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The Role of TNFRp55 and TNFRp75 in The Host Immune Response to *Mycobacterium tuberculosis.*

Roanne Shay Keeton

University of Cape Town

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Abbreviations

AFB	acid-fast bacilli
Ag	antigen
APC	antigen-presenting cell
BCG	Bacille Calmette Guerin
BMDM	bone marrow derived macrophage
CFU	colony forming units
CO_2	carbon dioxide
Cr3	complement receptor 3
CTL	cytotoxic T cells
DC's	dendritic cells
DCM	dendritic cell medium
ELISA	enzyme-linked Immunosorbant assays
FCS	fetal calf serum
FACS	fluorescently activated cell sorting
H&E	haemotoxylin and eosin staining
HIV	human immunodeficiency virus
IFNγ	interferon gamma IFNγ
IL-6	interleukin 6
IL-12	interleukin 12
iNOS	inducible nitrous oxide synthase
IV	intravenous infection
kD	kilodalton
LTα	lymphotoxin alpha

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LTβ	lymphotoxin beta
MHC	major histocompatibility complex
mAb	monoclonal antibody
M. africanum	*Mycobacterium africanum*
M. bovis	*Mycobacterium bovis*
M. cantetti	*Mycobacterium cantetti*
M. leprae	*Mycobacterium leprae*
M. microti	*Mycobacterium microti*
M. smegmatis	*Mycobacterium smegmatis*
M.tb	*Mycobacterium tuberculosis*
M. tuberculosis	*Mycobacterium tuberculosis*
MDR	multidrug-resistant
DLN	draining lymph node
NFκB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide
OADC	oleic acid-albumin-dextrose-catalase
PBS	phosphate-buffered saline
RNI	reactive nitrogen intermediates
RT	room temperature
NaN_3	sodium azide
PCR	polymerase chain reaction
SPF	specific pathogen free
TACE	tumor necrosis factor alpha converting enzyme
TBE	tris borate ethelyne diamino tetra acetic acid

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T_H	T-helper
Tm-TNFα	transmembrane tumor necrosis facter alpha
TNFα	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
WEHI	Walter and Eliza Hall Institute
WHO	world health organization
WT	wild type
XDR	extensively drug resistant
ZN	Ziehl-Neelson

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Abstract

Tumor necrosis factor alpha (TNFα) is critical for host protective immunity against *Mycobacterium tuberculosis* infection. TNFRp55 and TNFRp75 can both bind TNFα and conduct signaling, however the respective roles, in particular that of TNFRp75 in an *M. tuberculosis* aerosol inhalation infection was poorly defined. In this study the role of signaling through TNFRp55 and TNFRp75 was investigated using TNFR deficient mice in an aerosol inhalation *M. tuberculosis* infection model. This study showed that TNFRp75$^{-/-}$ mice are able to resist an acute *M. tuberculosis* infection with increased control of bacilli replication. Granuloma formation was dependent on TNFRp55 and independent of TNFRp75 signaling but with reduced pulmonary inflammation in the absence of TNFRp75 during *M. tuberculosis* infection. Enhanced protective immunity observed in TNFRp75$^{-/-}$ mice during acute *M. tuberculosis* infection was maintained during chronic infection with a significant increase in survival of TNFRp75$^{-/-}$ mice. An analysis of the mechanism underlying increased protective immunity in TNFRp75$^{-/-}$ mice found an increased activation of antigen presenting cells during *M. tuberculosis* infection with dendritic cells in particular showing higher IL-12p40 production *in vitro*. This enhanced dendritic cell IL-12p40 production was associated with an increase in the migration of dendritic cells to the lung draining lymph nodes and subsequently correlated with an increased number of activated T cells expressing CD4/CD44 in the lymph nodes of TNFRp75$^{-/-}$ mice. An increase in the number of activated lymph node T cells was associated with enhanced pulmonary *M. tuberculosis* specific IFNγ synthesis in TNFRp75$^{-/-}$ mice during infection. Shedding of TNFRp75 from the cell surface was shown to be induced during *M. tuberculosis* infection which resulted in