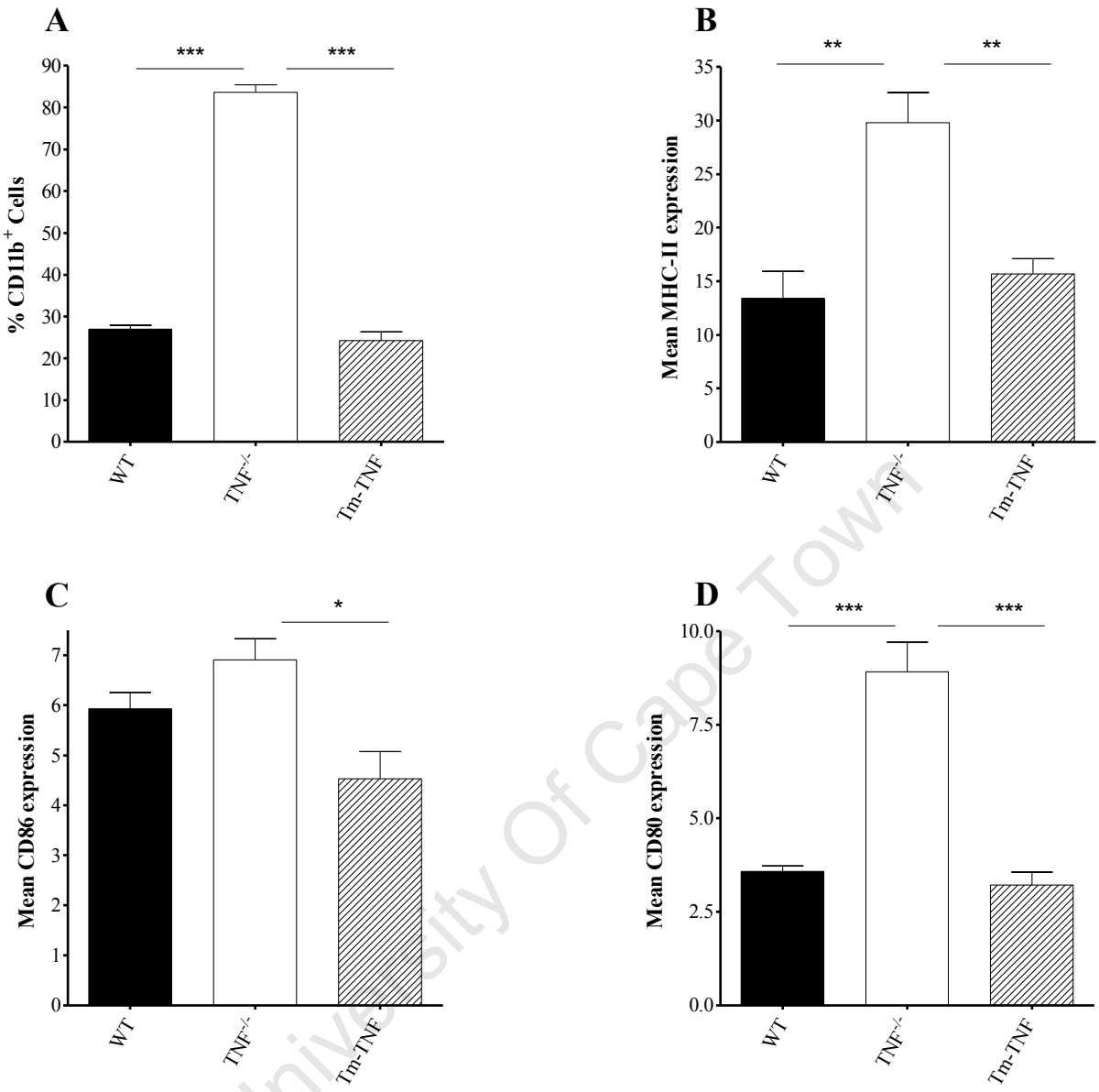


Figure 4.1.ii. Tm-TNF is sufficient to facilitate influx of CD11b$^+$ cells and cell activation. WT mice (black bars), TNF$^{-/-}$ (white bars) mice and Tm-TNF (striped bars) mice were challenged by aerosol inhalation infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lung single cell suspensions derived from respective mice groups at 35 days post-infection were stained for CD11b expression and analyzed by flow cytometry. The frequency of CD11b$^+$ cells (A) and the mean surface expression levels (MFIs) of MHC-II (B), CD86 (C), and CD80 (D) gated on CD11b$^+$ cell subpopulation was determined. The data are representative of 1 of 2 experiments performed and the results are expressed as mean ± SD of 4 animals/group. Significant differences (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$) were obtained using ANOVA.

Several studies have shown that the destructive pathology observed in TNF deficient mice involved presence of large quantities of activated Th1 cells at sites of *M. tuberculosis* infection (Saunders et al., 2005; Zganiacz et al., 2004). Lung single suspensions were generated from WT mice, $TNF^{-/-}$ mice and Tm-TNF mice 35 days post-infection and the numbers of $CD4^+$ T cells infiltrating the lung and their activation state reflected by cell surface expression of CD44 on $CD4^+$ T cells (gating strategy, Fig. 4.2.i) was determined by flow cytometry. We confirmed previous reports demonstrating that compared to WT mice, $TNF^{-/-}$ mice displayed significantly increased ($p < 0.01$) lung $CD4^+$ T cell numbers (Fig. 4.2.iiA) with an activated phenotype shown by the increased mean cell surface expression of CD44 molecule (Fig. 4.2.iiB) analyzed 35 days post-infection, in contrast, Tm-TNF mice displayed comparable $CD4^+$ T cell numbers (Fig. 4.2.iiA) infiltrating the lung with similar activation state to WT mice (Fig. 4.2.iiB). Thus dysregulated pulmonary $CD4^+$ T cell influx and hyperactivation is corrected by presence of Tm-TNF molecule. However, the increased frequency and the increased activation status of antigen presenting cells may facilitate the heightened Th1 immune response in complete absence of TNF.

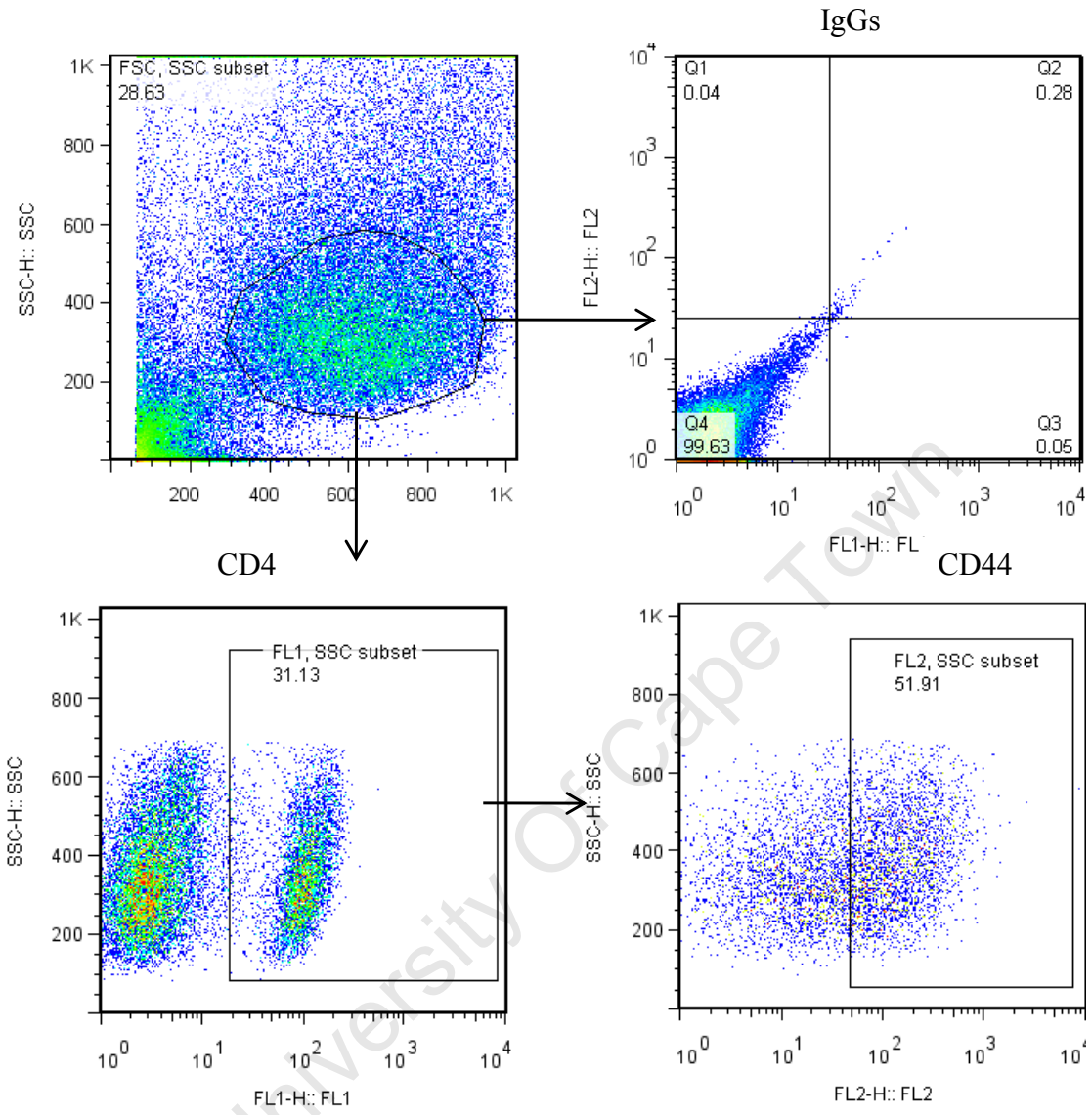


Figure 4.2.i. Gating strategy. Lung single cell suspensions derived from WT mice, $TNF^{-/-}$ mice and Tm-TNF mice were prepared 35 days after aerosol inhalation infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Cells were stained with irrelevant antibody (IgG) or anti-CD4 and the MFIs of CD44 gated on $CD4^{+}$ T cell subpopulation was determined by flow cytometry.

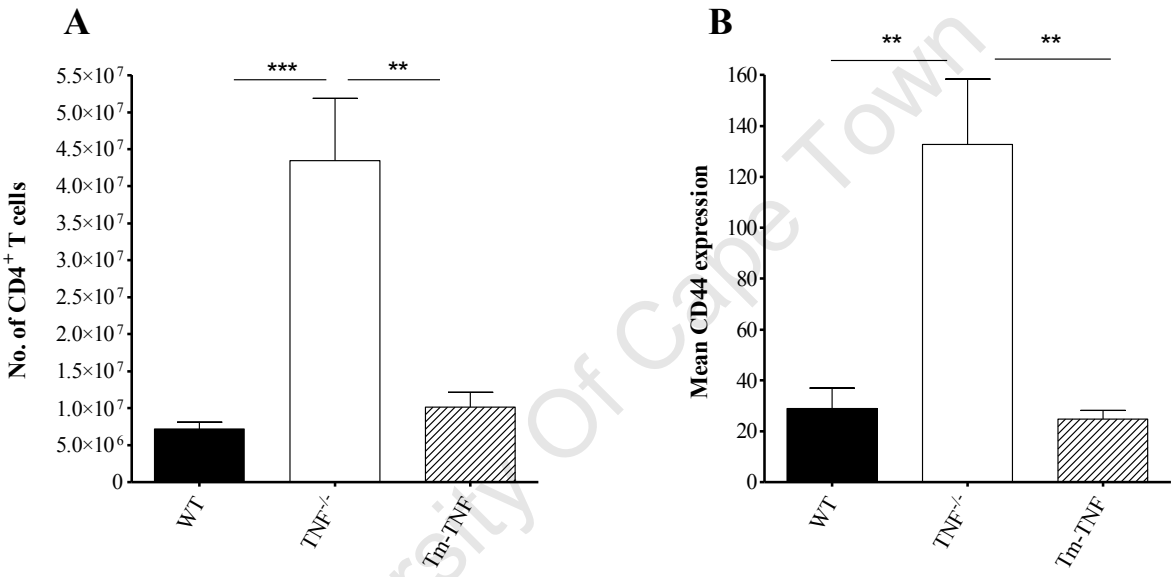


Figure 4.2.a: Tm-TNF mediates a controlled frequency and cellular activation by pulmonary CD4+ T cells. WT mice (black bars), $TNF^{-/-}$ (white bars) mice and Tm-TNF (striped bars) mice were challenged by aerosol inhalation infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lung single cell suspensions derived from respective mice groups at 35 days post-infection were stained for CD4 or CD44 expression then analyzed by flow cytometry. The frequency of $CD4^+$ T cells (A) and the mean surface expression levels (MFIs) of CD44 (B) gated on $CD4^+$ T cell subpopulation was determined. The data are representative of 1 of 2 experiments performed and the results are expressed as mean ± SD of 4 animals/group. Significant differences (** $p < 0.01$) were obtained using ANOVA.

Controlled pulmonary cytokine induction and iNOS expression during acute M. tuberculosis infection in Tm-TNF mice.

Previous studies have shown that both TNF and IFNγ are required for optimum macrophage activation and that IL-10 regulates Th1 responses during mycobacterial infection (Frankova and Zidek, 1998; Jacobs et al., 2000a). IL-12 has been implicated in previous reports as the key factor for the release of IFNγ by NK cells (Natural killer cells) and Th1 cells (Hsieh et al., 1993; Oswald et al., 1994). Furthermore, it has been shown that mice deficient of IL-12 are highly susceptible to mycobacterial infection due to impaired Th1 cytokine responses and defective granuloma formation (Cooper et al., 1997; Wakeham et al., 1998). We therefore examined pulmonary levels of IFNγ, IL-12p70 and IL-10 secretion as determinants of induction and regulation of Th1 immunity during acute *M. tuberculosis* challenge. Lungs were obtained from infected mice and cytokine production was assessed by ELISA at day 35 post-infection; at which point $TNF^{-/-}$ mice displayed susceptibility to infection. Data obtained showed that pulmonary IL-12p70 levels were significantly higher ($p < 0.05$) in $TNF^{-/-}$ mice compared to WT mice at day 35 post-infection, in contrast, Tm-TNF mice displayed comparable levels to WT mice (Fig. 5A). IFNγ levels were found to be 100x higher ($p < 0.001$) in $TNF^{-/-}$ mice compared to WT control mice at day 35 post-infection but in converse, the levels of IFNγ were similar between WT mice and Tm-TNF mice (Fig. 5B). Interestingly, the concentration of IL-10 was found to be significantly higher ($p < 0.01$) in moribund $TNF^{-/-}$ mice relative to WT mice 35 days post-infection but was comparable between Tm-TNF mice and WT mice (Fig. 5C). Therefore, these data suggest that cell surface TNF expression is sufficient to induce protective type 1 immune responses and that solTNF is

required for the negative regulation of this response and in its absence hyper immune activation which is not down regulated by IL-10, drive immunopathology. We therefore, investigated iNOS expression as a contributory factor to susceptibility of $TNF^{-/-}$ mice.

Previous reports have shown that TNF is central for macrophage activation that leads to mycobacterial killing through iNOS dependent and independent mechanisms (Appelberg et al., 1995; Bekker et al., 2001). Lung sections from infected WT mice, $TNF^{-/-}$ mice and Tm-TNF mice were stained for iNOS expression 33 days post-infection. Data obtained revealed that iNOS was expressed in all the respective mice and the expression pattern was associated with granuloma lesions (Fig. 5.D, E & F). These data suggest data iNOS induction on its own is insufficient for controlling mycobacterial growth if the granuloma structure is not properly formed.

Therefore, together the data presented here show that TNF deficiency mediates excessive inflammatory response that results in formation of pathological granulomas that cannot contain mycobacterial growth and this phenotype is corrected by the presence of Tm-TNF during acute *M. tuberculosis* infection.

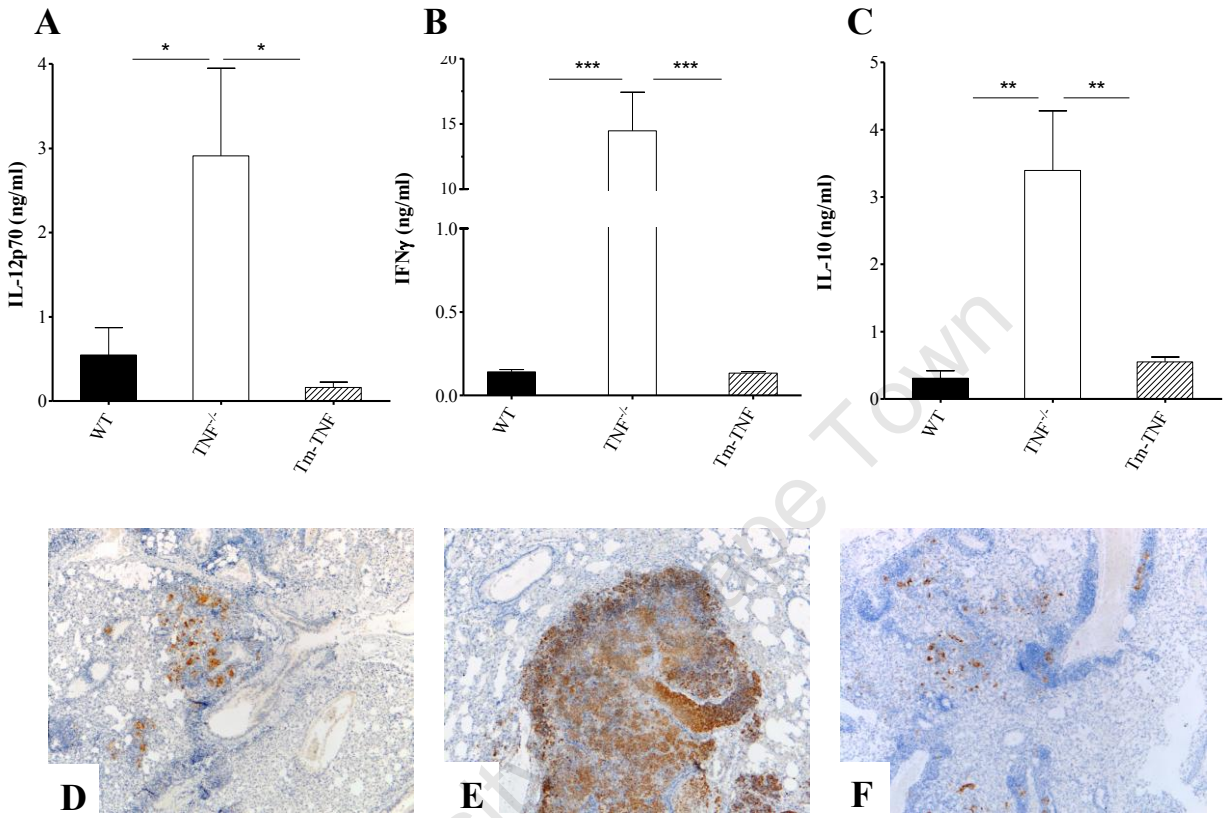


Figure 5. Pulmonary cytokine production is normal in the presence of Tm-TNF and iNOS is expressed. WT mice (black bars), $TNF^{-/-}$ mice (white bars) and Tm-TNF mice (striped bars) were infected with 100 CFUs *M. tuberculosis* H37Rv by aerosol inhalation and the concentration of IL-12p70 (A), IFNγ (B) and IL-10 (C) were measured by ELISA 33 days post-infection. Lung sections from WT mice (D), $TNF^{-/-}$ mice (E) and Tm-TNF mice (F) were removed 33 days post-infection and with polyclonal rabbit anti-mouse antibody. The brown stain represents iNOS expression by activated macrophages. Data are expressed as mean ± SD of 4 animals/group and represent 1 of 3 experiments performed. Significant differences (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$)) were obtained using ANOVA**.** Micrographs represent 4 animals/group and are shown at x32 magnification.

Section II: Tm-TNF and chronic *M. tuberculosis* infection

Summary

This study investigated the contribution(s) of solTNF and Tm-TNF during chronic *M. tuberculosis* infection. We report here that Tm-TNF was insufficient to control chronic infection with low dose aerosol inhalation challenge with virulent *M. tuberculosis* H37Rv as reported for the avirulent *M. bovis* BCG infection (Dambuza et al., 2008). We demonstrate that Tm-TNF mice displayed excess pulmonary and systemic inflammatory response following chronic exposure to *M. tuberculosis* with enhanced cell surface expression of MHC-II, CD80 and CD86 on $CD11b^{+}$ cells in the lungs. Also, we illustrate that granulomas formed in Tm-TNF mice appeared larger involving high proportions of the lung and iNOS expression pattern was dispersed compared to WT control mice resulting in failure to inhibit mycobacterial growth followed by demise of animals. In conclusion, Tm-TNF is not enough to sustain long term protection against chronic *M. tuberculosis* infection and we suggest that solTNF may be required to regulate excess inflammation at later stages of the infection.

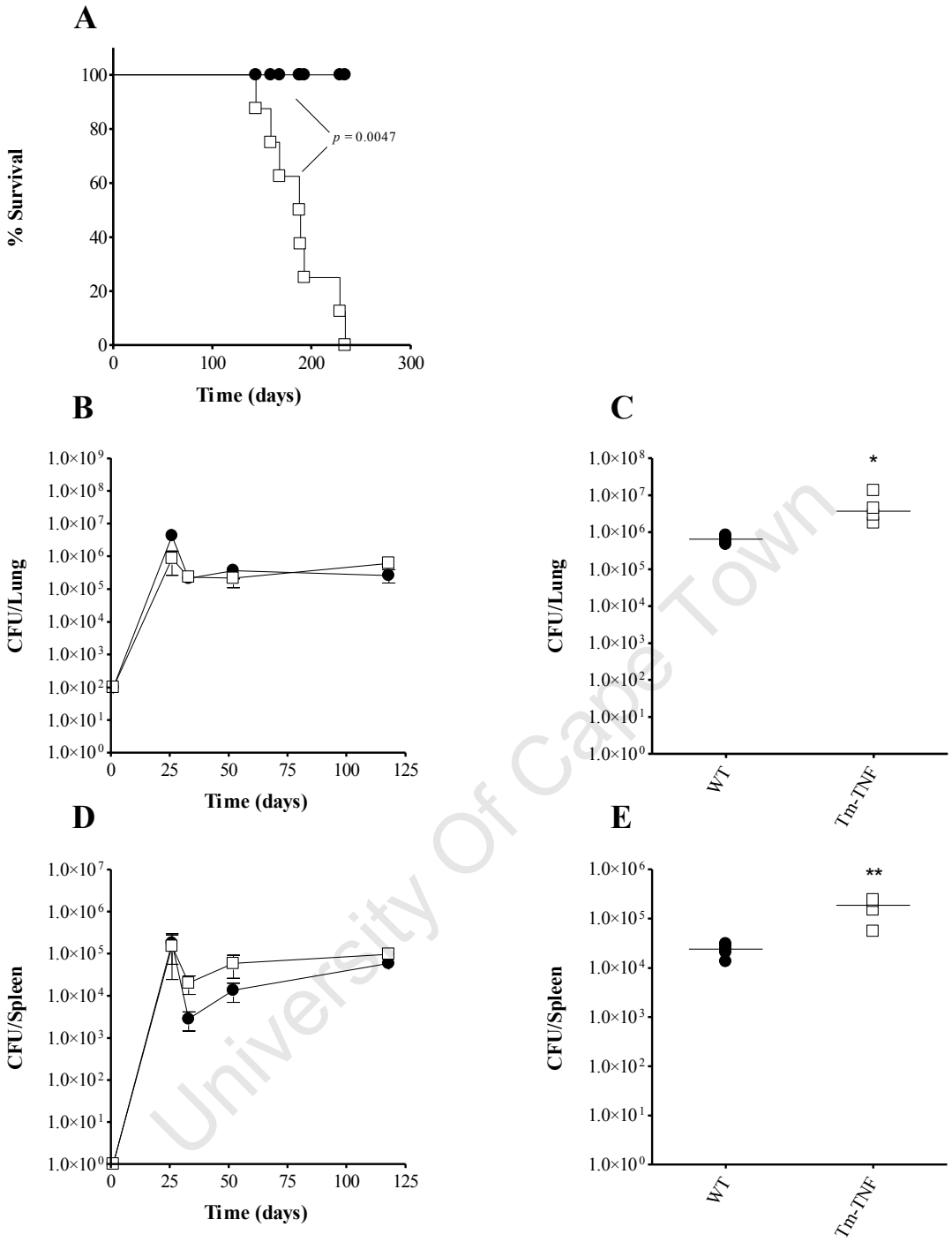
Results

Persistent virulent M. tuberculosis infection is lethal in Tm-TNF mice.

Experimental work conducted in our laboratory using a high dose intranasal challenge ($1x10^6$ CFUs) with non-virulent strain *M. bovis* BCG revealed that, despite a compromised immune response reflected by high bacilli burden in Tm-TNF mice tissues, chronic infection was not lethal. To determine whether Tm-TNF expression alone could provide protection against chronic infection with a virulent strain, WT mice and Tm-TNF mice were exposed to a low dose aerosol infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Mortality rates observed showed that persistent *M. tuberculosis* infection resulted in lethality in Tm-TNF mice with a mean survival time of 189 days compared to WT mice which displayed resistance to infection until the experiment was terminated 350 days post-infection (Fig. 1A). These data show that Tm-TNF does not afford long lasting protection against *M. tuberculosis* infection.

To dissect the components of Tm-TNF lethality to chronic infection, host immune responses were assessed. Firstly, the ability of mice to control bacilli growth during a chronic infection was investigated. WT mice and Tm-TNF mice were killed at specific time points during the infection period and the number of bacilli in the lungs and spleens were determined. Saunders et al., (2005), using the same Tm-TNF knock-in mouse, reported that membrane bound TNF was sufficient to induce mycobactericidal responses evidenced by equivalent bacilli burdens between WT mice and Tm-TNF mice 16 weeks post-infection. We report here that indeed, comparable to WT mice, Tm-TNF mice controlled bacilli numbers in the lungs (Fig. 1B) and spleens (Fig. 1D) for the first 118

days post-infection. However, by 154 days post-infection, there was a significant increase in bacilli numbers in the lungs ($p < 0.05$) and spleens ($p < 0.01$) (Fig. 1C & 1E, respectively) of Tm-TNF mice compared to WT mice. These data demonstrate that Tm-TNF signal alone is not sufficient to induce protective anti-mycobacterial immune responses required for the control of bacilli replication during chronic infection.



TNF mice (white squares) were infected with 100 CFUs/mouse *M. tuberculosis* H37Rv by aerosol inhalation. (A) Mortality rates, Tm-TNF mice succumb by day 189 post-infection. Data represent 1 of 2 experiments performed and are expressed as mean ± SD of 8 mice/group. Bacilli burdens were determined in the lungs (B & C) and spleens (D & E) of infected mice at time points indicated, where C and E represent samples obtained 154 days post-infection. Significant differences (*$p < 0.05$; **$p < 0.01$) were obtained using the Student's *t* test, for % survival the Log Rank test was used for curve comparison.

Uncontrolled inflammatory responses in Tm-TNF mice chronically infected with M. tuberculosis.

TNF is important for maintenance of protective immunity against chronic *M. tuberculosis* infection. A study conducted by Mohan et al*.,* (2001), revealed that TNF neutralization in mice chronically infected with *M. tuberculosis* resulted in histopathological features indicative of an enhanced inflammatory response in the lungs of infected mice which included increased cellularity, squamous metaplasia and granuloma disintegration. In another study, TNF blockade during chronic tuberculosis infection resulted in increased expression of pro-inflammatory cytokines and chemokines in the lungs (Chakravarty et al., 2008). To determine the role of Tm-TNF in mediating an inflammatory response in a persistent *M. tuberculosis* infection, organs were harvested from infected mice and weights were recorded as a surrogate marker of inflammation. Results obtained showed that during early infection (≤ 50 days post-infection), Tm-TNF mice controlled pulmonary inflammation similar to WT mice but displayed significant lung mass increase ($p < 0.05$) at 118 days and 154 days post-infection (Fig. 2A). The observed increased lung mass, particularly at day 118 did not correlate with an increased bacilli burden in the lungs of Tm-TNF mice at this time point (Fig. 1B). This finding was important as it suggested that the confounding factor for excess inflammation was not an increase in the number of bacilli in the lung. In addition, systemic inflammation reflected by splenomegaly was evident as early as 35 days post-infection and, although not significant, was found to be higher in Tm-TNF mice compared to WT mice (Fig. 2B) and further spleen weight increase was evident 118 days ($p < 0.05$) and 154 days post-

infection in Tm-TNF mice (Fig. 2B). These data show that Tm-TNF alone is not sufficient for control of inflammation during persistent *M. tuberculosis* infection.

We then asked whether granuloma structure was maintained as a correlate of susceptibility in Tm-TNF mice during chronic infection. Results obtained revealed that granuloma formation was characterized by distinctly defined structures in WT mice (Fig. 2C) whereas, granulomas form in Tm-TNF mice were larger in size with large areas of the lung tissue involved in inflammation (Fig. 2D) analyzed 154 days post-infection. These data support observations by previous reports (Fremond et al., 2005; Saunders et al., 2005). Therefore, these results suggest that Tm-TNF signal alone is not enough to maintain protective granuloma formation suggesting that solTNF may be required to control cellular infiltration in the lung during persistent *M. tuberculosis* infection.

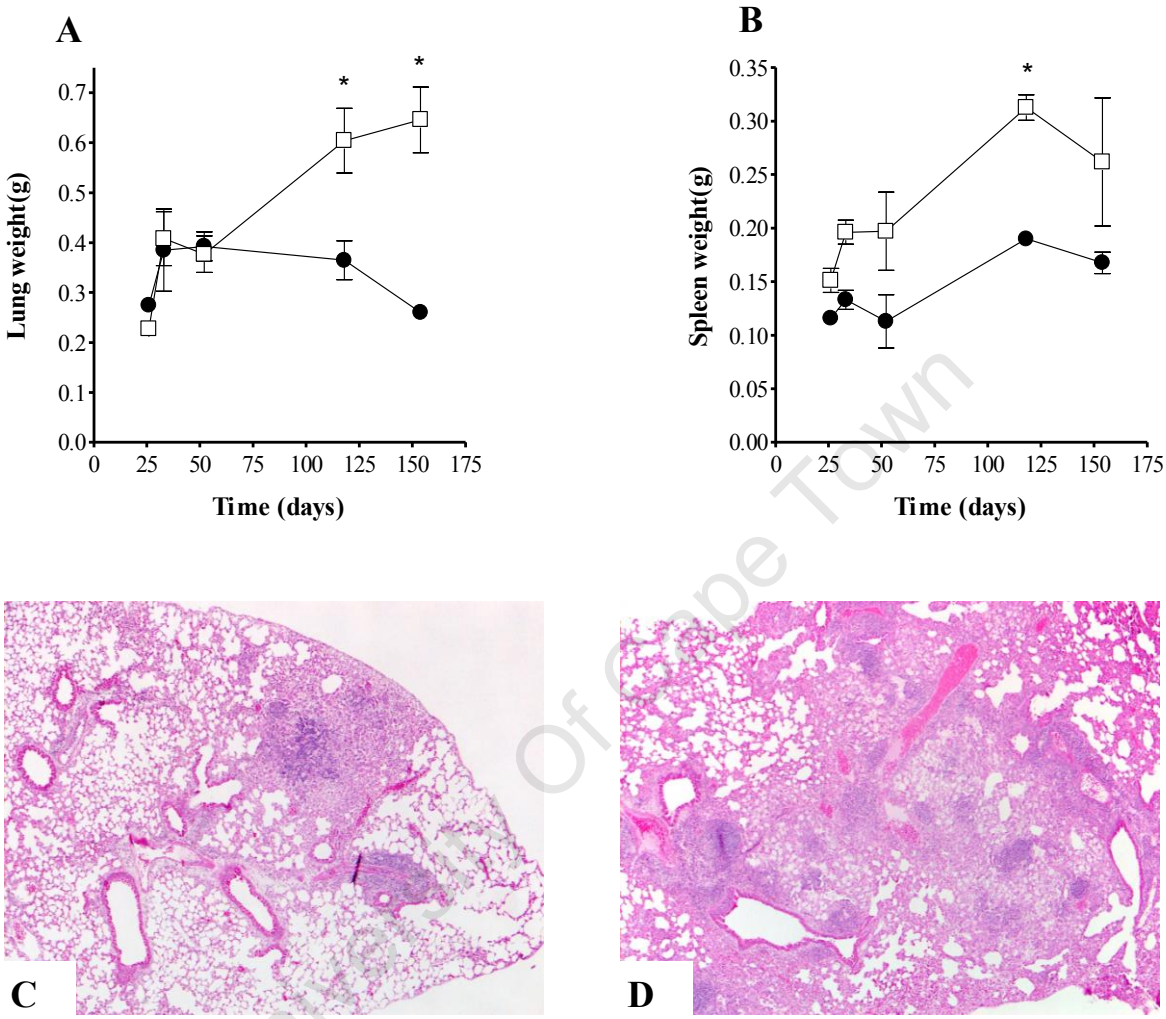


Figure 2. Tm-TNF is not sufficient to control inflammatory response during chronic M. tuberculosis infection. WT mice (black circles) and Tm-TNF mice (white squares) were exposed by aerosol inhalation to 100 CFUs/mouse *M. tuberculosis* H73Rv. (A) Lung and spleen (B) weights were recorded at time points shown to monitor inflammation. Lung sections were removed from WT mice (C) and Tm-TNF mice (D) 169 days post-infection and stained with haematoxylin and eosin. Data are expressed as the mean ± SD of 4 mice/group. Results represent 1 of 3 similar experiments. Significant differences (*$p < 0.05$) were determined by Student's *t* test. Micrographs represent 4 animals/group and shown at x32 magnification.

Enhanced activation of lung infiltrating CD11b^{+} cells in chronic M. tuberculosis infected Tm-TNF mice.

Next we compared the level of activation of lung infiltrating macrophages in WT mice and Tm-TNF mice during chronic *M. tuberculosis* infection. Previous studies have associated increased macrophage activation with the induction of severe tissue injury (Laskin and Pendino, 1995). In addition, Guler et al., (2004), showed that enhanced iNOS production induced tissue injury during mycobacterial infection. We therefore assessed the expression of MHC-II, CD80 and CD86 on CD11b^{+} cells as indicators of cellular activation and the level of iNOS expression in WT mice and Tm-TNF mice during chronic infection. Data obtained revealed that the frequency of CD11b^{+high} expressing cells (gating strategy, Fig. 3.1.i) was equivalent in both strains (Fig. 3.1.iiA). However, the analyses of cell surface expression levels of MHC-II (Fig. 3.1.iiB), CD86 (Fig. 3.1.iiC) and CD80 (Fig. 3.1.iiC) gated on CD11b^{+high} cells showed an increase ($p < 0.05$) in the mean fluorescence intensity for all three cellular markers in Tm-TNF mice compared to WT mice. iNOS expression pattern was compact and localized to translucent epitheloid macrophage cells confined to well-structured granulomas within lungs of WT mice (Fig. 3.2A). In contrast, lungs of Tm-TNF mice showed a dispersed staining pattern of iNOS expression reflecting the dissolute granuloma structure (Fig. 3.2B). These data indicate that Tm-TNF mediate an increased pulmonary macrophage activation which is not protective during the chronic phase of infection.

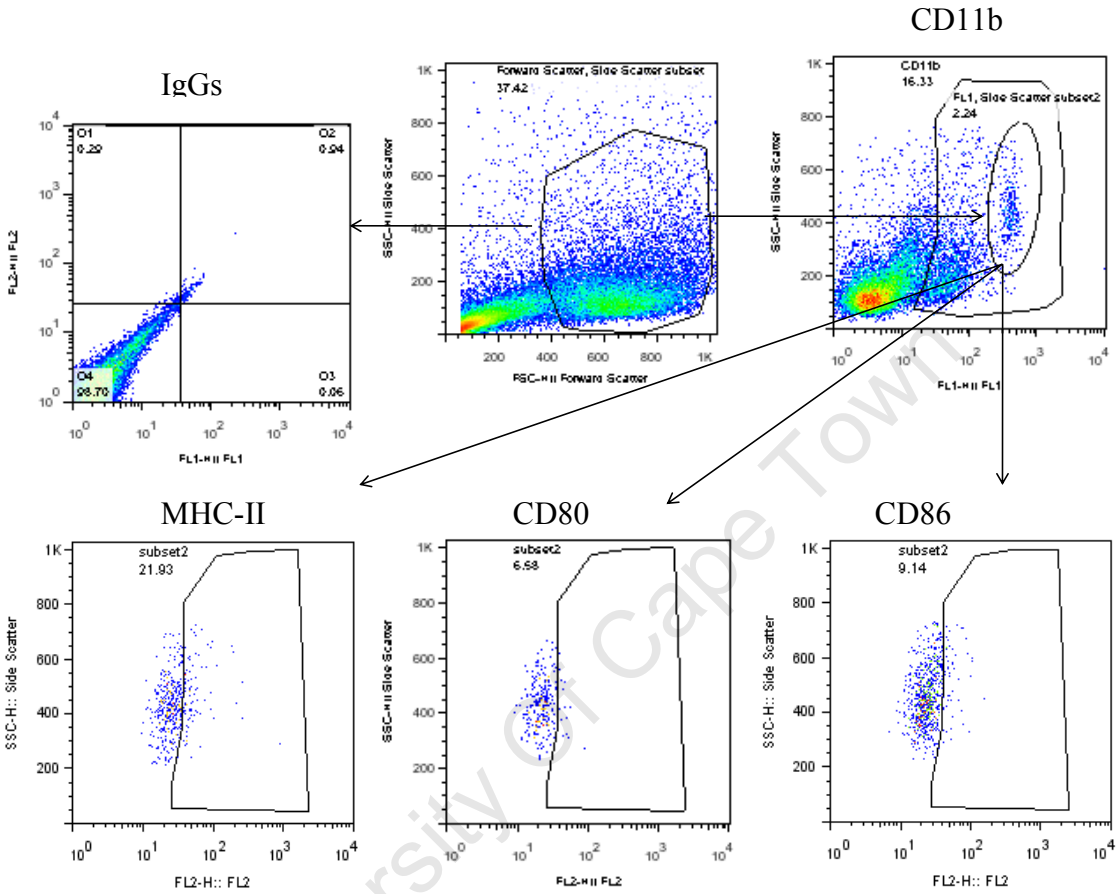
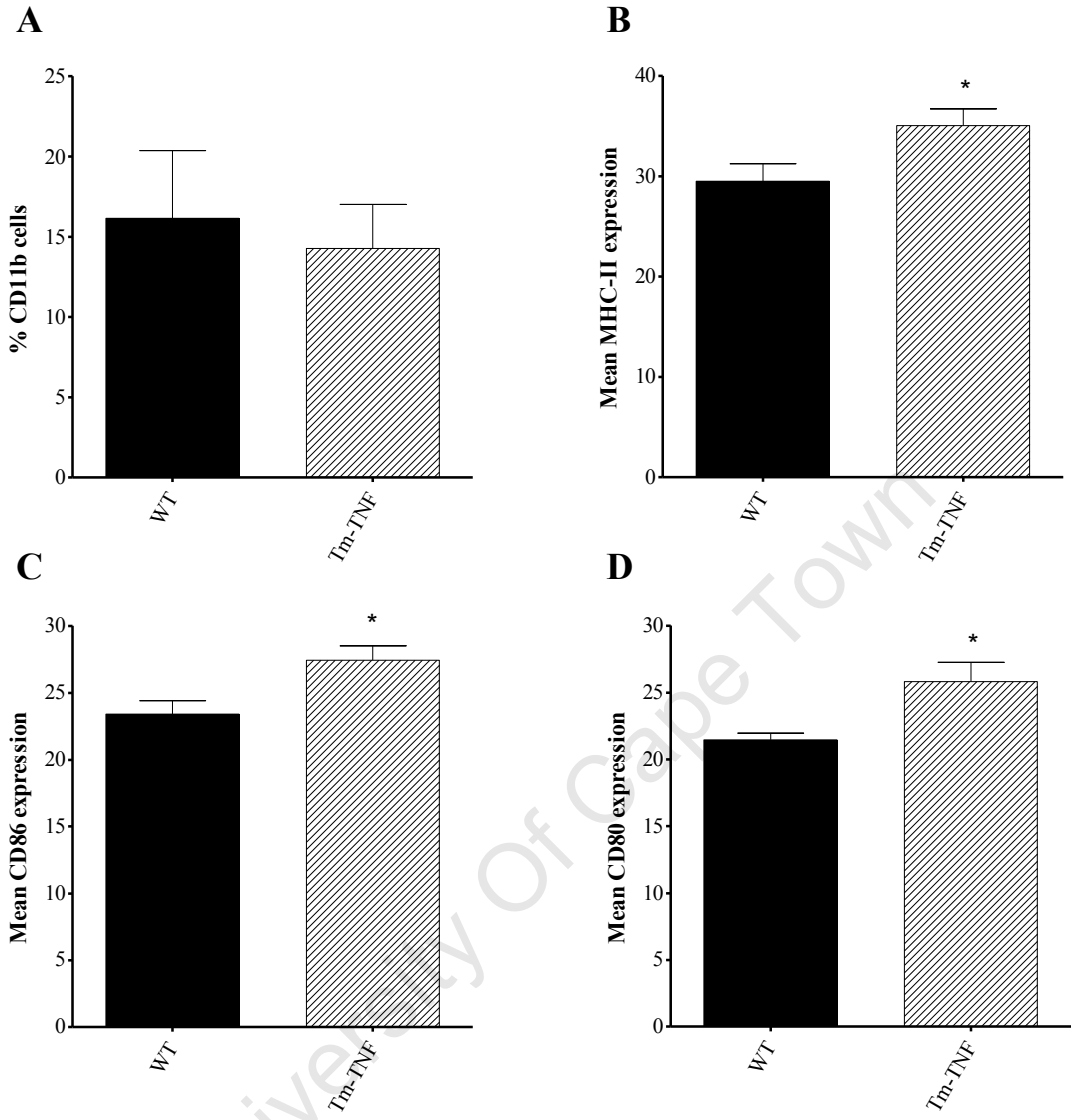


Figure 3.1.i. Gating strategy. Lung single cell suspensions derived from WT mice and Tm-TNF mice were prepared 169 days after aerosol infection with 100 CFUs/mouse *M. tuberculosis* H37Rv. Cells were stained with irrelevant antibody (IgG) or anti-CD11b and the MFIs of MHC-II, CD80 and CD86 gated on the $CD11b^{+\,high}$ subpopulation were determined by flow cytometry.



infection. WT mice (black bars) and Tm-TNF mice (striped bars) were exposed by aerosol inhalation to 100 CFUs/mouse *M. tuberculosis* H73Rv. The frequency of $CD11b^+$ cells (A) infiltrating the lung was determined at day 169 post-infection. The mean surface expression levels (MFIs) of MHC-II (B), CD86 (C) and CD80 (D) on $CD11b^+$ cells was analyzed by flow cytometry. Data represent 1 of 2 similar experiments and are expressed as the mean ± SD of 4 mice/group. Significant differences (*$p < 0.05$) were determined by Student's *t* test.

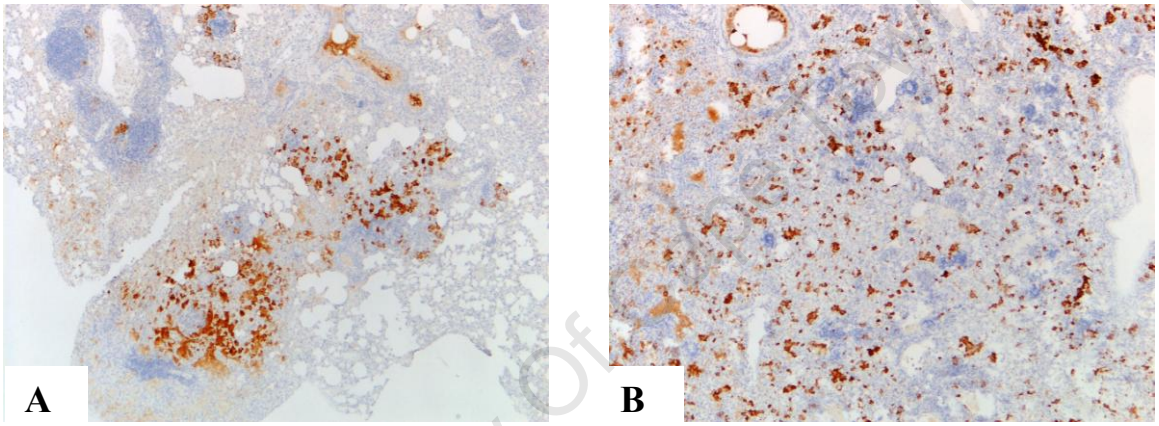


Figure 3.2. Enhanced iNOS expression in Tm-TNF mice during chronic infection. WT mice and Tm-TNF mice were challenged via aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lungs were obtained at day 169 post-infection and immunohistochemical staining was performed with polyclonal rabbit anti-mouse antibody. The brown stain is indicative of iNOS-positive macrophages. Micrographs represent 4 animals/group and shown at x32 magnification.

Increase in inflammatory cytokines mediates a non-protective immune response in Tm-TNF mice chronically infected with M. tuberculosis.

IL-12 and IFNγ have been shown to be important for protective immunity against *M. tuberculosis* infection because of the roles they have in activating and development of Th1 T cells (Cooper et al., 1993; Cooper et al., 1995). Additionally, IFNγ has been reported to collaborate with TNF for optimum macrophage activation for killing of intracellular pathogens including mycobacteria (Ding et al., 1988; Flesch and Kaufmann, 1990). Chakravarty and colleagues (2008) showed that in mice chronically infected with *M. tuberculosis*, TNF neutralization resulted in increased lung mRNA expression of IL-12p40 and IFNγ, suggesting an anti-inflammatory role for TNF. We quantified the concentration of IL-12p70 and IFNγ in relation to susceptibility observed in Tm-TNF mice during chronic *M. tuberculosis* infection. Lungs from infected mice were harvested at specific time points and the quantity of IL-12p70 and IFNγ was determined by ELISA. Shown in Figure 4A, the amount of IL-12p70 in Tm-TNF mice was slightly higher than WT mice with no significant difference attained at 154 days post-infection. In both groups, the levels of IL-12p70 declined by day 169 post-infection but Tm-TNF mice had significantly higher ($p < 0.01$) concentration compared to WT mice (Fig. 4A). IFNγ levels were found to be significantly higher in Tm-TNF mice compared to WT mice at 154 days ($p < 0.01$) and 169 days ($p < 0.05$) post-infection (Fig. 4B). These data support the earlier observation of increased inflammatory response in Tm-TNF mice (Fig. 2A). Next, we measured the level IL-10 because of its reported ability to down modulate Th1 responses (Gong et al., 1996). Results obtained showed comparable IL-10 levels at day 154 post-infection in both strains but, in contrast to WT mice, a significant rise ($p < 0.05$)

in IL-10 concentration was observed in Tm-TNF mice at day 169 post-infection (Fig. 4C). These data suggest that in absence of solTNF, increased IL-12p70 and IFNγ mediate excessive inflammation during the late stages *M. tuberculosis* infection and the elevated IL-10 production suggest a counter response by the host due to excess inflammation.

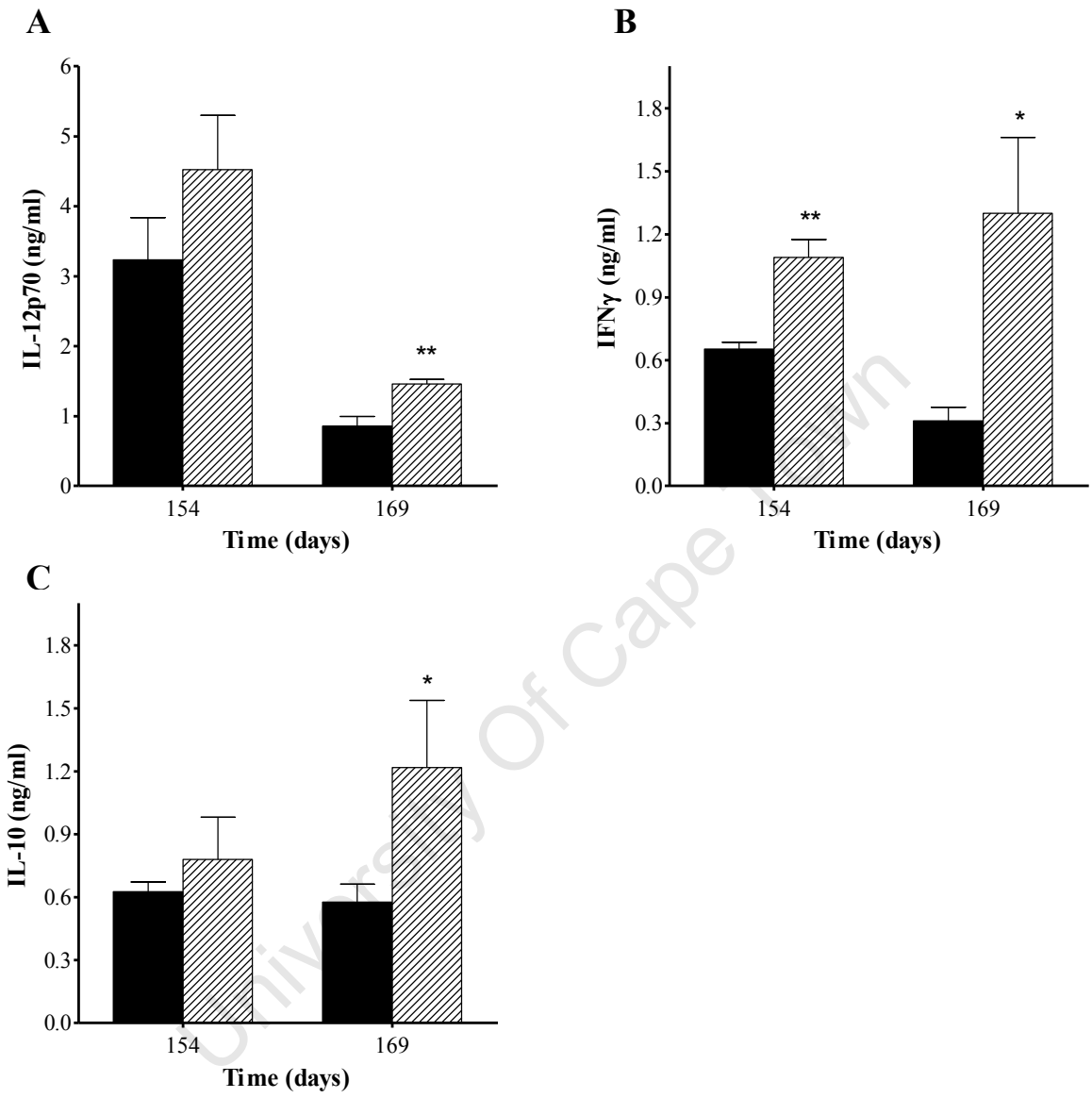


Figure 4. Increased cytokine production in Tm-TNF mice during chronic M. tuberculosis infection. WT mice (black bars) and Tm-TNF mice (striped bars) were challenged by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lungs were obtained from infected mice at 154 days and 169 days post-infection and the amount of IL-12p70, IFN_{γ} and IL-10 present in the homogenates was determined by ELISA. Data are a representation of 3 similar experiments and the values are expressed as mean ± SD of 4 animals/group. Significant differences (*$p < 0.05$; **$p < 0.01$) were determined by Student's *t* test.

Section III: Tm-TNF and *M. tuberculosis* reactivation murine model

Summary

This study investigated the contribution(s) made by solTNF and Tm-TNF in immune responses generated against reactivation tuberculosis. We employed a chemotherapy induced tuberculosis reactivation model, whereby groups of mice were challenged by aerosol infection with low dose *M. tuberculosis* for three weeks to establish infection followed by 25 mg/Kg INH and 25 mg/kg RIF treatment for six weeks in drinking water, after which therapy was terminated and tuberculosis reactivation was investigated. We demonstrate that complete absence of TNF results in host susceptibility to *M. tuberculosis* reactivation in the presence of established mycobacteria-specific immunity with mice displaying unrestricted bacilli growth and diffused granuloma structures compared to WT control mice. Interestingly, our data illustrate that bacterial re-emergence in Tm-TNF mice is contained during the initial phases of tuberculosis reactivation, sustaining immune pressure in a manner comparable to WT mice. We demonstrate that Tm-TNF mice however, show susceptibility to long term *M. tuberculosis* exposure associated with uncontrolled influx of immune cells in the lungs and reduced IL-12p70, IFNγ, enlarged granuloma structures, and failure to contain mycobacterial replication relative to WT mice. In conclusion, we demonstrate that both solTNF and Tm-TNF are required for maintaining immune pressure even after mycobacteria-specific immunity has been established.

Results

Tm-TNF protects mice from severe tuberculosis reactivation.

In a study performed by McCune et al., (1966), it was observed that immunizing mice with *M. tuberculosis* then reinfecting them preceding treatment with anti-tuberculous drugs resulted in mice reactivating with lower *M. tuberculosis* CFU numbers compared to control groups. This result was interpreted as the influence of the host's acquired immune resistance to mycobacteria. In this study, we investigated the contribution of two molecular forms of TNF, in particular Tm-TNF in host immunity in mice immunized by antecedent infection with *M. tuberculosis*. The model used entailed aerosol inhalation infection of WT mice, $TNF^{-/-}$ mice and Tm-TNF mice with 100 viable *M. tuberculosis* H37Rv bacilli. The infection was allowed to progress for 21 days before commencement of treatment with 25 mg/Kg INH and 25 mg/kg RIF in drinking water for 6 weeks to reduce bacilli numbers to at least less than 100 CFUs in the lungs after which treatment was withdrawn and tuberculosis reactivation was monitored.

Firstly, body weights were recorded throughout the infection period and body weight loss was interpreted as severe disease due to reappearing tuberculosis and correlated with susceptibility to infection. We found that that WT mice showed a steady increase in body weights over the duration of the infection in contrast to $TNF^{-/-}$ mice which displayed significantly lower body weights ($p < 0.05$) between 69 days and 116 days post-infection (Fig. 1A). $TNF^{-/-}$ mice also appeared sick with ruffled fur and hunched backs and eventually became moribund. In contrast, Tm-TNF showed an increase in body weights comparable to WT mice for the first 130 days post-infection (Fig. 1A) but significant

weight loss ($p < 0.05$) was recorded in Tm-TNF mice between 237 days and 265 days post-infection while the body weights in WT mice remained stable (Fig. 1A). For the remainder of the experimental period, Tm-TNF mice maintained slightly lower body weights relative to WT mice with no significant differences observed (Fig. 1A) but exhibited no physical signs of severe disease apparent in $TNF^{-/-}$ mice. These data indicate that complete absence of TNF renders mice susceptible to severe reactivating tuberculosis which is alleviated by the presence of Tm-TNF.

Secondly, culturable bacilli numbers in lungs and spleens of infected mice were determined at specific time points to investigate the effect of pre-existing immunity on recrudescence mycobacteria in WT mice, $TNF^{-/-}$ mice and Tm-TNF mice. Bacilli burdens in WT mice were reduced by 3.5 $\log_{10}$ in the lungs (Fig. 1B) and by 2.5 $\log_{10}$ in the spleens (Fig. 1C) after exposure to 6 weeks INH-RIF treatment. Withdrawal of treatment resulted in spontaneous *M. tuberculosis* reactivation with bacilli burdens reaching up to 4 $\log_{10}$ in lungs and 2.5 $\log_{10}$ in the spleens (Fig. 1B & C, respectively). In the contrary, INH-RIF treatment in $TNF^{-/-}$ mice reduced bacilli burdens by 6.5 $\log_{10}$ in the lungs (Fig. 1B) and 4.5 $\log_{10}$ in the spleens (Fig. 1C) after 6 weeks exposure. The observed significant reduction in ($p < 0.05$) bacterial burdens in the lungs of $TNF^{-/-}$ mice relative to WT mice after the 6 weeks INH-RIF treatment regimen (Fig. 1B) is consistent with published data on the hypothesis that release of immune pressure is intimately associated with improved antibiotic-mediated mycobacterial clearance (Horne, 1960). Within 70 days post-treatment, mycobacteria reappeared in $TNF^{-/-}$ mice and bacilli burdens reached at least 6 $\log_{10}$ in the lungs (Fig. 1B) and 4 $\log_{10}$ in the spleens (Fig. 1C).

Interestingly, Tm-TNF mice responded to INH-RIF treatment in a manner comparable to WT mice and showed a similar slow kinetic in the rate of reactivation comparable to that of WT mice, however, by 259 days post-treatment, bacterial burdens in the lungs of Tm-TNF mice increased significantly ($p < 0.05$) by 2 $\log_{10}$ compared to 1 $\log_{10}$ increase observed in WT mice (Fig. 1B). Bacilli burdens in the spleens of Tm-TNF mice were found to be comparable to WT mice in all the time points investigated (Fig. 1C). These data show that Tm-TNF plays a role in early bacterial killing mechanisms even after the host has encountered *M. tuberculosis* previously. And, although Tm-TNF confers protection against recrudescence *M. tuberculosis*, solTNF is crucial for long term control of the infection.

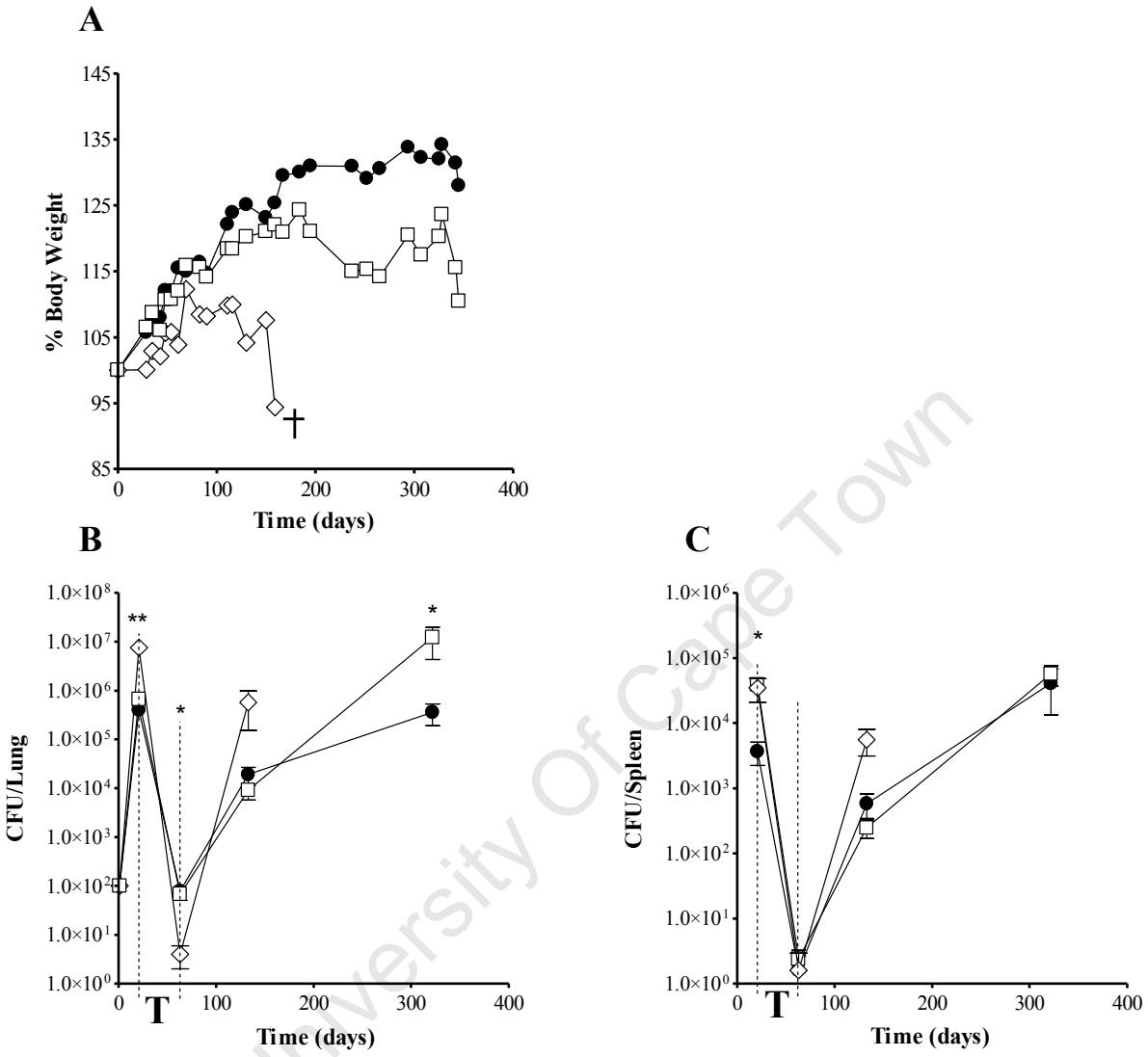


Figure 1. Progression of M. tuberculosis infection in the presence of specific immunity. WT mice (black circles), $TNF^{-/-}$ mice (white diamonds) and Tm-TNF mice (white squares) were treated for 6 weeks with 25 mg/Kg INH and 25 mg/kg RIF in drinking water subsequent to 3 weeks aerosol infection with 100 *M. tuberculosis* H37Rv. (A) Body weights were recorded throughout the course of the infection period and bacterial burdens in lungs (B) and spleens (C) were enumerated at time points indicated above. Data are representative of two experiments and data points are expressed as the mean ± SD of 5 mice/group (for CFUs). The body weight study consisted of between 6 – 16 mice/group. Significant differences (*$p < 0.05$; **$p < 0.01$) were determined by Student's *t* test for comparisons between two groups and ANOVA for comparisons between three groups.

Abnormal inflammatory response in the absence of total TNF during tuberculosis reactivation is delayed in Tm-TNF mice.

TNF has previously been shown to be at the apex of inflammatory responses (Algood et al., 2005). To determine whether Tm-TNF was sufficient in mediating an inflammatory response during tuberculosis reactivation, mouse lung weights were recorded at specific time points during the infection period as a surrogate marker of inflammation. Compared to lung weights determined at day 21 post-infection, WT mice displayed no change in lung weights after exposure to INH-RIF for 6 weeks however; an increase in lung weights was observed during later stages of disease (322 days post-infection) (Fig. 2.1A) which was consistent with the increase in bacilli burdens at this time point (Fig. 1B). In sharp contrast, by 21 days post-infection, $TNF^{-/-}$ mice already displayed significantly ($p < 0.05$) higher lung weights compared to WT mice (Fig. 2.1A). Paradoxically, 63 days post-infection at the end of the therapy period, the lung weights had increased significantly ($p < 0.001$) compared to WT mice (Fig. 2.1A) but did not correlate with the decreased number of culturable bacilli at this time point (Fig. 1B). Susceptibility of $TNF^{-/-}$ mice was confirmed with a further significant increase ($p < 0.001$) in lung weights noted at 113 days post-infection, 50 days after cessation of therapy. In contrast, inflammation in Tm-TNF mice was similar to WT mice during early infection as they maintained consistent lung weights at 21 days, 63 days and 133 days post-infection (Fig. 2.1A). However, control of inflammation was not sustained as a significant increase ($p < 0.01$) in lung weights was evident in Tm-TNF mice at day 322 post-infection compared to WT mice (Fig. 2.1B).

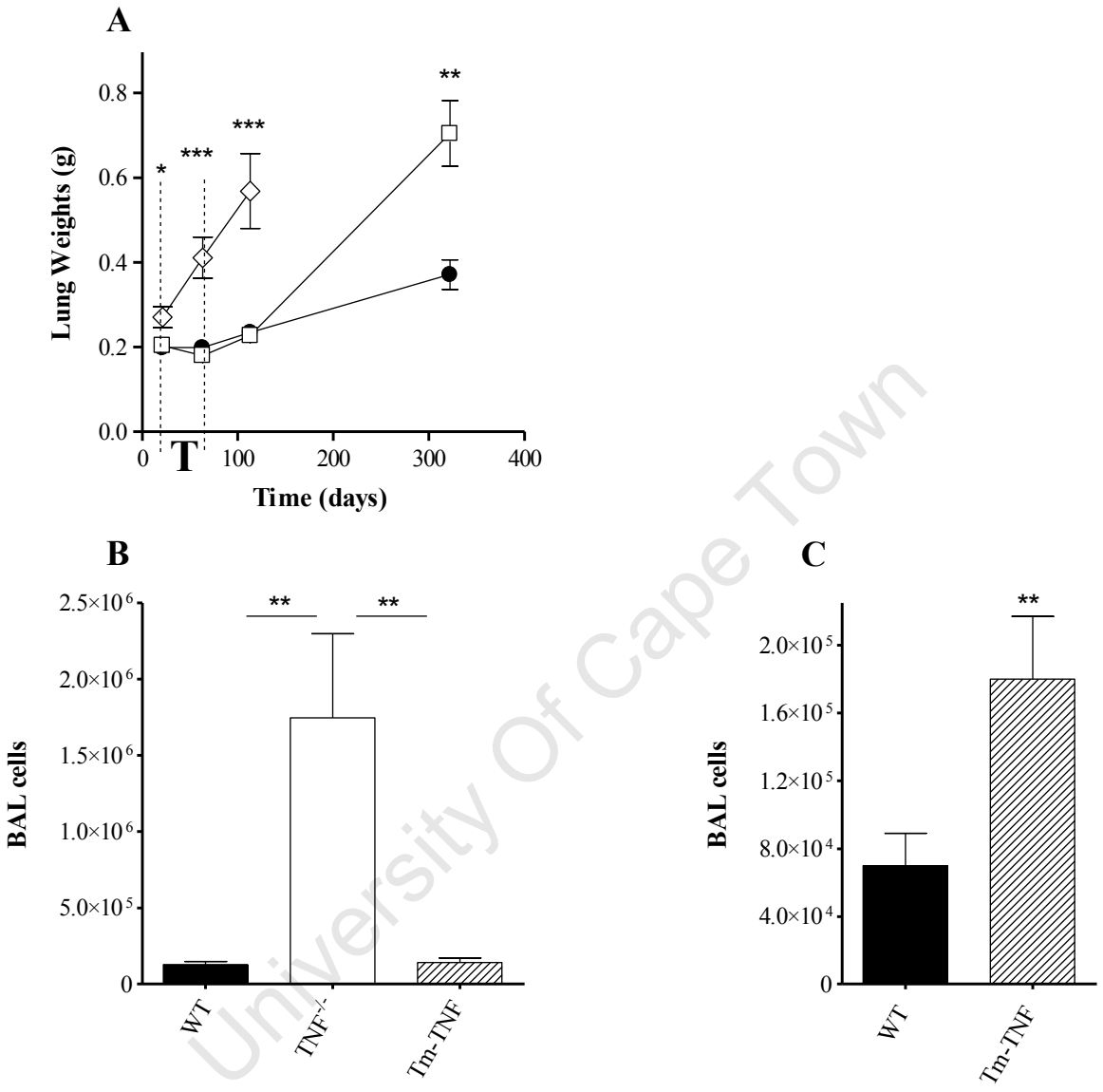


Figure 2.1: Induction of excessive inflammation in the absence of solTNF during reactivation of M. tuberculosis. WT (black circles), $TNF^{-/-}$ (white diamonds) and Tm-TNF (white squares) mice were exposed by aerosol inhalation infection to 100 CFUs/mouse *M. tuberculosis* H37Rv for 3 weeks preceding chemotherapy with 25 mg/Kg INH and 25 mg/kg RIF for 6 weeks in drinking water. (A) Lung weights were measured at specific time points and BAL derived cell numbers were determined 77 days (B) and 378 days (C) post-infection. Data are representative of 1 of 2 experiments performed and are expressed as mean ± SD of 5 mice/group. Significant differences (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) were determined by Student's *t* test for comparisons between two groups and ANOVA for comparisons between three groups.

To quantify inflammation in the respective mouse strains, the number of cells in BAL (bronchoalveolar lavage) fluid was determined after infection. The cellularity in the lavage fluid was found to be significantly higher ($p < 0.01$) in $TNF^{-/-}$ mice compared to WT mice, whereas, Tm-TNF mice had number of cells comparable to WT mice at day 77 post-infection (Fig. 2.1B) confirming that the control of inflammation during early infection was membrane TNF dependent. Consistent with lung weights data, Tm-TNF mice displayed significantly increased ($p < 0.01$) number of cells in lavage fluids relative to WT mice analyzed at day 378 post-infection (Fig. 2.1C). Together, these data indicate that control of early inflammation is mediated primarily by Tm-TNF but solTNF is required for regulation of inflammation during chronic infection. However, the lack of control of inflammation appears to be strongly associated with the onset of tuberculosis reactivation.

We next asked whether granuloma structures were formed in the presence of Tm-TNF molecule during tuberculosis reactivation. Studies performed Mohan et al., (2001), illustrated that TNF was required for maintenance of granuloma structure during persistent *M. tuberculosis* infection whereby upon treatment with TNF-neutralizing antibody, mice displayed severe histopathology marked with excessive inflammation and loss of structured granulomas. Lung sections were obtained from infected WT mice, $TNF^{-/-}$ mice and Tm-TNF mice 133 days post-infection and granuloma structures were analyzed. Depicted in Fig. 2.2A, WT mice displayed small compact lesions with tight lymphocytic wedges and a high degree of clear airway spaces. In sharp contrast, $TNF^{-/-}$ mice showed larger lesions with inflammation occupying larger areas of the lung with

some evidence of necrosis (Fig. 2.2C). These findings were congruent with earlier published findings from our group (Botha and Ryffel, 2003).

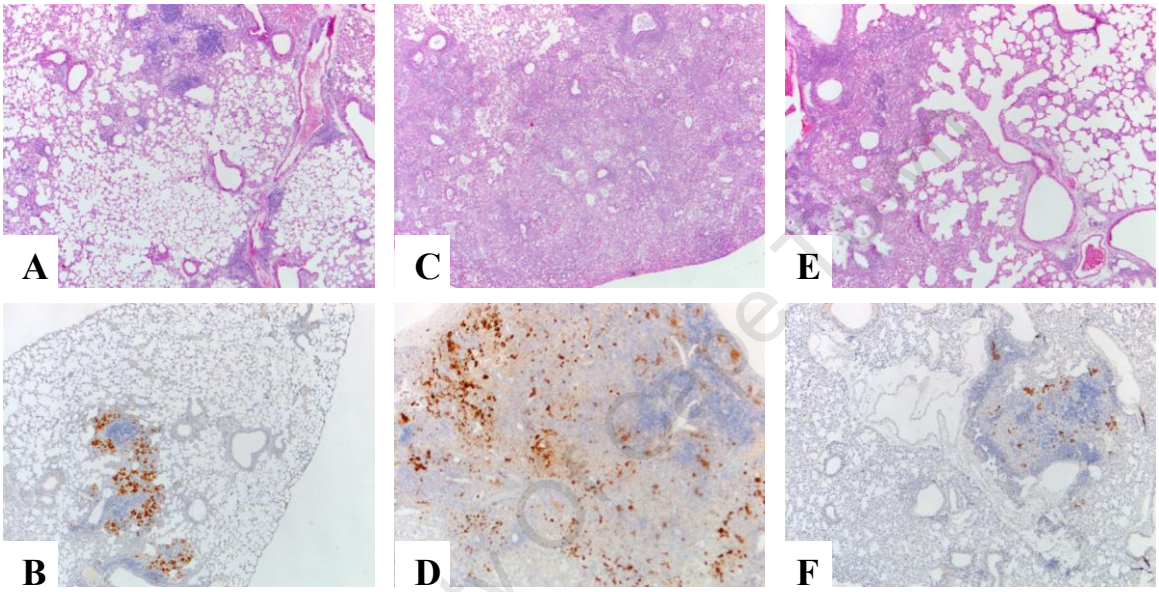


Figure 2.2. Tm-TNF contributes to protective granuloma formation during M. tuberculosis reactivation. WT mice (A & B), $TNF^{-/-}$ mice (C & D) and Tm-TNF mice (E & F) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv for 3 weeks preceding chemotherapy with 25 mg/Kg INH and 25 mg/kg RIF for 6 weeks in drinking water. Lung sections were removed 113 days post-infection and stained with haematoxylin and eosin (A, C & E) to determine the granulomatous response. Sections B, D and F were stained with polyclonal rabbit anti-mouse antibody (see *Materials and methods*). The brown stain represents iNOS expression by activated macrophages. Micrographs represent 4 animals/group and shown at x32 magnification.

In converse, granuloma lesions observed in Tm-TNF were comparable to that of WT mice (Fig. 2.2E) analyzed 133 days post-infection. However, by day 322 post-infection, in contrast to WT granulomas (Fig. 2.3A), Tm-TNF displayed larger lesions with excess inflammatory response and interstitial pneumonia (Fig. 2.3C). These observations demonstrate that Tm-TNF does not afford long term maintenance of protective granuloma structure in the absence of solTNF resulting in malformed lesions that associate with failure to inhibit *M. tuberculosis* growth. We therefore determined whether effector macrophage anti-mycobacterial function was intact in Tm-TNF mice.

Studies have shown that a cell mediated mycobacterial killing function is achieved through production of toxic reactive intermediates (RNI) via the enzymatic action of macrophage iNOS (Chan et al., 1995; Flynn et al., 1993). Previously, Flynn et al., (1998), demonstrated that inhibition of iNOS in mice chronically infected with *M. tuberculosis* resulted in reactivation of tuberculosis disease with increased organ bacillary burdens and extensive granulomatous response. In view of these findings we determined iNOS expression immunohistochemically in lung tissue sections of infected WT mice, $TNF^{-/-}$ mice and Tm-TNF mice. Results obtained demonstrated that iNOS expression pattern was largely within the confinement of granuloma lesions in WT mice (Fig. 2.2B) in contrast to $TNF^{-/-}$ mice where the pattern was dispersed and associated with the diffused granuloma lesions in these mice (Fig. 2.2D) analyzed 133 days post-infection. Tm-TNF mice displayed similar iNOS expression at day 133 days post-infection, however, later during infection (day 322 post-infection), in contrast to WT mice (Fig. 2.3B), more iNOS expression was evident in Tm-TNF with a more random distribution

and some evidence of lung tissue destruction (Fig. 2.3D). Therefore, these observations indicate data iNOS induction on its own is insufficient for controlling mycobacterial growth if the granuloma structure is not properly formed and the increased iNOS expression may be harmful to the host. This is in with line published observations by Guler et al., (2004); showing that iNOS$^{-/-}$ mice were protected from tissue damage in *M. bovis* BCG infected mice.

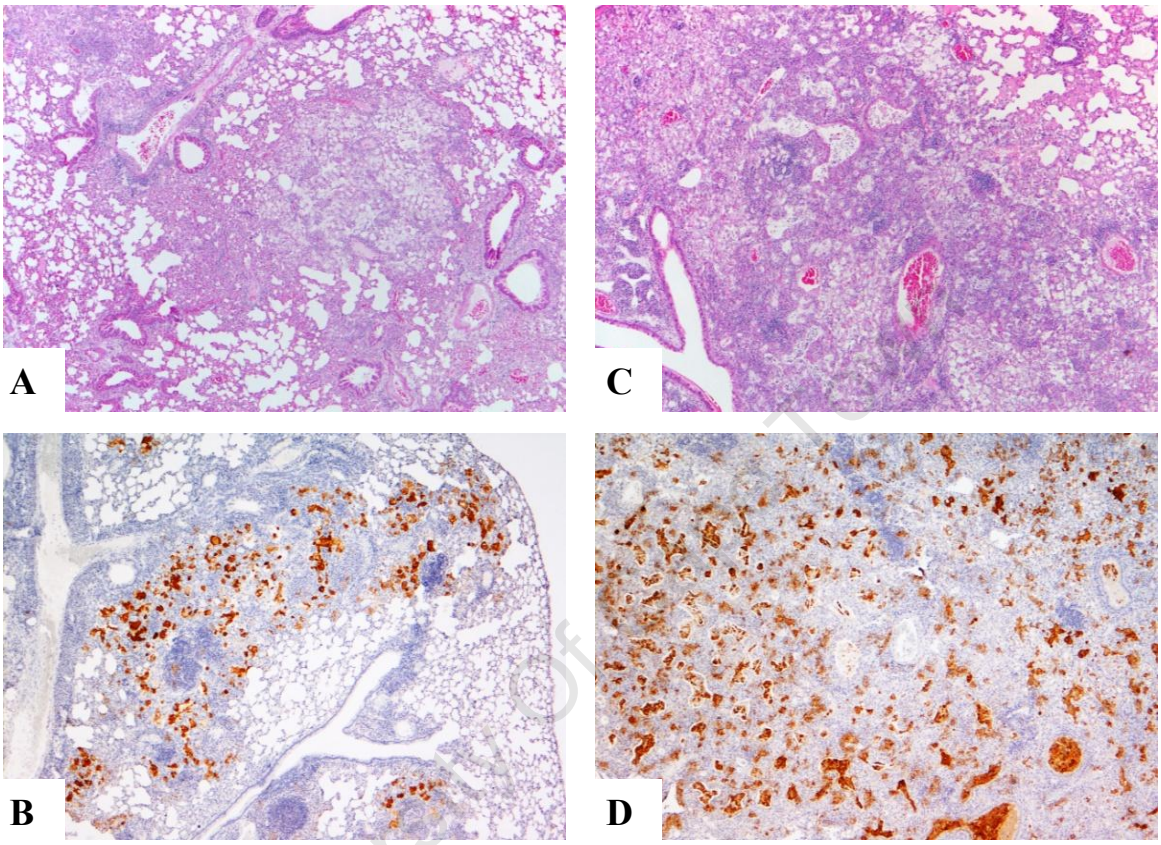


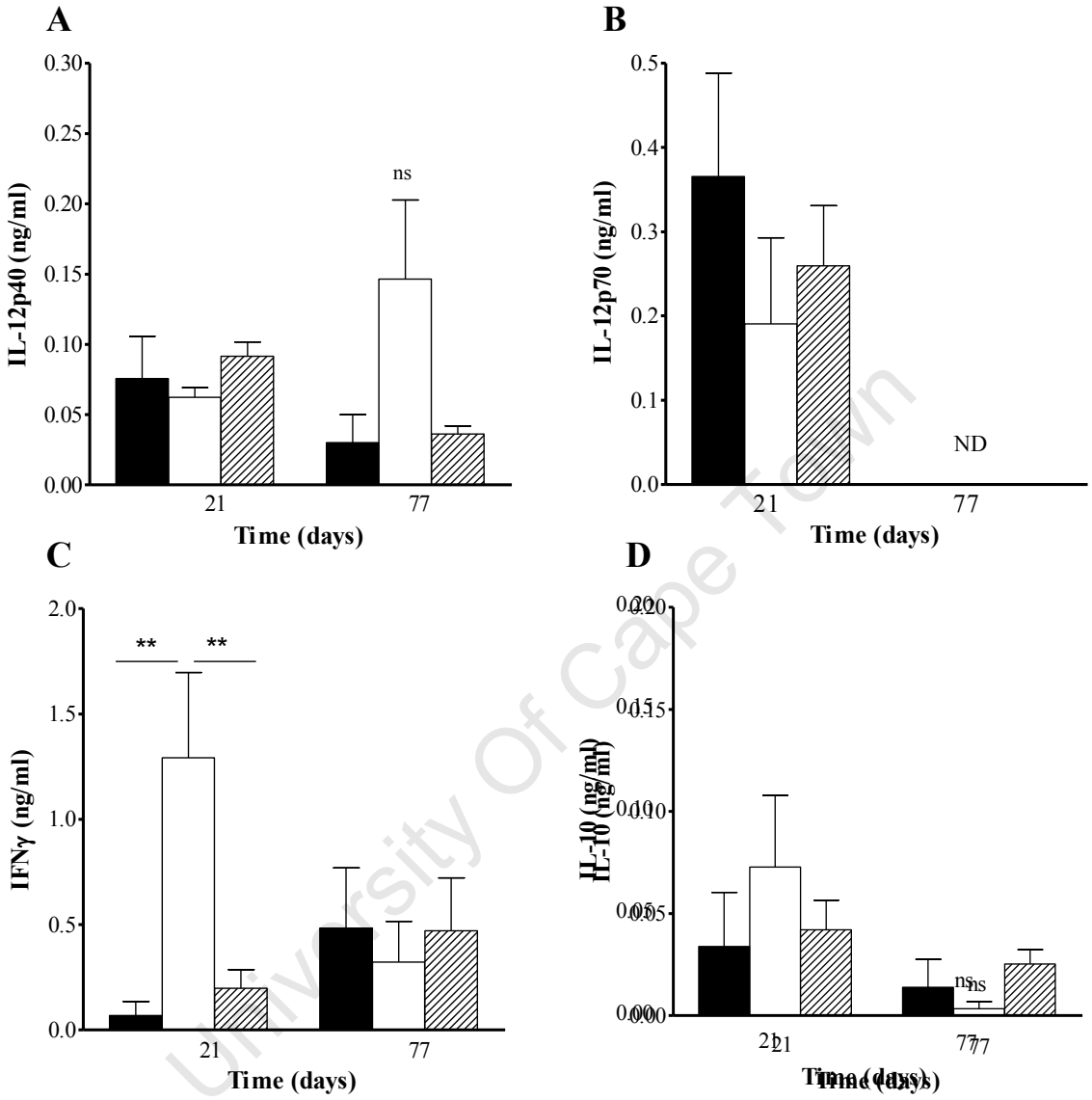
Figure 2.3. Tm-TNF is not sufficient to maintain bactericidal granuloma structures during late phase M. tuberculosis reactivation. WT mice (A & B) and Tm-TNF mice (C & D) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv for 3 weeks preceding chemotherapy with 25 mg/Kg INH and 25 mg/kg RIF for 6 weeks in drinking water. Lung sections were removed 322 days post-infection and stained with haematoxylin and eosin (A, C & E) to determine the granulomatous response. Sections B and D indicate iNOS-positive activated macrophages. Micrographs represent 4 animals/group and shown at x32 magnification.

Breakdown in protective cytokine induction in Tm-TNF mice during late stages of M. tuberculosis reactivation.

Next we assessed immune responses in infected mice prior to and subsequent to INH-RIF treatment. IFNγ and IL-12 levels were quantified because of the reported functions they have in generating and maintaining protective immunity against *M. tuberculosis* infection. Shown in Fig. 3.1A, IL-12p40 levels were comparable in all the strains 21 days post-infection. After exposure to INH-RIF treatment, although not significant, an elevation of IL-12p40 was observed in $TNF^{-/-}$ mice compared to WT mice quantified at day 77 post-infection. Tm-TNF mice displayed a decreasing trend in IL-12p40 levels that were comparable to that of WT mice at day 77 post-infection (Fig. 3.1A). There was no significant difference observed in the expression level of IL-12p70 although there was a decreasing trend in $TNF^{-/-}$ mice and Tm-TNF mice compared to WT mice 21 days post-infection (Fig. 3.1B). No IL-12p70 could be detected in all the strains at day 77 post-infection (Fig. 3.1B). IFNγ was found to be significantly higher ($p < 0.01$) in $TNF^{-/-}$ mice compared to WT mice 21 days post-infection (Fig. 3.1C). In contrast, Tm-TNF mice showed IFNγ levels comparable to WT mice 21 days post-infection (Fig. 3.1C). No differences were observed 77 days post-infection in IFNγ levels in all the strains (Fig. 3.1C). Next we quantified the levels of immunoregulatory cytokine, IL-10. Results obtained showed that IL-10 production was slightly higher in $TNF^{-/-}$ mice compared to WT mice 21 days post-infection but was statistically insignificant, whereas, in Tm-TNF mice the IL-10 values were found to be comparable to that of WT mice (Fig. 3.1D). The concentration of IL-10 measured 77 days post-infection did not vary in all the strains (Fig. 3.1D). Together these data indicate that Tm-TNF mice behave in similar manner to

WT mice with respect to pulmonary expression levels of cytokines investigated and the elevated IFNγ in absence of total TNF after acute *M. tuberculosis* infection correlate with excessive inflammation observed in these mice. And, the reduced cytokines expression levels post INH-RIF treatment is associated with reduced bacilli burdens with exception to IL-12p40 in TNF deficient mice which correlates with excess inflammation.

Studies by Feng et al., (2005), demonstrated that continuous IL-12 production is necessary for maintenance of pulmonary IFNγ-producing effector $CD4^+$ T cells and subsequent bacilli control during chronic *M. tuberculosis* infection suggesting that interruption of IL-12 signal transduction contribute to development of reactivation of tuberculosis. Therefore, we quantified pulmonary IL-12 and IFNγ production during tuberculosis reactivation. Results obtained showed comparable amounts of IL-12p40 (Fig. 3.2A), IL-12p70 (Fig. 3.2B), and IFNγ (Fig. 3.3C) in both WT mice and Tm-TNF mice assessed 133 days post-infection. However, there was a significant decrease in IL-12p70 ($p < 0.01$) and IFNγ ($p < 0.05$) production in Tm-TNF compared to WT mice 322 days post-infection (Fig. 3.2B & C, respectively) which associated with susceptibility of Tm-TNF mice to *M. tuberculosis* reactivation at this time point. IL-12p40 production was maintained to be equivalent levels in both strains 322 days post-infection (Fig. 3.2A). The concentration of anti-inflammatory IL-10 was found to be comparable measured 133 days post-infection but increased in WT mice by day 322 post-infection and remained significantly lower ($p < 0.05$) in Tm-TNF mice. These data suggest that Tm-TNF is not enough to sustain protective cytokine induction in post-infection *M. tuberculosis* immunity and this phenotype is associated with lethality of these mice.



Tm-TNF mice (striped bars) were exposed by aerosol infection to 100 CFU *M. tuberculosis* H37Rv strain for 3 weeks then treated with 25 mg/Kg INH and 25 mg/kg RIF for 6 weeks in drinking water. Bronchoalveolar lavage was performed 21 days and 77 days post-infection and the level of IL-12 p40, IL-12p70, IFN_{γ} and IL-10 present in the fluid was determined by ELISA. Data represent 1 of 2 similar experiments and values are expressed as mean ± SD of 5 animals/group. Significant differences (*$p < 0.05$; **$p < 0.01$, ns: $p > 0.05$) were determined by ANOVA.

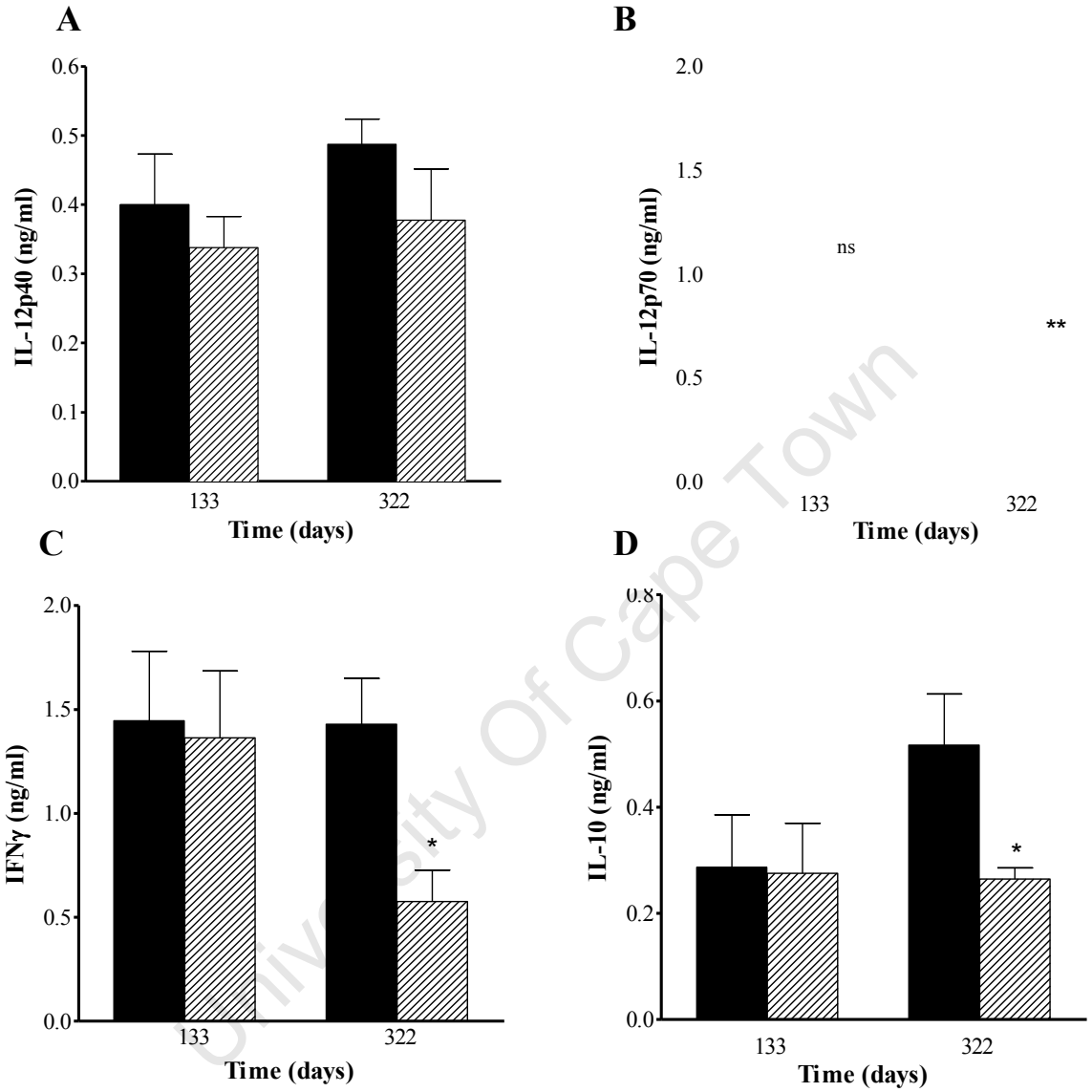


Figure 3.2. Decrease in pulmonary cytokine production is associated with increased susceptibility observed in Tm-TNF mice during M. tuberculosis reactivation. WT (black bars) and Tm-TNF mice (striped bars) were exposed by aerosol inhalation to 100 CFUs/mouse *M. tuberculosis* H37Rv for 3 weeks preceding chemotherapy with 25 mg/Kg INH and 25 mg/kg RIF for 6 weeks in drinking water. Lungs were obtained 133 days and 322 days post-infection and the level of IL-12 p40, IL-12p70, IFN_{γ} and IL-10 present in the homogenates was determined by ELISA. Data represent 1 of 2 experiments performed and values are expressed as mean ± SD of 5 animals/group. Significant differences (*$p < 0.05$; **$p < 0.01$, ns: $p > 0.05$) were determined by Student's *t* test.

Discussion

It has been established that TNF signaling is vital for control of mycobacterial infections (Bean et al., 1999; Botha and Ryffel, 2003; Flynn et al., 1995; Jacobs et al., 2000b; Kindler et al., 1989). Specifically, TNF signaling through the p55TNFR (Flynn et al., 1995), was shown to be important in protective immune responses in mycobacterial infections rather than p75TNFR signaling (Jacobs et al., 2000). In another study, challenging $TNF^{-/-}$ mice with recombinant BCG expressing TNF reconstituted granuloma structure formation and immune responses but this was not observed in p55TNFR deficient mice, supporting the critical role of TNF-p55TNFR signaling in mycobacterial specific immunity (Bekker et al., 2001).

In this study, we investigated the contribution(s) made by the two molecular forms of TNF: solTNF and Tm-TNF in generating protective immunity or immunopathology in different phases of *M. tuberculosis* infection. We used a genetically manipulated knock-in mouse model where the endogenous *TNF* allele was replaced with a non-sheddable cell surface TNF molecule that is under normal expression and regulation with functionality equivalent to its WT counterpart but is resistant to TACE mediated cleavage (Ruuls et al., 2001). Our data confirm that complete absence of TNF results in rapid weight loss, unrestricted mycobacterial replication in the lungs and spleens and death with a mean survival time of 44 days after challenge with *M. tuberculosis* H37Rv. In contrast, we show that Tm-TNF is sufficient to induce protective immune responses, reflected by the ability of Tm-TNF mice to control mycobacterial growth and maintenance of body weight in a manner similar to WT control mice. Tm-TNF however, is insufficient to maintain chronic *M. tuberculosis* infection displaying increased bacilli

burdens and death in contrast to WT mice and these observations are supported by previous reports (Fremond et al., 2005; Saunders et al., 2005). Moreover, our data illustrate that TNF is important for control of recrudescence of *M. tuberculosis* infection. We found that in our drug-induced tuberculosis reactivation model; although there was some degree of inhibition of bacterial replication relative to bacterial replication rate observed during the primary infection, the immune response mounted in $TNF^{-/-}$ mice was insufficient to inhibit propagation of mycobacteria after cessation of antibiotic treatment. This observation confirmed previous findings by Botha and Ryffel (2003), and support previous findings showing that treatment of chronically infected mice (Mohan et al., 2001) or latently infected humans (Dimakou et al., 2004) with TNF neutralizing antibodies results in reappearance of tuberculosis. By contrast, in Tm-TNF mice, mycobacterial replication progressed slowly during tuberculosis reactivation in comparison to primary infection and this rate was comparable to that observed in WT mice indicating that at least in the context of bacilli control, antigen specific effector memory responses mounted were equivalent in both strains. However, Tm-TNF was observed to be insufficient for control of long term mycobacterial containment resembling the outcome of chronic infection. Together, these observations demonstrate that Tm-TNF can elicit mycobacteria-specific recall responses that are able to suppress re-emergence of *M. tuberculosis* infection but there is a requirement of solTNF for maintenance of immune pressure during persistent infection.

Granuloma formation is an underlying characteristic feature of protective immunity and its absence is usually associated with mycobacterial dissemination and lethality (Bean et al., 1999; Emile et al., 1997; Saunders et al., 1999). Establishment of granulomas

requires cell migration to sites of infection and is associated with TNF dependent chemokine induction and regulation of adhesion molecule expression (Hickey et al., 1997; Mulligan et al., 1993; Roach et al., 2002). Previous studies described disruptive granuloma formation or delayed of granuloma initiation in the absence of TNF signaling (Bean et al., 1999; Flynn et al., 1995; Jacobs et al., 2000b). We show that Tm-TNF supports early granuloma formation during attenuated and virulent mycobacterial challenge (Dambuza et al., 2008). However it is clear from comparative bacterial burdens in WT mice and Tm-TNF mice that granulomas established in the presence of Tm-TNF only, are inferior in bactericidal potential. This is evident from the onset after *M. bovis* BCG infection and becomes apparent during chronic infection after *M. tuberculosis* challenge. Interestingly, in contrast to the abnormal pulmonary inflammation of $CD4^+$ T cells and $CD8^+$ T cells observed in the absence of total TNF during *M. bovis* BCG infection (Dambuza et al., 2008), enhanced recruitment of both lymphocytic subsets were stimulated by Tm-TNF also supported by previous reports (Fremond et al., 2005; Saunders et al., 2005). This may imply that solTNF has an inhibitory function during inflammation and that the two molecular forms have distinct roles during mycobacterial infection. The observation during attenuated *M. bovis* BCG challenge is supported by our findings during virulent *M. tuberculosis* challenge. In the latter case, our data demonstrate the absence of solTNF is characterized by enhanced inflammation during persistent infection and leads to formation of large granulomas that occupy larger areas of the lung tissue. Previously Zganiacz et al., (2004), proposed that TNF is a negative regulator of Th1-type inflammatory responses and found enhanced inflammation of both $CD4^+$ T cells and $CD8^+$ T cells in $TNF^{-/-}$ mice after *M. bovis* BCG

infection. In addition to their observations, we propose that Tm-TNF has a cellular recruitment function and that solTNF has an important role in mediating inhibitory responses during Th1-type inflammation and this holds true for chronic infection and drug-induced reactivation tuberculosis. In other studies, clinical relevance of Tm-TNF function in cellular recruitment has been illustrated through its ability to induce inflammation and contributing to chronic arthritis (Georgopoulos et al., 1996) and EAE (Ruuls et al., 2001). The concept of different functions of the molecular forms of TNF is not unprecedented. Separate functions for Tm-TNF and solTNF were also described by Birkland et al., (1992), who showed that solTNF and membrane TNF expressed on $CD4^+$ T cells differentially activates anti-leishmanial mechanisms in macrophages. Furthermore, additional studies found that cells insensitive to lysis by solTNF can be killed by Tm-TNF (Peck et al., 1989), and growth of retrovirally transduced tumours was inhibited by solTNF but not Tm-TNF (Karp et al., 1992).

IFNγ is essential for mediating Th1 protective immunity against mycobacterial infections (Cooper et al., 1993; Flynn et al., 1993), but in the absence of TNF does not afford protection during mycobacterial infections. In contrast, excessive levels of IFNγ promote tissue necrosis leading to host lethality (Zganiacz et al., 2004). In this study we found significantly higher pulmonary IFNγ production during late stage infection in $TNF^{-/-}$ mice challenged with *M. tuberculosis*. Although WT mice and Tm-TNF mice produced similar levels of pulmonary IFNγ during early infection, chronic *M. tuberculosis* infection was associated with significantly higher production of IFNγ in Tm-TNF mice. We therefore propose that Tm-TNF is capable of regulating type 1 immune responses during early *M. tuberculosis* challenge but that host immunity requires solTNF during persistent

infection to control onset of IFNγ mediated pathology. In addition, we found that during early stages of tuberculosis reactivation, the levels of IFNγ were comparable between Tm-TNF mice and WT mice supporting further the notion that the emerging mycobacteria-specific immune responses are equivalent between the two strains. However, a different scenario was observed at later stages of disease reactivation, in which we found that relative to WT control, Tm-TNF mice displayed a decline in IFNγ levels. In this context, we suggest that the mechanisms that govern protection collapse during tuberculosis reactivation and may involve reduced macrophage-T lymphocyte contact required in the case of Tm-TNF to induce a signal.

With regards to activation of macrophages, IFNγ-mediated signal during infection results in pathogen control. However uncontrolled IFNγ production can cause detrimental effects and contribute to tissue damage (Parfrey et al., 1999; Zganiacz et al., 2004). Similarly, sustained macrophage activation, characterized by increased MHC Class II expression has been associated with promoting tissue injury (Huang et al., 2000). We found that in contrast to WT mice, $TNF^{-/-}$ mice displayed increased macrophage activation characterized by enhanced surface expression levels of MHC Class II, CD80 and CD86 on recruited pulmonary $CD11b^{+}$ cells. This was further demonstrated by increased pulmonary IL-12p70 levels. Furthermore, there was an increased frequency of $CD4^{+}$ T cells with an enhance activation phenotype reflected by enhanced cell surface expression of CD44 which associated with elevated levels of pulmonary IFNγ in $TNF^{-/-}$ mice. It was interesting to note that a similar phenotype is observed in Tm-TNF mice during chronic *M. tuberculosis* infection closer to their death. It is therefore plausible that sustained macrophage activation was maintained by high IFNγ levels contributing to

tissue injury. solTNF may therefore have an inhibitory function in regulating macrophage activation during persistent *M. tuberculosis* infection. In fact, Watanabe and Jacob (1991) found that TNF antagonizes IFNγ mediated upregulation of MHC class II on macrophages *in vivo*. Furthermore, we found enhanced levels of iNOS expression present in lungs of *M. tuberculosis* infected Tm-TNF mice which may have contributed to local tissue necrosis. This is supported by findings which demonstrated that BCG induced iNOS expression promoted tissue damage whereas $iNOS^{-/-}$ mice were protected (Guler et al., 2004).

Studies have illustrated that both TNF and IL-12 are required to mediate protective immunity in part through maintaining the structural integrity of granulomas to limit spread of bacilli (Bean et al., 1999; Cooper et al., 1997; Ehlers et al., 2000; Wakeham et al., 1998). In the absence of either cytokines, structural integrity is compromised during mycobacterial challenge. Similarly, both cytokines are required to control infection during persistent infection (Botha and Ryffel, 2003; Feng et al., 2005; Mohan et al., 2001). In this context, we investigated expression of both IL-12p70 and IL-12p40 during tuberculosis reactivation. Our data show relative to WT control mice, there was significant reduction of pulmonary IL-12p70 and not IL-12p40 levels in Tm-TNF mice during *M. tuberculosis* reactivation closer to their demise. Our results are interesting in view of recent data which showed that *M. bovis* BCG infection reduces IL-12 production from host cells (Gagliardi et al., 2005), and suggest that solTNF may regulate IL-12 production *in vivo*. Here we propose that collapse of protective immunity in Tm-TNF mice during late phase reactivation is possibly due to reduced cell-cell contact required in the case of membrane expressed TNF to induce relevant signals, we suggest that this is

partly associated with enlarged granulomatous response that does not allow for sufficient macrophage-T cell interactions leading to decreased IL-12p70-dependent IFNγ production.

It is interesting to speculate on *M. tuberculosis* virulence factors that compromise Tm-TNF mediated immunity. Genes encoded within the RD1 are obvious candidates because of their absence from all *M. bovis* BCG strains. ESAT-6 and CFP-10 are located within RD1 and have been implicated in regulating immune responses. Studies supporting this suggestion demonstrated that deletion of RD1 from *M. tuberculosis* H37Rv significantly attenuated virulence to assume growth characteristics and granuloma induction potential analogous to that of *M. bovis* BCG (Lewis et al., 2003). Similarly, reintroduction of RD1 into *M. bovis* BCG restored virulence and pathogenicity to rival that of *M. tuberculosis* H37Rv (Majlessi et al., 2005). It is therefore likely that genes encoded within RD1 may have contributed to mycobacterial virulence that resulted in compromised immunity and eventual lethality in Tm-TNF mice during pathogenic challenge.

IL-10 is an inhibitory cytokine required to regulate inflammation in several infectious diseases resulting in improved pathogen control (Moore et al., 2001). Previously, studies found enhanced mycobacterial clearance in the absence of IL-10 whereas a limited or no role for IL-10 was also described (Jacobs et al., 2000a; North, 1998; Roach et al., 2001). In this study we found enhanced IL-10 production associated with end stage disease in $TNF^{-/-}$ mice relative to WT mice and Tm-TNF mice during acute *M. tuberculosis* challenge. Similarly, we found enhanced pulmonary IL-10 production in Tm-TNF mice during persistent *M. tuberculosis* infection. We therefore propose that increased IL-10 production promotes host susceptibility to acute *M. tuberculosis* infection in total absence

of TNF and likewise support bacterial persistence in the absence of solTNF during chronic infection. However, our data demonstrates further that low pulmonary IL-10 levels were not beneficial with respect to *M. tuberculosis* reactivation as observed for Tm-TNF mice. In this context, our findings contradict studies of Turner et al., (2002), who showed that overexpression of IL-10 in chronically but not acutely infected C57BL/6 mice resulted in reactivation of disease. Interestingly, these authors also found that susceptibility of mice was associated with reduced IL-12 and TNF levels. We suggest here, that during *M. tuberculosis* reactivation, excess pulmonary inflammation over longer periods possibly depletes IL-10 by a mechanism presently unknown and Tm-TNF mice eventually succumb to pathology induced by the vigorous immune responses to persistent *M. tuberculosis*.

In conclusion, data presented here illustrate that TNF mediated immunity against *M. tuberculosis* infections requires both Tm-TNF and solTNF. Tm-TNF protects mice against acute *M. tuberculosis* infection and is adequate to elicit immune pressure during tuberculosis reactivation; solTNF is however necessary for the down modulation of the Th1 immune responses during *M. tuberculosis* infections. The characterization of the molecular forms of TNF in disease outcome will inform design of better therapeutic interventions for human subjects suffering from TNF-mediated autoinflammatory disorders who become susceptible to intracellular pathogens due to administration of the currently used anti-TNF therapies i.e. etanercept, infliximab, and adalimumab; these biologics block both Tm-TNF and solTNF (Agnholt et al., 2003; Mitoma et al., 2004; Mitoma et al., 2005; Scallon et al., 2002). Recently, research laboratories have tested

new approaches of solely targeting the soluble form of TNF while sparing Tm-TNF and it was found that mice were protected from the experimental model of arthritis (Spohn et al., 2007; Steed et.al., 2003), Parkinson‘s disease (McCoy et al., 2006) and endotoxin-induced liver inflammation (Olleros et al., 2009) and still retained immunity against *M. tuberculosis*, *M. bovis* BCG or *L. monocytogenes* infections.

4 Persistent p55TNFR signaling in *M. tuberculosis* infection and the limiting role of p75TNFR

Summary

The pleiotropic activities of TNF are mediated by two structurally related but functionally distinct type-1-transmembrane receptors, p55TNFR and p75TNFR expressed in most cell types (Aggarwal et al., 1985; Smith and Baglioni, 1989; Tsujimoto et al., 1985). The recent generation of a nonsheddable p55TNFR ($p55^{\Delta NS}$) has provided insights into the roles played by both the soluble and the membrane-bound p55TNFR in pathogenesis of infectious, inflammatory and autoimmune diseases (Xanthoulea et al., 2004). We hypothesized that sustained p55TNFR cell surface expression will lead to enhanced TNF-p55TNFR interaction resulting in enhanced immune activation. Here, our data demonstrate that persistent p55TNFR cell surface expression does not afford better protection to *M. tuberculosis* infection, reflected by comparable containment of bacilli replication and mortality rates. We observed a transient elevation in the frequency of pulmonary $CD11b^{+}$/MHC-II^{+} cells in $p55^{\Delta NS}$ mice relative to WT mice and comparable lung recruitment of $CD4^{+}$ T cells in both strains but CD44 cell surface expression was reduced in $p55^{\Delta NS}$ mice compared to WT mice. We also show that pulmonary IL-12p70 and TNF concentrations were elevated whereas IFNγ levels were reduced in $p55^{\Delta NS}$ mice relative to WT mice. Furthermore, we propose that enhanced p75TNFR release in $p55^{\Delta NS}$ mice limit TNF signaling. We demonstrate using a double mutant mouse strain that in the absence of p75TNFR, $p55^{\Delta NS}$ mice controlled bacterial replication better that WT control mice.

Results

Sustained p55TNFR surface expression in p55$^{\Delta NS}$ peritoneal elicited cells after M. tuberculosis infection.

The literature is replete with studies that demonstrate loss of TNFRs after infection or immunization *in vitro* and *in vivo*. TNFR cleavage from the surface coincides with the generation of TNF and its functional significance has been associated with the regulation of TNF mediated effects (Engelmann et al., 1989; Engelmann et al., 1990a; Engelmann et al., 1990b; Olsson et al., 1989). Studies by Xanthoulea et al., (2004), showed that upon activation with PMA, peritoneal exudate cells derived from p55$^{\Delta NS}$ mice expressed a nonsheddable p55TNFR. Here we investigated the relationship of p55TNFR expression and *M. tuberculosis* infection. Thioglycollate elicited macrophages from WT mice or p55$^{\Delta NS}$ mice were exposed to *M. tuberculosis* H37Rv for 20 minutes and p55TNFR cell surface expression was determined by flow cytometry. p55TNFR cell surface expression was detected in both the WT and p55$^{\Delta NS}$ unstimulated macrophages (Fig. 1.1). However, exposure to *M. tuberculosis* H37Rv resulted in loss of p55TNFR surface expression on WT macrophages (reflected by reduced Mean Fluorescent Intensity) compared to the elevated and sustained expression noted in p55$^{\Delta NS}$ macrophages (Fig. 1.1). This experiment investigated cleavage, as opposed to internalization of p55TNFR; hence soluble p55TNFR accumulation in the supernatants of WT and p55$^{\Delta NS}$ cultures was quantified by ELISA. Detectable levels of soluble p55TNFR were present in unstimulated culture supernatants of thioglycollate elicited macrophages from WT mice, which increased significantly ($p < 0.01$) after LPS (Fig. 1.2A) or viable *M. tuberculosis* H37Rv (Fig. 1.2B) activation. No soluble p55TNFR was detected in supernatants of

p55$^{\Delta NS}$ macrophages before or after stimulation (Fig. 1.2). These data show that *in vitro,* macrophage activation with *M. tuberculosis* H37Rv does not induce receptor shedding in p55$^{\Delta NS}$ macrophages.

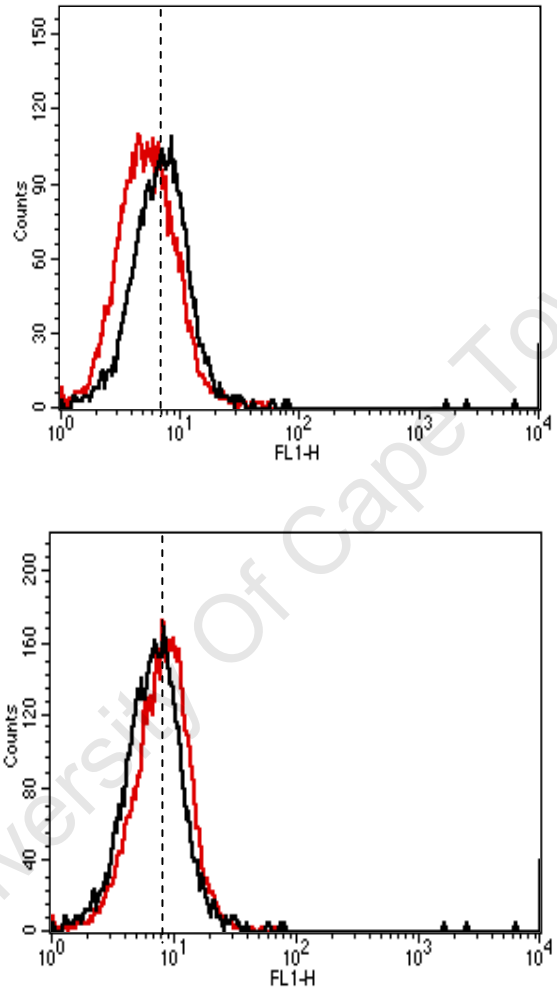


Figure 1.1. Sustained p55TNFR cell surface expression in p55$^{\Delta NS}$ cells. Thioglycollate elicited peritoneal macrophages from p55$^{\Delta NS}$ and WT were stained with anti-p55TNFR before stimulation (black histogram) or after 20 min stimulation with *M. tuberculosis* H37Rv (MOI = 2:1) and analyzed by flow cytometry to determine p55TNFR cell surface expression (red histogram, loss in Mean Fluorescent Intensity is indicative of receptor shedding). Results represent 1 of 2 experiments performed.

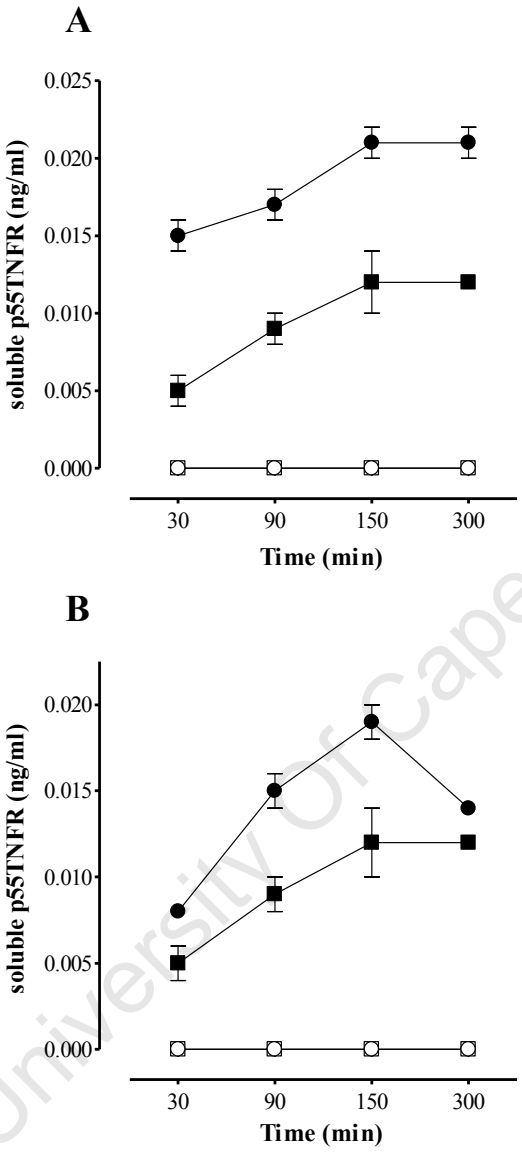


Figure 1.2. Activation induced soluble p55TNFR cleavage in WT but not* p55$^{\Delta NS}$ *cells. Thioglycollate elicited peritoneal macrophages were isolated from WT mice (black squares represent resting cells and black circles represent stimulated cells) and p55$^{\Delta NS}$ mice (white squares represent resting cells and white circles show stimulated cells) and stimulated with 100 ng/ml LPS (A) or *M. tuberculosis* H37Rv (MOI = 2:1) (B). Soluble p55TNFR accumulation in the supernatants was measured at time points indicated above by ELISA. Data are expressed as mean ± SD of 5x10^5 cells/well performed in triplicates and results are representative of 1 of 2 experiments performed.

Sustained expression of surface p55TNFR does not enhance mycobactericidal response.

The abrogation of TNF-p55TNFR signaling either genetically or with neutralizing antibodies has been reported to result in susceptibility to mycobacterial and other intracellular infections (Bean et al., 1999; Bekker et al., 2001; Flynn et al., 1995; Kindler et al., 1989). However, dysfunctional TNF regulation has also been reported to have a central role in pathogenesis of autoimmune disorders such as rheumatoid arthritis and Crohn's disease (Dimakou et al., 2004). The specific functions attributable to soluble and membrane TNFRs in various infections and disease states is still not fully understood. In both mice and humans, interference of the shedding of p55TNFR showed enhanced sensitivity to TNF mediated immunopathology (Jesus et al., 2008). Xanthoulea et al., (2004), reported enhanced innate immune activation in $p55^{\Delta NS}$ mice, which mediated increased resistance to *L. monocytogenes* infection *in vivo* and suggested that persistently expressed membrane p55TNFR conferred enhanced protective antibacterial immunity. In consideration of these results, we addressed the *in vivo* functional role of membrane bound p55TNFR and receptor shedding during *M. tuberculosis* H37Rv infection. WT mice and $p55^{\Delta NS}$ mice were exposed to a low dose aerosol inhalation infection of 100 CFUs/mouse *M. tuberculosis* H37Rv and pulmonary bacilli numbers as well as the extent of extra pulmonary dissemination were assessed. Results obtained showed that $p55^{\Delta NS}$ mice controlled acute phase infection with comparable kinetics to WT mice reflected by equivalent bacilli numbers in the lungs (Fig. 2.1A), spleens (Fig. 2.1B) and livers (Fig. 2.1C) at all the time points investigated.

To determine the effect of sustained p55TNFR expression on disease outcome during chronic infection, infected $p55^{\Delta NS}$ mice and WT mice were monitored for > 500 days. Bacilli numbers were maintained at equivalent levels in both strains in the lungs (Fig. 2.2A) and spleens (Fig. 2.2B) at every time point investigated. Mortality rates between both strains were also found to be comparable (Fig. 2.2C). These data indicate that persistent p55TNFR surface expression does not result in enhanced effector anti-mycobacterial mechanisms.

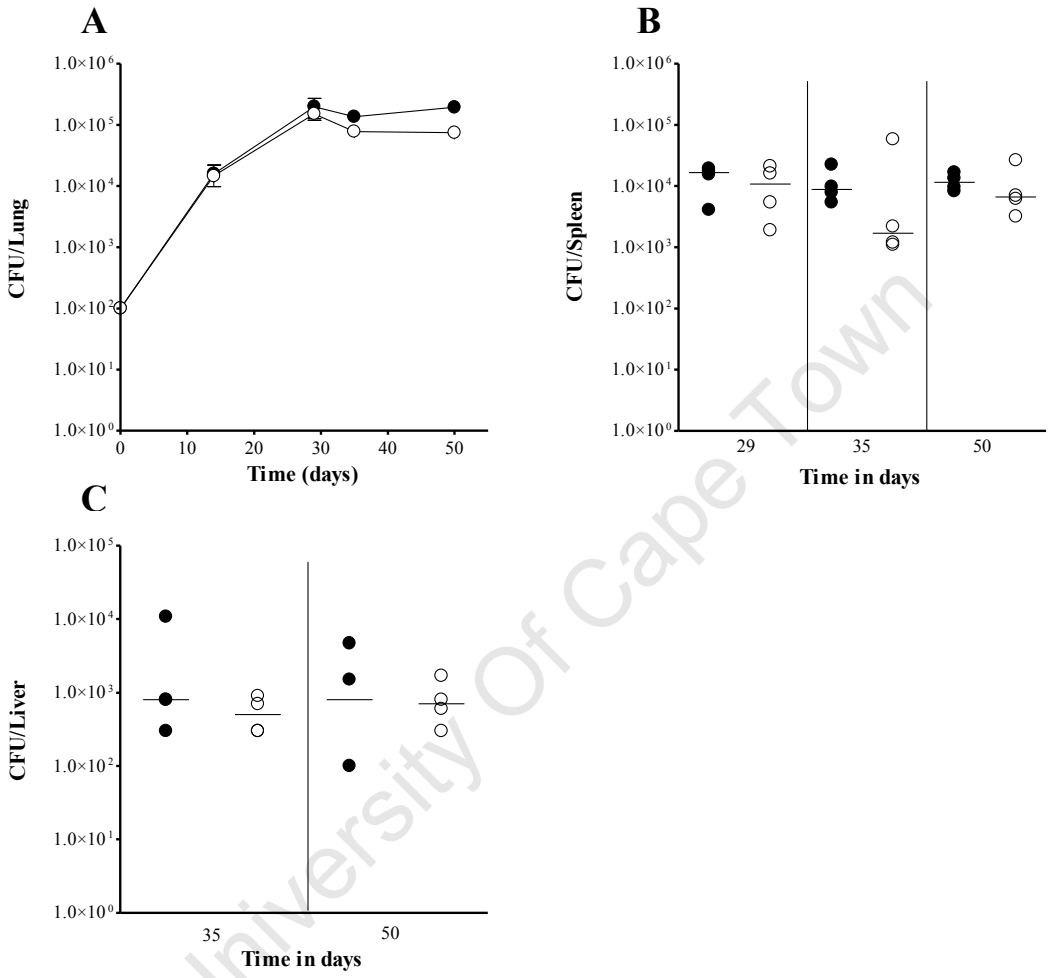


Figure 2.1. Persistent membrane p55TNFR expression does not enhance control of mycobacterial replication. WT mice (black circles) and $p55^{\Delta NS}$ mice (white circles) were challenged by aerosol inhalation with 100 CFUs/mouse with *M. tuberculosis* H37Rv. Bacilli numbers were enumerated in the lungs (A), spleens (B) and livers (C) at time points indicated above. Data are representative of 3 similar experiments and results are expressed as mean ± SD of 4 animals/group.

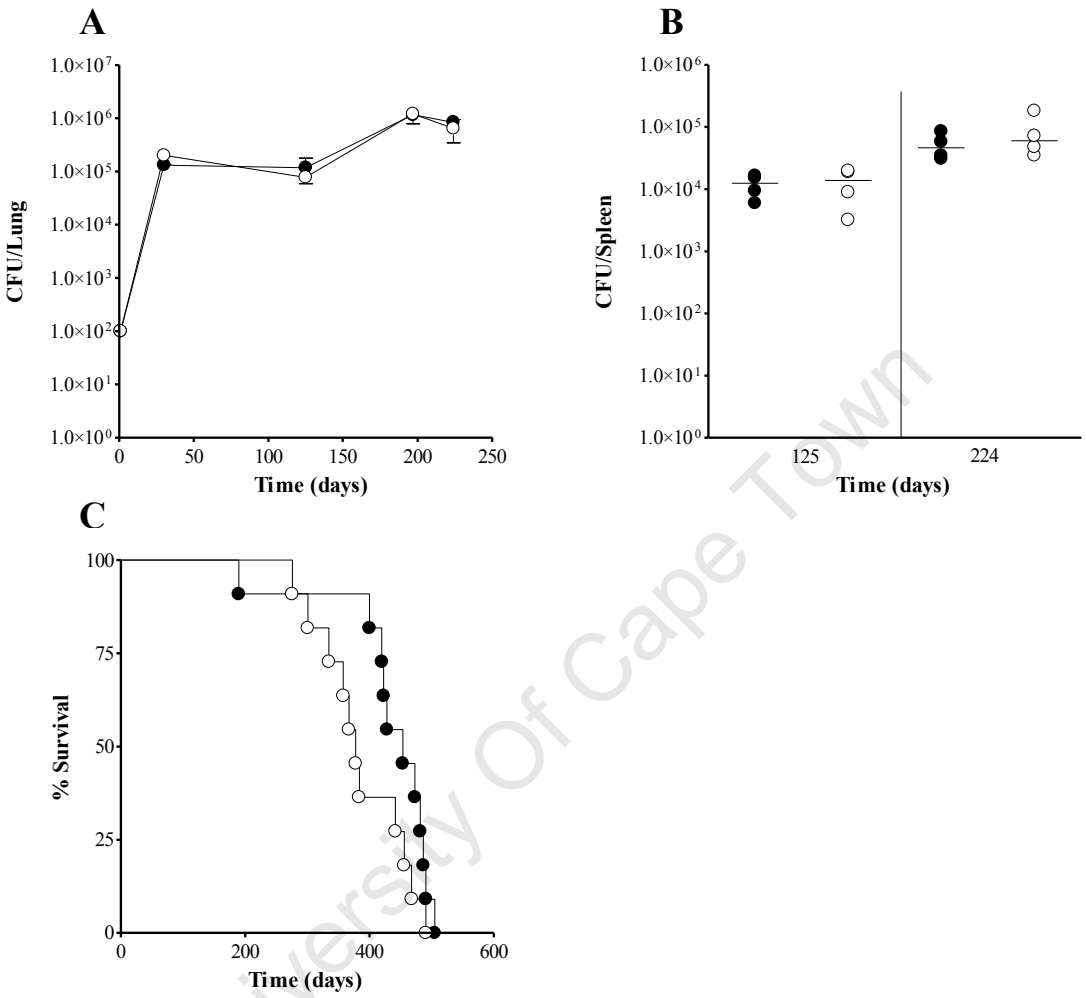


Figure 2.2. Control of M. tuberculosis replication is equivalent in p55$^{\Delta NS}$ mice and WT mice during chronic infection. WT mice (black circles) and p55$^{\Delta NS}$ mice (white circles) were challenged by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv**.** Bacilli numbers in the lungs (A) and spleens (B) were enumerated at time points indicated. Data are expressed as mean ± SD of 3- 4 animals/group. (C) Mortality rates were monitored over the infection period (n =11). Results represent 1 of 2 similar experiments.

Transient enhanced CD11b cell recruitment to the lung in p55$^{\Delta NS}$ infected mice.

To understand how persistent membrane p55TNFR expression affects macrophage function *in vivo,* CD11b^{+} cell recruitment to the lung and activation state of the cells was assessed in WT mice and p55$^{\Delta NS}$ mice after *M. tuberculosis* H37Rv infection. Single cells suspensions were generated from whole lungs and stained for CD11b, MCH-II or CD80 expression. Figure 3A shows that the total cell counts were moderately higher in p55$^{\Delta NS}$ mice compared to WT mice 14 days post-infection. However, 21 days later, cell numbers in WT mice increased at least two fold in contrast to p55$^{\Delta NS}$ mice which remained significantly low ($p < 0.05$) (Fig. 3A). These results suggest that persistent cell surface p55TNFR expression may negatively regulate cellular migration into the lung.

Flow cytometric analyses of pulmonary cells revealed that there was a significant increase ($p < 0.01$) in the frequency of CD11b^{+} cells in p55$^{\Delta NS}$ compared to WT mice at day 14 post-infection which increased to equivalent levels in both strains by day 21 post-infection (Fig. 3B). We then analyzed the percentage of a subpopulation of CD11b^{+} cells that expressed the MHC-II molecule. p55$^{\Delta NS}$ mice displayed a significantly higher ($p < 0.05$) CD11b^{+}/MHC-II^{+} frequency compared to the WT mice 14 days post-infection but, by 21 days post-infection, both groups had comparable increased levels of infiltrating CD11b^{+}/MHC-II^{+} cells (Fig. 3C). The analysis of cell surface expression of MHC-II gated on CD11b^{+} cells was found to be equivalent in both strains at 14 days and 21 days post-infection (Fig. 3D). The expression of co-stimulatory molecule CD80 gated on CD11b^{+} cells was also analyzed and was found to be equivalent in both strains at 14 days and 21 days post-infection (Fig. 3E). These data show that persistent membrane

p55TNFR expression facilitates an increased but transient recruitment of $CD11b^+$/MCH-II^+ cells *in vivo* but does not enhance the expression levels of MHC-II and CD80 molecules. Nonetheless, we postulated that the increased frequency of $CD11b^+$/$MHC\text{-}II^+$ cells in $p55^{\Delta NS}$ mice might drive enhanced *M. tuberculosis* H37Rv specific type 1 immune response. Therefore in view of this, the influx and activation state of $CD4^+$ T cells in the lungs of infected WT mice and $p55^{\Delta NS}$ mice was then evaluated. The frequency of $CD4^+$ T cells in both strains was found to be equivalent analyzed at 14 days and 21 days post-infection (Fig. 4A). In addition, the frequency of $CD4^+$/$CD44^+$ T cells was also found to be comparable in both strains determined 14 days and 21 days post-infection (Fig. 4B). Interestingly, the cell surface expression of CD44 gated on $CD4^+$ T cells was found to be significantly lower ($p < 0.05$) in $p55^{\Delta NS}$ mice compared to WT mice at day 14 post-infection and increased in both strains by day 21 post-infection, but the expression was lower in $p55^{\Delta NS}$ mice relative to WT mice with no statistical difference attained (Fig. 4C). These data illustrate that despite the comparable $CD4^+$ T cell recruitment to lung of WT mice and $p55^{\Delta NS}$ mice, persistent p55TNFR expression in $CD4^+$ T cells might play a suppressive role in T cell activation.

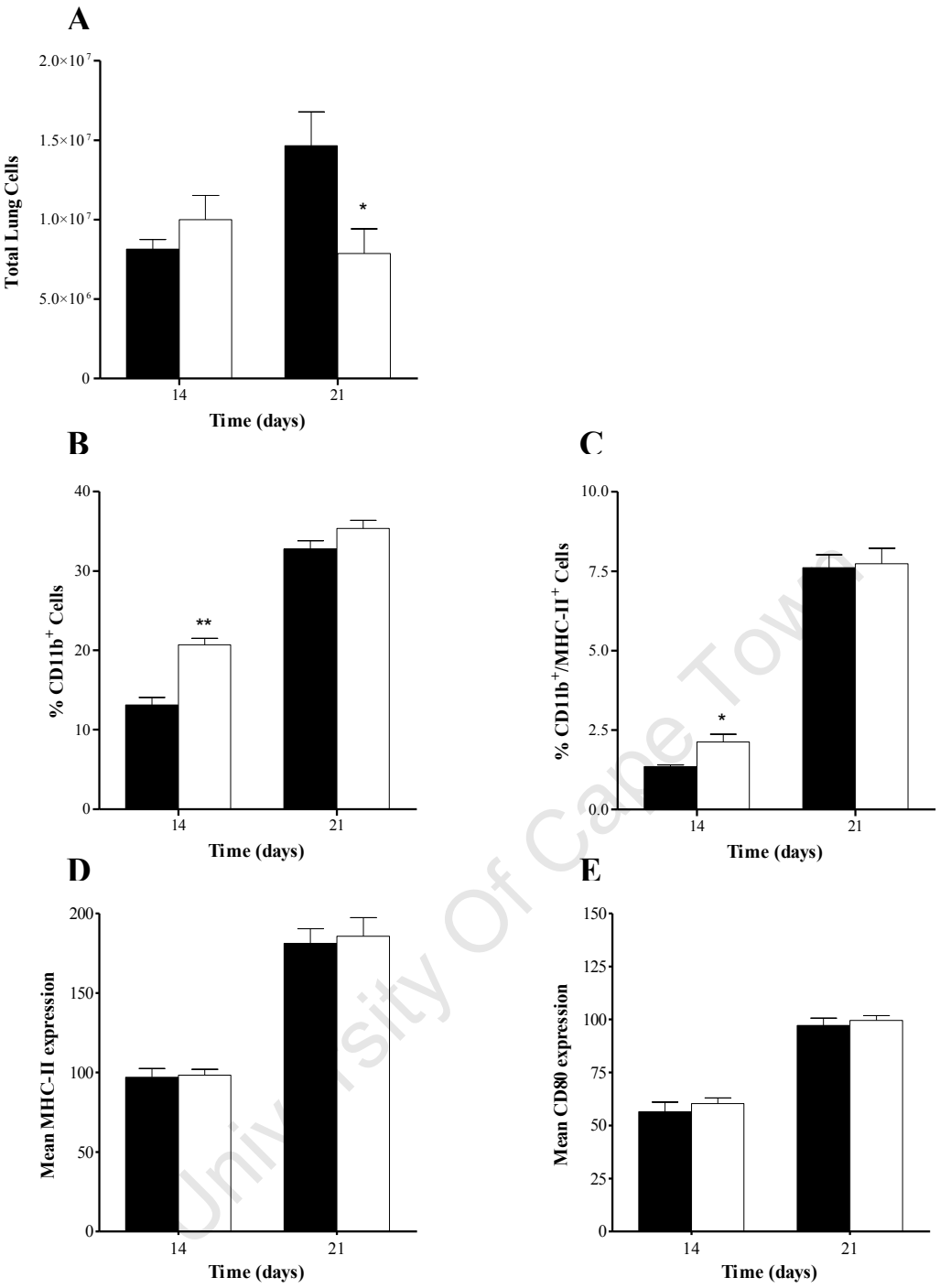


Figure 3. Transient elevation of CD11b$^+$/MHC-II$^+$ cells in the lungs of p55$^{\Delta NS}$ mice compared to WT controls. WT mice (black bars) and p55$^{\Delta NS}$ mice (white bars) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. (A) Lung single cell were generated at day 14 and day 21 post-infection and stained for CD11b, MHC-II or CD80 then analyzed by flow cytometry. Percentages of CD11b$^+$ (B) and CD11b$^+$/MHC-II$^+$ (C) cells in the lung and MFIs of cell surface expression of MHC-II (D) and CD80 (E) gated on CD11b$^+$ cells. Data are representative of 1 of 3 experiments performed and results are expressed as mean ± SD from 4 mice/group. Significant differences (*$p < 0.05$; **$p < 0.01$) were determined by Student's *t* test.

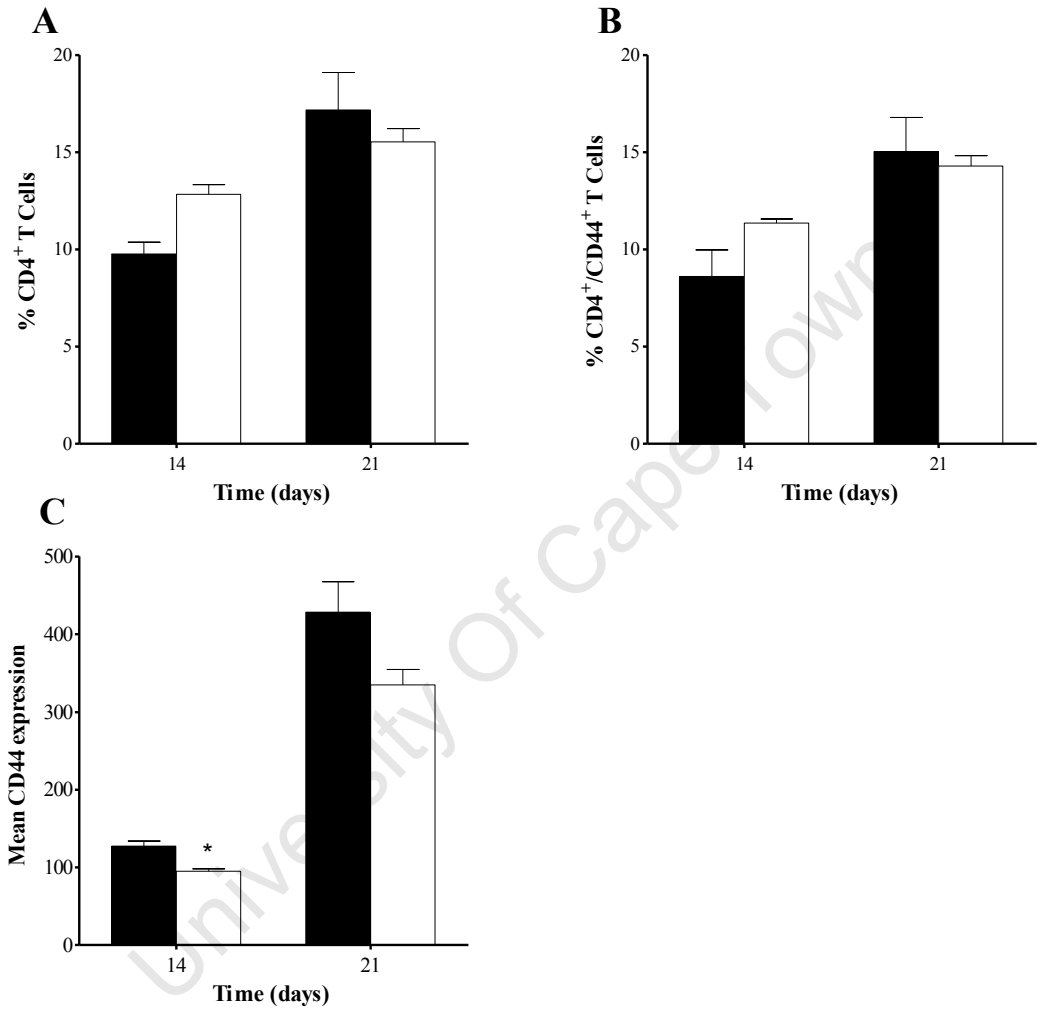


Figure 4: Persistent membrane p55TNFR expression does not improve CD4+ T cell recruitment into the lung but CD4+ T cell activation is reduced. WT mice (black bars) and $p55^{\Delta NS}$ mice (white bars) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Single cell suspensions were generated from lung tissue at day 14 and day 21 post-infection and cells were stained with anti-CD4 and anti-CD44. (A) Percentages of $CD4^+$ T cells and $CD4^+/CD44^+$ T cells (B) as well MFIs of cell surface expression of CD44 (C) gated on $CD4^+$ T cells were analyzed by flow cytometry. Data represent 1 of 3 experiments performed and results are expressed as mean ± SD of 4 mice/group. Statistical difference (*$p < 0.05$) was determined by the Student's *t* test.

Persistent p55TNFR expression has varying effects on macrophage and T lymphocyte function in acute M. tuberculosis H37Rv-infected mice.

IL-12p70, TNF and IFNγ are known critical mediators of anti-mycobacterial immunity (Cooper et al., 1993; Cooper et al., 1997; Feng et al., 2005; Flynn et al., 1993; Frankova and Zidek, 1998). To determine the influence of persistent p55TNFR expression on cytokine production, lungs from *M. tuberculosis* H37Rv infected WT mice and $p55^{\Delta NS}$ mice were obtained at specific time points and the concentration of cytokines was measured by ELISA. The concentration of IL-12p70 in $p55^{\Delta NS}$ mice was higher at 14 days and 21 days post-infection but was not statistically different from WT mice declining to equivalent levels in both strains by day 28 post-infection (Fig. 5A). Although TNF levels were equivalent at day 14 post-infection for both strains, higher concentrations were subsequently measured at day 21 and day 28 post-infection in $p55^{\Delta NS}$ mice with significant difference at day 21 ($p < 0.05$) (Fig. 5B). These data suggest that macrophage activation at least with respect to IL-12p70 production is moderately enhanced in $p55^{\Delta NS}$ mice relative to WT mice.

An inverse trend was observed when comparing the kinetics of IFNγ production. $p55^{\Delta NS}$ mice displayed lower concentrations with significant differences observed at day 21 ($p < 0.05$) and day 28 ($p < 0.01$) post-infection (Fig. 5C) compared to WT mice. Next, we analyzed the levels of the immunoregulatory cytokine, IL-10. Here, WT mice and $p55^{\Delta NS}$ mice showed comparable IL-10 concentrations at all the time points investigated (Fig. 5D). Together, these data suggest that persistent p55TNFR expression is associated with reduced pulmonary IFNγ production which is not regulated by IL-10.

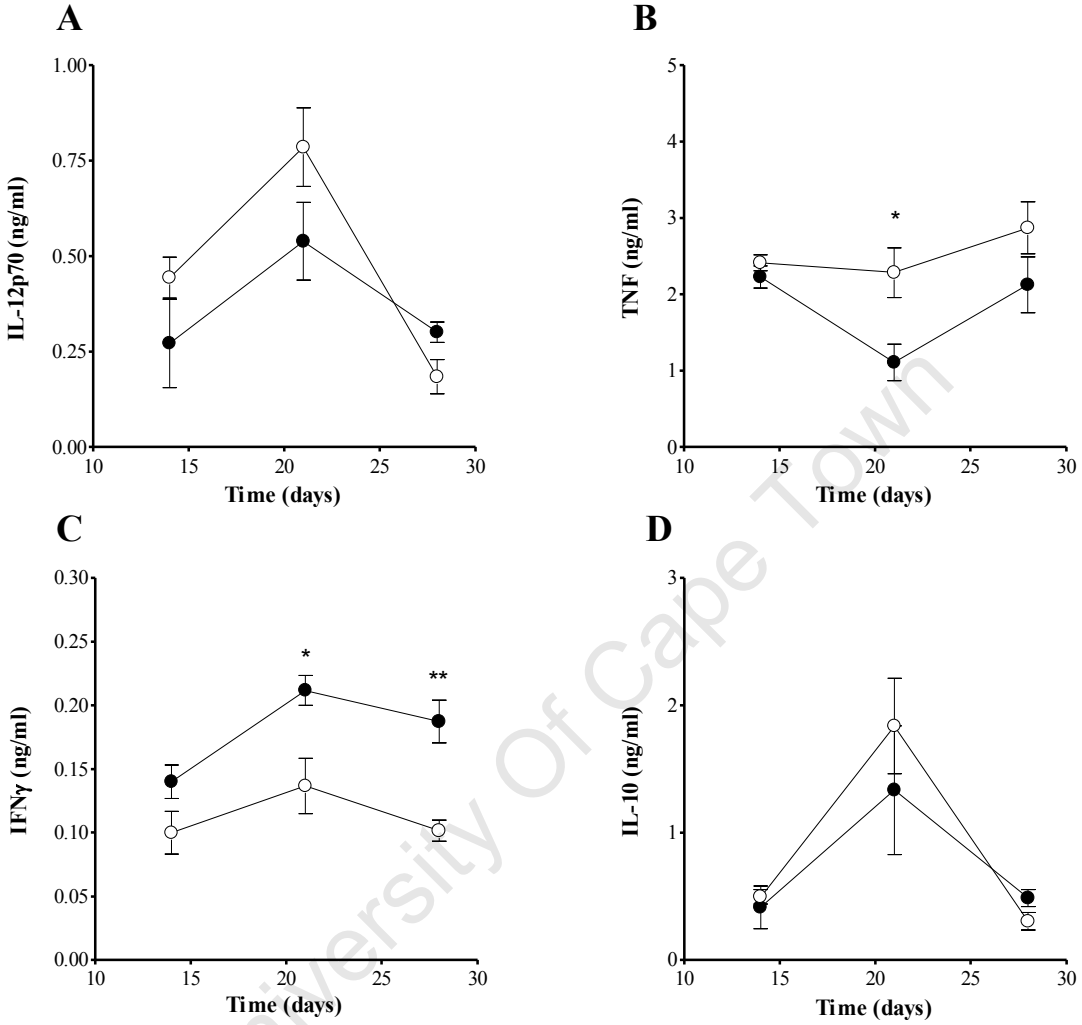


Figure 5. Enhanced pulmonary TNF and IL-12p70 but reduced IFNγ production in p55$^{\Delta NS}$ mice. WT mice (black circles) and p55$^{\Delta NS}$ mice (white circles) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lungs were isolated and homogenized at time points indicated and the levels of IL-12p70 (A), TNF (B), IFNγ (C) and IL-10 (D) were quantified by ELISA. Data are representative of 1 of 3 experiments performed and the results are expressed as mean ± SD from 4 mice/ group. Statistical difference (*$p < 0.05$; **$p < 0.01$) was determined by Student's *t* test.

We next analyzed memory antigen-specific T cell IFNγ induction in WT mice and $p55^{\Delta NS}$ mice. Whole mediastinal lymph node cells were isolated 28 days post-infection and single cell suspensions were generated then restimulated with either viable *M. tuberculosis* H37Rv, or mycobacteria-specific ESAT-6. Depicted in Fig. 6, the concentration of IFNγ was found to be equivalent in both strains after 72 h restimulation with *M. tuberculosis* H37Rv or ESAT. TCR/CD3 ligation of T cells with anti-CD3 and co-stimulatory anti-CD28 resulted in comparable IFNγ levels in both strains (Fig. 6). These data suggest that *in vivo,* lymphocyte antigen-specific memory responses are not affected by persistent p55TNFR expression.

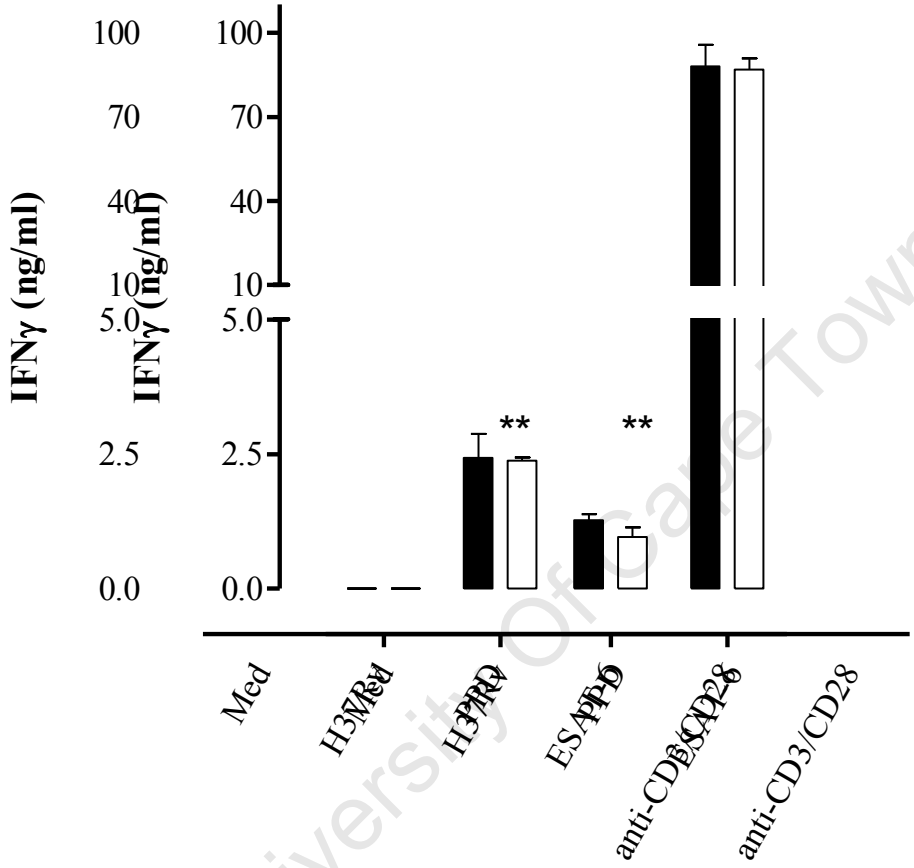


***Figure 6. Assessment of T cell IFNγ antigen-specific recall responses in* p55$^{\Delta NS}$**. WT mice (black bars) and p55$^{\Delta NS}$ mice (white bars) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv and whole mediastinal lymph nodes cells were isolated 28 days post-infection and restimulated with either viable *M. tuberculosis* H37Rv (MOI = 2:1) or 5 µg/ml anti-CD3/anti-CD28 or 10 µg/ml ESAT-6 for 72 h, and IFNγ concentration was quantified in the supernatants by ELISA. Results are representative of 1 of 2 experiments and data are expressed as mean ± SD of 5x10^5 cell/well plated in duplicates.

Persistent membrane p55TNFR expression is associated with enhanced production of pro-inflammatory mediators from macrophages in vitro.

We hypothesized that the inability to shed p55TNFR might result in enhanced TNF signaling. Results obtained from our *in vivo* studies showing that *M. tuberculosis* H37Rv bacilli growth was controlled in $p55^{\Delta NS}$ mice in a manner similar to WT mice (Fig. 2.1 & 2.2) were therefore interesting and warranted further investigation. To determine how sustained membrane p55TNFR expression influences macrophage activation, macrophages from WT mice and $p55^{\Delta NS}$ mice were prestimulated with equimolar IFNγ concentrations for 4 h and TNF and nitric oxide (NO) levels were quantified following stimulation with LPS or *M. tuberculosis* H37Rv. Consistent with published data (Xanthoulea et al., 2004), stimulating $p55^{\Delta NS}$ derived macrophages with 100 ng/ml LPS resulted in increased TNF concentration in a time dependent manner with a significant difference ($p < 0.05$) observed at 300 min compared to WT derived macrophages (Fig. 7.1A). A similar result was obtained when macrophages were challenged with *M. tuberculosis* H37Rv, however, TNF concentrations were found to be 10 fold less when compared to LPS stimulation (Fig. 7.1B). These data suggest that persistent p55TNFR expression positively enhance macrophage TNF production during LPS or *M. tuberculosis* stimulation.

Previous studies have established that TNF and IFNγ synergize to optimally activate macrophages to produce the effector molecule Nitric Oxide (NO) for mycobacterial killing (Ding et al., 1988; Flesch and Kaufmann, 1990). Therefore, we quantified the amount NO in supernatants of activated macrophages and it was observed that NO level

was significantly enhanced ($p < 0.05$) in $p55^{\Delta NS}$ derived macrophages relative to WT controls after 48 hours stimulation with LPS (Fig. 7C). Similar results were attained when macrophages were stimulated with *M. tuberculosis* H37Rv, with significant NO increase ($p < 0.001$) observed as early as 24 h after stimulation and a further increase in NO synthesis ($p < 0.05$) was observed 48 h after stimulation in $p55^{\Delta NS}$ mice compared to WT mice (Fig. 7D). Therefore, the increased synthesis of TNF and NO in $p55^{\Delta NS}$ macrophages points to a heightened state of cellular activation.

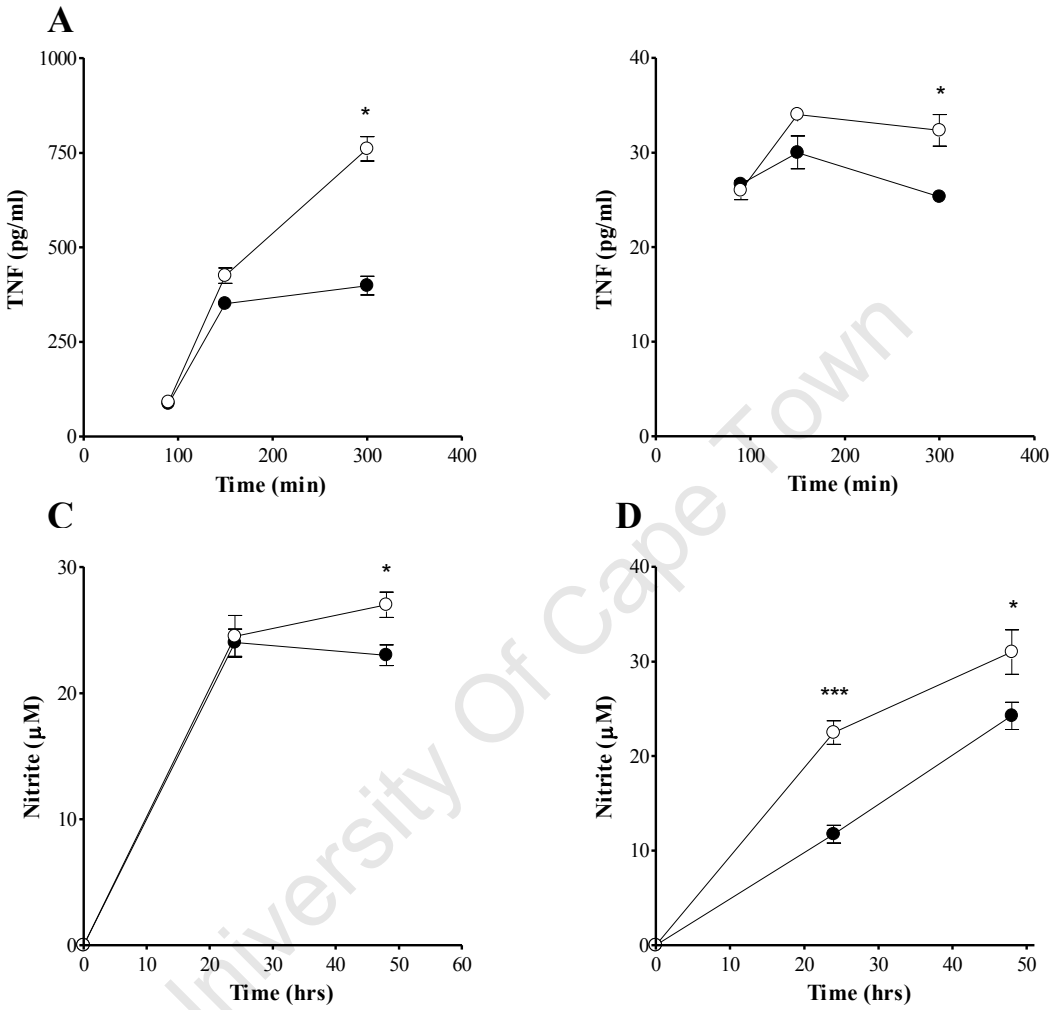


Figure 7.1. Enhanced TNF and Nitric Oxide production in peritoneal exudate cells expressing a non-sheddable form of p55TNFR. Bone marrow derived macrophages were isolated from WT mice (black circles) and $p55^{\Delta NS}$ mice (white circles) were prestimulated with 100 U/ml IFN_{γ} for 24 h prior stimulation with (A & C) 100 ng/ml LPS or (B & D) *M. tuberculosis* H37Rv, MOI = 2:1. The kinetics of soluble TNF accumulation in the supernatants was measured by ELISA (A & B), and the production of nitrite (pulmonary nitrate produced is reduced by nitrite reductase to nitrite) was quantified using Griess reagent (C & D). Results represent 1 of 3 similar experiments and data are expressed as mean ± SD of $5x10^5$ cells/well plated in triplicates. Statistical significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) was determined by Student's *t* test.

Elevated levels of soluble p75TNFR in p55$^{\Delta NS}$ macrophages: possible limiting factor of TNF bioavailability

Soluble TNFRs are thought to modulate TNF responsiveness. However, there is controversy about the role mediated by these receptors -whether they serve as agonists or antagonists of TNF effects. Nonetheless, both TNFRs are proteolytically cleaved from the cell surface in the presence of inflammatory stimuli and the levels of soluble p75TNFR greatly exceed those of soluble p55TNFR. This increased shedding implies a more dominant role for p75TNFR in down regulating TNF driven responses (Peschon et al., 1998). To determine whether there is a difference in p75TNFR shedding between WT and p55$^{\Delta NS}$ derived macrophages, the levels of soluble p75TNFR were quantified in the supernatants after cells were stimulated with 100 ng/ml LPS or viable *M. tuberculosis* H37Rv bacilli at MOI of 2:1. Results obtained showed that p55$^{\Delta NS}$ derived macrophages produced significantly higher levels ($p < 0.01$) of soluble p75TNFR when stimulated with either LPS (Fig. 7.2A) or *M. tuberculosis* H37Rv (Fig. 7.2B) for 300 minutes. These data indicate that defective p55TNFR shedding results in significant increased release of soluble p75TNFR as a compensatory mechanism with potential to modulate TNF activity. To determine whether this increased soluble p75TNFR had an effect on TNF bioactivity, TNF mediated cytotoxicity was evaluated using WEHI cells. Interestingly, the amount of bioactive TNF was comparable in both strains (Fig. 7.2C). The results therefore indicate that even though there is significantly increased total TNF in p55$^{\Delta NS}$ mice (Fig. 7.1B); increased soluble p75TNFR normalizes the concentrations of bioactive TNF.

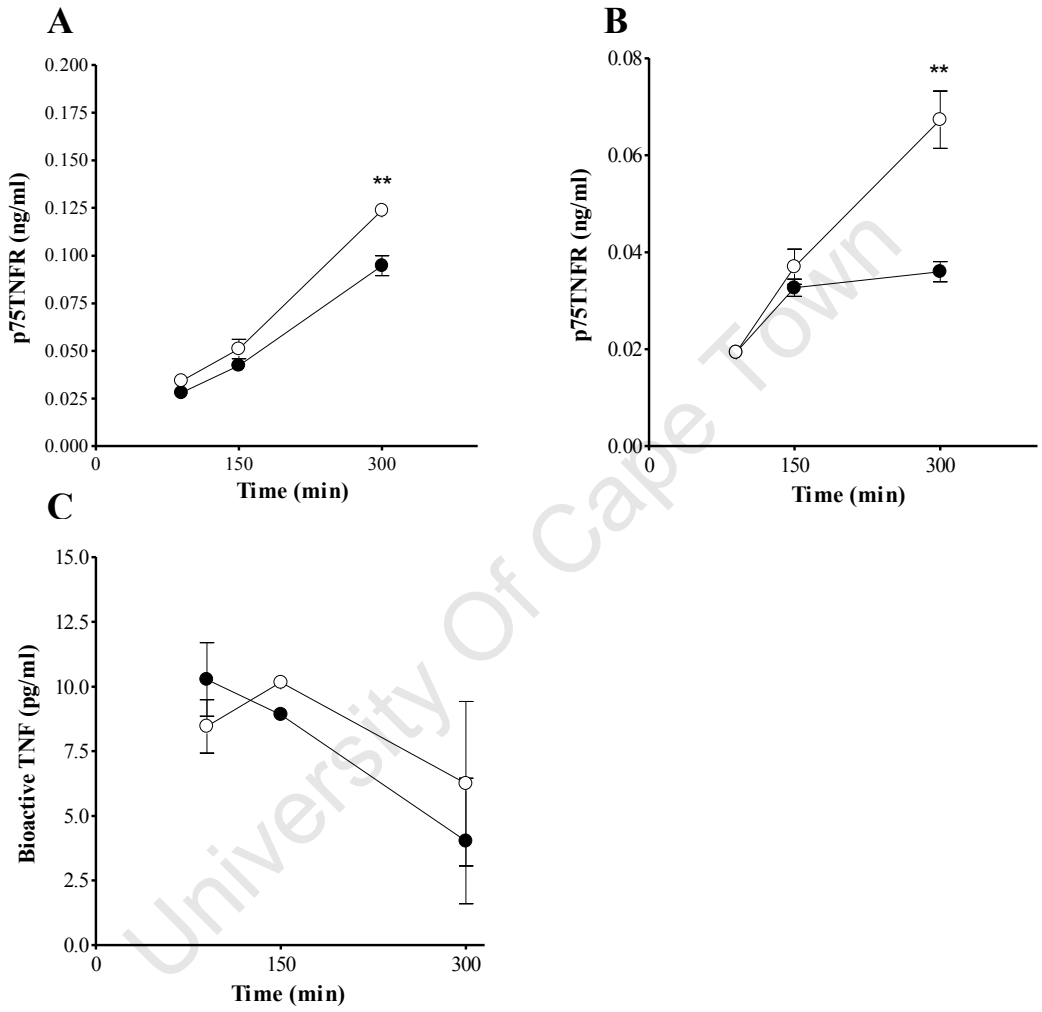


Figure 7.2 Increased soluble p75TNFR correlates with equimolar bioactive TNF in WT and p55$^{\Delta NS}$. Bone marrow derived macrophages from WT mice (black circles) and p55$^{\Delta NS}$ mice (white circles) were stimulated with 100 ng/ml LPS (A) or *M. tuberculosis* H37Rv MOI = 2:1 (B) and the levels of soluble p75TNFR were quantified by ELISA. (C) Bioactive TNF was measured by cytotoxicity assay using TNF sensitive WEHI cell line. Data represent 1 of 2 experiments performed and results are expressed as mean ± SD of 5x10^5 cells/well plated in triplicates. Significant differences (**$p < 0.01$) were obtained by Student's *t* test.

p75TNFR deficiency in the presence of persistent p55TNFR expression results in improved bacterial clearance.

We postulated that increased p75TNFR shedding in $p55^{\Delta NS}$ mice resulted in neutralization of TNF bioactivity to levels equivalent to that of WT mice despite the higher levels of TNF synthesis observed in this strain. To test this postulate, we investigated immune responses in $p55^{\Delta NS}$ in the absence of p75TNFR. We generated a double mutant $p55^{\Delta NS}$-$p75^{-/-}$ mouse strain for comparative challenge studies. Here, we infected WT mice, $p55^{\Delta NS}$ mice, $p75^{-/-}$ mice and $p55^{\Delta NS}$-$p75^{-/-}$ mice with 100 CFUs/mouse *M. tuberculosis* by aerosol inhalation and the lungs and spleens were harvested at day 28 post-infection to compare bacilli burdens in the respective mouse strains. Results obtained showed that there was no statistical difference in bacilli burdens between WT control mice and $p55^{\Delta NS}$ mice in the lungs (Fig. 8A) and spleens (Fig. 8B) at day 28 post-infection. Mice deficient for p75TNFR were found to have reduced bacilli numbers in the lungs (Fig. 8A) and spleens (Fig. 8B) relative to WT mice at day 28 post-infection but no statistical difference was attained, in contrast, $p55^{\Delta NS}$-$p75^{-/-}$ mice displayed a significant reduction in bacilli burdens in the lungs ($p < 0.01$) and more impressively in the spleens ($p < 0.01$) (Fig. 8A & B, respectively). These data indicate that persistent p55TNFR expression in the absence of p75TNFR mediate an enhanced mycobactericidal response *in vivo* and imply that p75TNFR shedding play a dominant role in modulating TNF bioavailability.

We next compared granuloma structure formation in WT mice, $p55^{\Delta NS}$ mice, $p75^{-/-}$ mice and $p55^{\Delta NS}$-$p75^{-/-}$ 28 days post-infection. We found that there was no discernable

differences in granuloma structures that were formed in all the mouse strains investigated (Fig. 9.1); in addition, iNOS expression pattern was comparable in all the groups (Fig. 9.2). Thus, these results illustrate that p75TNFR is not required for granuloma structure formation and induction of iNOS.

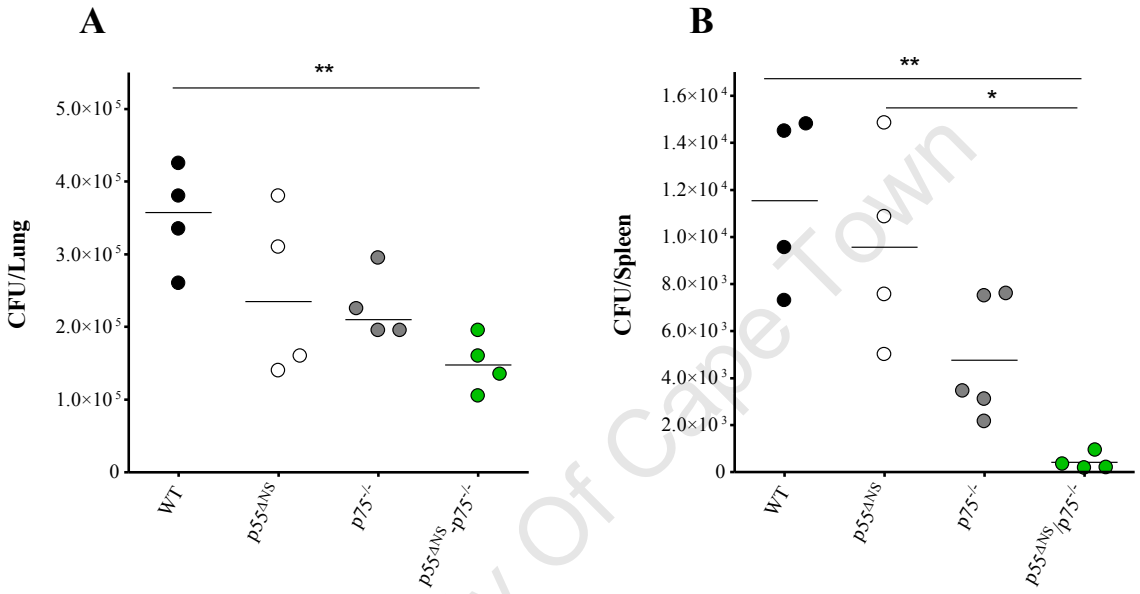


Figure 8. Absence of p75TNFR in the presence of persistent p55TNFR expression improves mycobactericidal responses in vivo. WT mice (black circles), $p55^{\Delta NS}$ mice (white circles), $p75^{-/-}$ mice (grey circles) and $p55^{\Delta NS}$-$p75^{-/-}$ mice (green circles) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Bacilli numbers were enumerated in the lungs (A) and spleens (B) at day 28 post-infection. Data are representative of 1 of 2 experiments and results are expressed as mean ± SD of between 4-5 mice/group. Statistical differences (*$p < 0.05$; **$p < 0.01$) were determined by ANOVA.

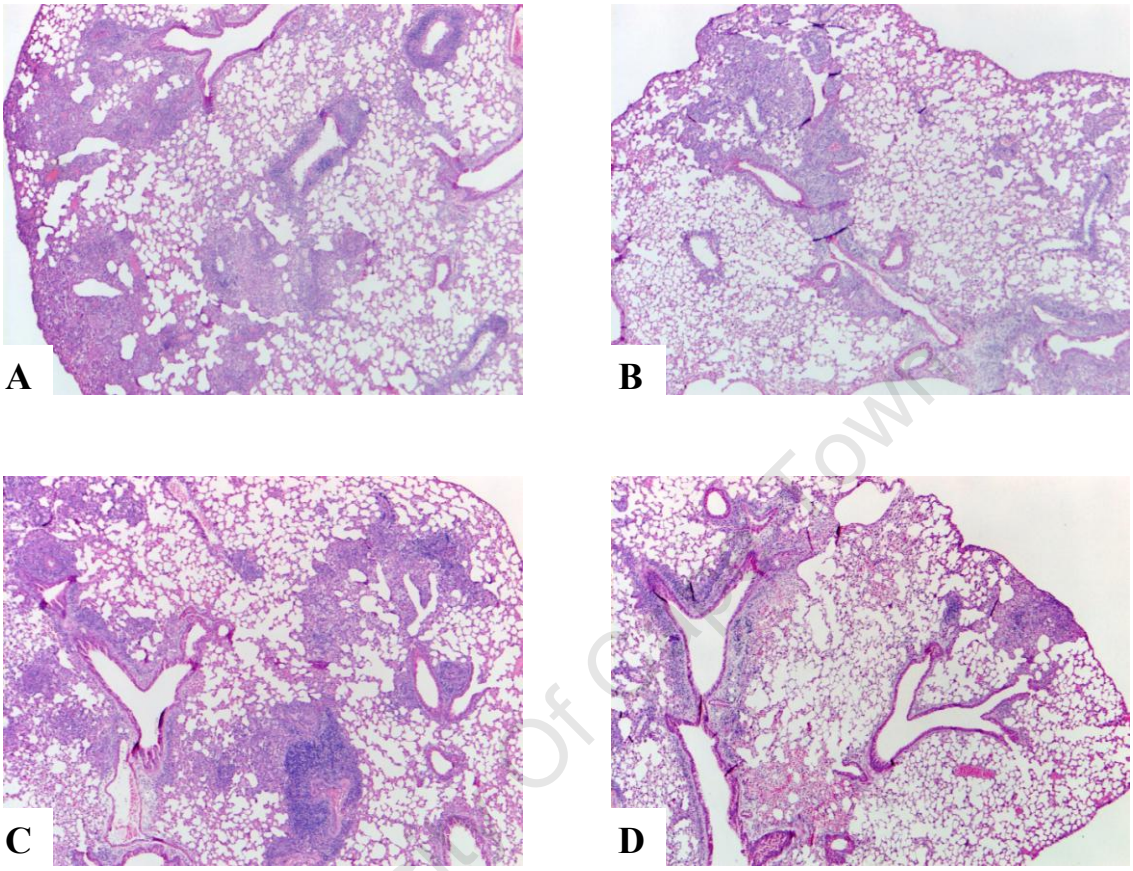


Figure 9.1. p75TNFR is not required for granuloma structure formation in M. tuberculosis infection.. WT mice (A), $p55^{\Delta NS}$ mice (B), $p75^{-/-}$ mice (C) and $p55^{\Delta NS}$-$p75^{-/-}$ mice (D) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lung sections were removed at day 28 post-infection and stained with haematoxylin and eosin. Micrographs are representative of 5mice/group shown at x32 magnification.

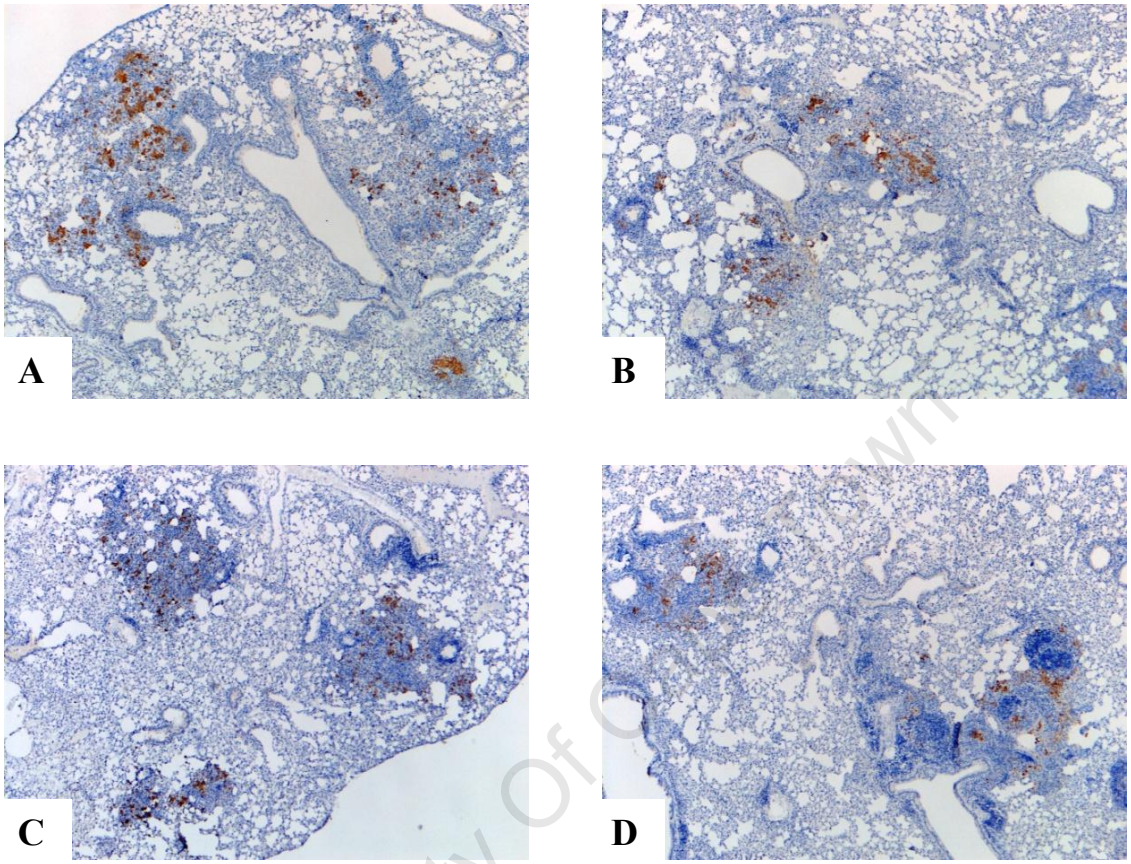


Figure 9.2. iNOS expression is similar in all the strains after M. tuberculosis infection. WT mice (A), $p55^{\Delta NS}$ mice (B), $p75^{-/-}$ mice (C) and $p55^{\Delta NS}$-$p75^{-/-}$ mice (D) were infected by aerosol inhalation with 100 CFUs/mouse *M. tuberculosis* H37Rv. Lung sections were removed at day 28 post-infection and stained with polyclonal rabbit anti-mouse antibody (see *Materials and methods*), the brown stain is indicative of iNOS-positive epitheloid macrophages. Micrographs are representative of 5mice/group shown at x32 magnification.

Discussion

Several studies have shown that TNF mediate protective effects in immunity against invading pathogens (Bean et al., 1999; Florido and Appelberg, 2007; Garcia et al., 1997; Jacobs et al., 2000b; Kindler et al., 1989; Roach et al., 2002) but is also one the chief mediators in pathogenesis of endotoxic shock (Rothstein and Schreiber, 1988) and its neutralization with antibodies results in alleviation of autoimmune inflammatory disorders such as rheumatoid arthritis and Crohn's disease (Dimakou et al., 2004; Sichletidis et al., 2006). The widespread expression of the TNFRs on different cell types and tissues reported by several researchers (Aggarwal et al., 1985; Smith and Baglioni, 1989; Tsujimoto et al., 1985) show that TNF can interact with a vast array of target cells and hence TNF-TNFR mediated effects are tightly regulated. TNF mediated activities are regulated by multiple regulatory mechanisms and one important way is thought to be proteolytic shedding of TNFRs from cell surfaces resulting in soluble TNFRs that can bind TNF thereby competing with cell surface receptors thus limiting TNF function in target cells (Engelmann et al., 1989; Engelmann et al., 1990a; Engelmann et al., 1990b; Olsson et al., 1989). However, the *in vivo* significance of TNFR shedding has remained vague. McDermott et al., (1999), reported that in humans, different missense mutations on the p55TNFR gene that associate with defective receptor shedding strongly associate with autosomal dominant periodic fever syndromes known as TRAPS which include recurrent episodes of fever, myalgia, rash, abdominal pain, conjunctivitis and arthritis and are thought to be primarily caused by irregular innate immune function in these patients.

In this study, we investigated the functional relevance of persistent p55TNFR cell surface expression and p55TNFR shedding during infection with *M. tuberculosis* H37Rv using a mutant mouse strain ($p55^{\Delta NS}$) with p55TNFR wild type receptor functionality but have defective shedding capability (Xanthoulea et al., 2004). We show using an *in vitro* macrophage culture system that p55TNFR is released from the cell surface prior to and after cell activation with either LPS or viable *M. tuberculosis* H37Rv bacilli and this observation is in agreement with previous reports which also showed in other systems that soluble TNFRs are generated after exposure to stimuli (Lantz et al., 1990; Leeuwenberg et al., 1994; Porteu and Nathan, 1990). Moreover, our results show both the constitutive and *M. tuberculosis*-activation induced shedding are inhibited in $p55^{\Delta NS}$ derived cells and this result correlates with the presence of cell surface p55TNFR which coincides with previous studies which used PMA to stimulate cells using a similar mutant mouse (Xanthoulea et al., 2004) confirming persistency of cell surface receptor expression.

Studies have shown that interference of TNF-p55TNFR signaling pathway either by gene deletion of ligand or receptor or by neutralizing antibodies leads to susceptibility to mycobacterial and other intracellular pathogens (Bean et al., 1999; Jacobs et al., 2000b; Kaneko et al., 1999; Pfeffer et al., 1993; Rothe et al., 1993b) but the characteristic role(s) of both soluble p55TNFR and the membrane bound p55TNFR were not clearly defined *in vivo* during challenge with pathogens. We hypothesized that persistent cell surface p55TNFR expression will result in enhanced TNF-p55TNFR interaction leading to enhanced immune responses. Our data demonstrate that persistent cell surface p55TNFR expression does not lead to enhanced protection against *M. tuberculosis* H37Rv infection

in both the acute and chronic phases of the infection. We found that mice expressing the mutant persistent cell surface receptor succumbed to infection with similar kinetics to control mice and that bacterial clearance was equivalent in both strains suggesting that host bactericidal mechanisms were not improved. These results were surprising because an earlier study by Xanthoulea et al., (2004), showed that challenging $p55^{\Delta NS}$ mice with increasing doses of *L. monocytogenes* results in improved host resistance in mutant mice relative to control animals suggesting that defective p55TNFR shedding enables enhanced antibacterial host mechanisms and that pathogens may evoke p55TNFR shedding as an immune escape mechanism.

Nevertheless, we investigated the host immune responses in $p55^{\Delta NS}$ mice to better describe what happens during *M. tuberculosis* infection. We found that pulmonary cellular recruitment does not increase over the course of infection in $p55^{\Delta NS}$ mice relative to control animals, this outcome was interesting because TNF is known as the master orchestrator of all stages of inflammation from recruitment of inflammatory cell, limitation of the process to its termination with repair of inflammation induced damage through promotion of fibroblast growth (Vilcek et al., 1986) and neovascular formation (Frater-Schroder et al., 1987). Closer inspection of the lung cell populations during early infection revealed that there is a transient increase in the frequency of $CD11b^{+}/MHC\text{-}II^{+}$ expressing cells in mice persistently expressing p55TNFR but the activation state of these cells determined by mean fluorescent intensity of the cell surface expression levels of MCH-II and co-stimulatory molecule CD80 is not enhanced compared to WT controls. However, by virtue of the observed transient increase of pulmonary $CD11b^{+}/MHC\text{-}II^{+}$ cells and the increasing trend of lung IL-12p70 levels; which is reported to play a pivotal

role in the generation and activation of Th1 immune responses (Hsieh et al., 1993; Oswald et al., 1994), we hypothesized heightened type 1 immune induction in mice expressing persistent cell surface p55TNFR. Other studies have demonstrated importance of IL-12 showing that in its absence mice are susceptible to infection with mycobacteria and this was associated with impaired type 1 cytokine responses as well as malformed granuloma structures (Cooper et al., 1997; Wakeham et al., 1998). We found that persistent p55TNFR expression is associated with a decrease of total pulmonary IFNγ levels and this observation correlated with a decrease in $CD4^+$ T cell activation state reflected by a decrease in mean fluorescent intensity of CD44 expression on $CD4^+$ T cells although the total frequency of pulmonary $CD4^+$ T cells was equivalent in both strains. As a possible mechanism known to down regulate Th1 immune responses (Gong et al., 1996), we investigated the kinetics of pulmonary IL-10 levels and interestingly we found equivalent concentrations in both strains, suggesting other mechanisms that modulate IFNγ production might be at play. One such mechanism explores the potent TNF-TNFR immunomodulatory effects and has been elegantly demonstrated by Isomaki and colleagues (2001), using T cell hybriboma clones which were exposed chronically to TNF resulting in down regulation of TCRδ and expression of the TCR/CD3 complex at the cell surface and ultimately leading to T cell hyporesponsiveness reflected by decrease in antigen-specific proliferation and suppression of cytokine responses. In addition, the authors demonstrate that this T cell hyporesponsiveness is mediated through p55TNFR and is TNF dependent as IL-1 which shares some signaling pathways with TNF does not induce the suppressive effects on the T cell hybridomas. Another study by Cope et al., (1997), also shows that in a model of chronic inflammation involving TCR transgenic

mice that persistent p55TNFR signaling leads to down modulation of T cell responses. Therefore, the results obtained suggest that persistent p55TNFR expression have different outcomes with regards to macrophage and $CD4^+$ T cells activation and this is further corroborated by results presented by Xanthoulea et al., (2004), which demonstrate macrophage hyperactivation following stimulation with LPS and the authors suggest presence of a positive feed-back loop operating between cell surface p55TNFR and TNF mRNA transcription and/or stabilization. These outcomes however, have not been demonstrated in *M. tuberculosis* H37Rv infections.

It was intriguing though, that the observed reduced levels of IFNγ did not result in less resistance to challenge with *M. tuberculosis* in $p55^{\Delta NS}$ mice possibly because the IFNγ levels were still sufficient to induce protective effects, moreover, we found that cells derived from lung draining lymphoid tissue of these mice produced IFNγ similar to WT controls after 72 h stimulation with either mycobacteria-specific ESAT-6 antigen, or live *M. tuberculosis* bacilli, or stimulation with anti-CD3/CD28. Although commonly used, this *ex-vivo* method does not take into account when comparing the different mouse strains the number of peptide/MHC complexes on antigen presenting cells or the level of costimulatory molecules at the T cell-antigen presenting cell interface which may influence the amplitude of the T cell activation and therefore, the results obtained may be difficult to interpret (Kupfer, A., and Singer, S.J., 1989; Kupfer et.al.,1989; Wülfing, C., and Davis, M.M., 1998; Wülfing et al., 1998).

Regarding possibilities of TNF-TNFR immunomodulatory effects in our system, we focused our attention on TNF neutralizing potential of soluble TNFRs as a way of mechanism that mediates TNF available for inducing physiological responses. Several

reports have indicated that soluble TNFRs are natural inhibitors of TNF and were shown be present in blood and urine (Engelmann et al., 1989; Engelmann et al., 1990b; Novick et al., 1989). Pinckard and colleagues (1997), showed that both the TNFRs are constitutively shed in the circulation and their levels increase during the course of different disease states (Diez-Ruiz et al., 1995) and after stimulation with TNF (Lantz et al., 1990). More recently, van Mierlo et al., (2008), identified p75TNFR shedding to be a novel mechanism by which T regulatory cells down modulates inflammatory mediators. Our data show that p55TNFR is released in WT macrophages after stimulation with either LPS or viable *M. tuberculosis* H37Rv bacilli which contradicts the observation reported by Balcewicz-Sablinska et al., (1998). In their *in vitro* culture system soluble p55TNFR levels were not significantly elevated after stimulation of human alveolar macrophages with either avirulent *M. tuberculosis* H37Ra or virulent *M. tuberculosis* H37Rv, however our results do support p55TNFR release shown in other studies (Aderka et al., 1991; Carpenter et al., 1995; Pinckard et al., 1997). Furthermore, we show that more p75TNFR is released after macrophage activation with either LPS or viable *M. tuberculosis* H37Rv compared to p55TNFR release, this phenomenon has been reported elsewhere (Aderka et al., 1991; Carpenter et al., 1995; Pinckard et al., 1997). Moreover, we show that persistent p55TNFR leads to further release of p75TNFR after macrophage stimulation with both stimulants used, LPS or viable *M. tuberculosis* H37Rv bacilli. This could be explained by the increased TNF levels in macrophages expressing persistent cell surface p55TNFR and this result coincides with previous reports (Xanthoulea et al., 2004) but another possibility is receptor cross-regulation suggested by Higuchi and Aggarwal (1992).

TNF, IL-1 (Joyce and Steer, 1995)and IL-10 (Balcewicz-Sablinska et al., 1998; Joyce et al., 1994) were reported to influence release of p75TNFR. In this context, it appears that p75TNFR shedding may be regulated by multiple mechanisms. In addition, the enzymatic regulatory mechanisms that influence metalloprotease TNF-α-converting enzyme (Peschon et al., 1998) and carboxy-peptidase cathepsin-D (Coyne et al., 1999) established as key players of p75TNFR release need further investigation to determine whether their activity during inflammatory processes and infections is affected.

We demonstrate that the increased p75TNFR expression in $p55^{\Delta NS}$ macrophages is associated with TNF bioactivity that is equivalent to WT control macrophages and we associated this with the similar outcome of *M. tuberculosis* infection in $p55^{\Delta NS}$ mice and WT mice. Many studies have shown TNF-TNFR complexes and the therapeutic benefits for down modulating excess TNF using anti-TNF antibodies or administration of soluble TNFRs have been realized as well as the consequences of complete TNF neutralization. We investigated genetic deletion of p75TNFR in mice persistently expressing p55TNFR to determine whether control of mycobacterial infection is enhanced. We reasoned that the resultant double mutant mouse strain $p55^{\Delta NS}$-$p75^{-/-}$ will allow for better TNF-p55TNFR signaling which should enhance immune responses against *M. tuberculosis* H37Rv and lead to better clearance because of more biologically active TNF. We found that after challenging mice with *M. tuberculosis* H37Rv, absence of p75TNFR alone results in better bacterial control relative to WT control mice and this outcome is slightly better than $p55^{\Delta NS}$ mice. Our data further demonstrate more significant bacterial control is observed in mice expressing persistent p55TNFR in absence of p75TNFR ($p55^{\Delta NS}$-$p75^{-/-}$) relative to WT mice and in addition, the granulomas that are formed seem slightly

smaller consequently due to reduced bacilli burdens. It must be noted that p75TNFR is redundant with regards to granuloma structure formation because in p75TNFR deficient mice the bactericidal granulomas are formed.

Although we show that p75TNFR shedding controls bioactive TNF levels, our results do not exclude the possibility that *M. tuberculosis* itself use p75TNFR shedding as an immune evasion mechanism and this line of thought is supported by Balcewicz-Sablinska et al., (1998), showing decrease in macrophages apoptosis after challenge with *M. tuberculosis* and the authors suggest that virulent mycobacterial strains achieve this by inducing IL-10-dependent p75TNFR shedding.

The role of signaling through p75TNFR in host immunity must not be ignored. To date, there is controversy regarding the *in vivo* functional role of p75TNFR, Allendoerfer and Deepe Jr., (2000), show a protective function against infection with fungus such as *H. capsulatum*, where deficiency of p75TNFR leads to reduced IFNγ production and treatment with this cytokine restores protective immunity. Furthermore, infections with the intracellular parasite *T. brucei* result in reduced infection-associated pathology in absence of p75TNFR (Magez et al., 2004). By contrast, Peschon et al., (1998), showed that p75TNFR does not play a role in host immunity after *L. monocytogenes* infection.

In conclusion, our results confirm previous reports that show that signaling through p55TNFR is important for control of *M. tuberculosis* infection and p75TNFR signaling is redundant. We demonstrate clearly that p55TNFR shedding is subservient to p75TNFR shedding in attenuating TNF activities. Therefore, the enhanced immune response at least with regard to *M. tuberculosis* infection is a factor of TNF

bioavailability and specific targeting of soluble p75TNFR might be beneficial for improving immune responses towards *M. tuberculosis* infections.

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Appendix

General Solutions

All the chemical reagents used were purchased from Merck Laboratory Supplies (Germany) or Sigma-Aldrich (St. Loius, USA) unless otherwise stated.

Difco Middlebrook 7H10 agar
19 g of 7H10 agar was dissolved in distilled H_2O with 5 ml glycerol and the volume was made up to 900 ml with distilled H_2O. The solution was autoclaved at 121°C for 10 minutes and was allowed to cool to 55°C before adding 100 ml OADC. Seven miles per side was poured on sterile Sterilin duplicate petridishes. Agar was allowed to solidify at room temperature before storage at 4°C.

Formalin
100 ml formaldehyde (40% w/v) and
900 ml PBS (pH 7.4) were mixed and stored at room temperature in the dark.

0.9% Saline
9 g NaCl was dissolved in
100 ml distilled H_2O and autoclaved at 121°C for 30 minutes. The solution was stored at room temperature.

Tween 80 saline
0.04% Tween 80 and
0.9% saline was dissolved in distilled H_2O and autoclaved at 121°C for 30 minutes.
The solution was stored at room temperature.

10x PBS
80 g NaCl,
2.4 g KH_2PO_4,
2 g KCl and
14.4 g $Na_2HPO_4.2H_2O$ were dissolved in
900 ml distilled H_2O,
The pH was adjusted to 7.4 with HCl and the volume was made up to 1000 ml with distilled
H_2O then sterilized by filtering through a 0.45 µm filter (Millipore Corporation, Bedford, USA). The solution was stored at room temperature.

Flow cytometry solutions

10% NaN_3
10 g NaN_3 was dissolved in
100 ml distilled H_2O and sterilized by filtration through a 0.45 µm filter (Millipore Corporation, Bedford, USA). The solution was stored at 4°C.

FACS buffer
1 g BSA (Boeringer Mannhain, Germany) and
0.1 g NaN3 were dissolved in

900 ml 1x PBS.
The pH was adjusted to 7.4 and the volume was made up to 1000 ml then sterilized through using a 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

FACS blocking buffer
3.75 µl normal rat serum (heat inactivated),
3.75 µl normal mouse serum (heat inactivated), and
0.9375 µl anti-CD32/CD16c (anti-FCγRIII/II) (clone 2.4G2, Pharmingen) were made up to 150 µl with FACS buffer.

FACS fixation buffer
4 g NaOH was dissolved in
100 ml 1x PBS,
20 g paraformaldehyde was added, the volume was made up to 1000 ml and the pH was adjusted to 7.2. The solution was sterilized by filtering through 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C in the dark.

The following antibodies purchased from Pharmingen were used in this study : anti-CD4-FITC (H129.19), anti-CD11a-PE (M17/4), anti-CD44-PE (IM7), anti-CD86-PE (GL1), anti-CD80-PE (16-10A1), anti-CD120b-PE (HM 102), anti-CD62L-PE (MEL-14), anti-CD69-PE (H1.2F3), anti-I-A/I-E-PE (M5/14.15.2), anti-CD11b-FITC, anti-CD49a-PE, anti-IgG 2a-FITC (R35-95), anti-IgG 2a-PE (R35-95) and, anti-IgG-PE (G70-204) .

ELISA solutions and brief protocol

Coating buffer
0.2 g NaN_3 was dissolved in

800 ml 1x PBS. The pH was adjusted to 7.2 and the volume was made up to 1000 ml then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Dilution buffer
10 g BSA (Boeringher Mannheim, Germany) and
0.3 g NaN_3 were dissolved in

800 ml 1x PBS. The pH was adjusted to 7.4; the volume was made up to 1000 ml then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Reagent diluent
0.1% BSA (Boeringher Mannheim, Germany) and
0.05% Tween 20 were dissolved in saline containing
20 mM Trizma base and
150 mM NaCl. The pH was adjusted to 7.4 then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Blocking buffer 1
1% BSA (Boeringher Mannheim, Germany),
5% Sucrose and
0.05% NaN_3 were dissolved in 1x PBS, the pH of the solution was adjusted to 7.4 then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Blocking buffer 2
40 g BSA (Boeringher Mannheim, Germany),
0.2 g NaN_3 were dissolved in
800 ml 1x PBS. The pH was adjusted to 7.4 then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

20x Wash buffer
20 g KCl,
20 g KH_2PO_4,
144 g NaH_2PO_4, and
800 g NaCl were dissolved in 4.5 L of distilled H_2O.
50 ml Tween 20 and
100 ml 10% NaN_3 were added. The volume was made up to 5 L then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at room temperature.

Substrate buffer
0.2 g NaN_3 and
0.8 g $MgCl_2$ were dissolved in 700 ml distilled H2O,
97 ml liquefied diethanolamine was added then the volume was made up to 1000 ml. The pH was adjusted to 9.8 and the solution was then sterilized through filtering using 0.45 µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

ELISA protocol

Supernatants from organ homogenates or from cultured cells were harvested and assayed for cytokine concentrations using commercially available ELISA reagents for TNF, IFNγ, IL-10, IL-12p40, IL-12p70, p55TNFR and p75TNFR (R&D Systems, Germany and BD PharMingen, San Die). Briefly, Nunc Maxisorp 96 well plates (Nalge Nunc International, Naperville, IL, USA) were coated with 50 µl of capture antibody at 2 µg/ml except for TNF and IFNγ (coated with 1 µg/ml and 4 µg/ml capture antibody, respectively) overnight at 4°C. Wells were washed with 1x wash buffer and blocked with 300 µl of block buffer for 1 h at 37°C. After washing the wells, 50 µl of samples or 50 µl appropriate recombinant mouse standards at 2 fold dilutions (diluted in Reagent diluent) at a starting concentration of 2 ng/ml except for TNF which was added in 3 fold serial dilutions (diluted in dilution buffer) at a starting concentration of 100 ng/ml were incubated for 2 h at 37°C. Wells were washed with 1x wash buffer and 50 µl of appropriate biotinylated detection antibody at recommended concentrations were added and incubated for 2 h at 37°C. Wells were washed with 1x wash buffer and incubated in 50 µl of streptavidin-alkaline phosphatase (R & D Systems, Minneapolis, USA) at 1: 1000 dilution for at least 45 min at room temperature, for TNF ELISA, wells were incubated at 1: 10000 with alkaline phosphatase-labelled goat anti-rabbit (BD PharMingen, San Diego, USA) for at least 2 h at room temperature. Wells were washed with 1x wash buffer and 50 µl of p-nitrophenyl phosphate substrate (Boehringher Mannheim, Germany) at 1 mg/ml was added, the yellow colour was allowed to develop and absorbance was read at 405 nm on a VERSAmax Tunable Microplate Reader (Molecular Devices Corporation, California, USA) and for data analysis, SoftMax Pro 4.3 software (Molecular Devices Corporation, California, USA) was used.

Histology reagents and brief protocol

E1 Mayers Haematoxylin
1 g haematoxylin was dissolved in
800 ml distilled H_2O.
50 g aluminium ammonium sulphate was added and dissolved.
0.2 g sodium iodate,
1 g citric acid and
50 g chloral hydrate were added in the specified order, dissolving each reagent prior addition of the next compound. The volume was made up to 1000 ml with distilled H_2O then passed through Whatmann filter paper no. 1 and stored in the dark at room temperature.

Wegert's Haematoxylin

Solution a
1 g haematoxylin was dissolved in
100 ml absolute alcohol

Solution b
4 ml 30% aqueous ferric chloride (anhydrate), and
1 ml concentrated HCl were added to
95 ml distilled H_2O. Equal volumes of solutions a and b were mixed before use.

Eosin
150 ml of 1% Eosin was added to
75 ml 1% Phloxine solution. The solution was filtered through Whatmann filter paper no. 1 and stored at room temperature.

Carbol Fuchsin
10 ml 6% Basic fuchsin (in absolute alchohol) and
90 ml 5% carbolic acid were mixed and passed through a Whatmann filter paper no. 1. The solution was stored at room temperature.

Loeffers' Methylene Blue
1 ml 1% KOH,
99 ml distilled H2O and
3 ml 0.8% Methylene Blue (in absolute alcohol) were mixed and filtered through Whatmann filter paper no. 1 and the solution was stored at room temperature.

Paraffin wax embedding
Tissue samples were dehydrated using an automated tissue processor (LEICA TP1020, Wetzlar, Germany) and were embedded in wax (LEICAEG1140C, Wetzlar, Germany) in the following way:
70% alcohol, 30 min,
96% alcohol, 45 min (2x),
100% alcohol, 45 min (4x),
Xylol, 60 min (2x)
Wax (55°C - 60°C), 45 min (2x) with vacuum

Sections of 2 µm – 3 µm in size were cut using a microtome (Leica RM-2125RT or SM 2000R, Heidelberger, Strasse) then floated onto glass slides and fixed overnight at 37°C. The wax was removed from tissue section by incubating at 60°C for 2 - 18 h. Prior to staining, tissues were rehydrated in the following way:
Xylol, 3 min,
Xylol, 1 min (2x),
Absolute alcohol, 1 min (2x)
96% alcohol, 1 min (2x)
70% alcohol, 1 min (2x),
Water, 1 min

Haematoxylin and Eosin staining protocol (H&E)

Mice were euthanized by carbon dioxide inhalation at specific time points. Rehydrated tissue was immersed for 8 min in Haematoxylin, rinsed with water then immersed in 1% acid alcohol. Subsequently, tissues were rinsed in running water for 30 min then counterstained by soaking in 1% Eosin for 2 min, followed by a quick wash in water and dehydrated by 10 sec immersions in 70% alcohol, 96% alcohol and xylol, in that order. Sections were mounted using entellen (Merck, Germany).

Ziehl-Neelson staining protocol (ZN)

Rehydrated tissues were soaked in Carbol Fuschin solution, flamed and then allowed to cool for 5 min. The flaming and cooling procedure was repeated followed by rinsing in water. The slides were subsequently soaked in 1% acid alcohol for 30 sec and rinsed in water, followed by staining with 25% H_2SO_4 for 20 min and then rinsing under running water. Tissues were then stained with Methylene Blue for 1 min then followed by a quick rinse with water and subsequently dehydrated by 10 sec immersions in 70% alcohol, 96% alcohol and xylol, in that order. Sections were mounted using entellen (Merck, Germany).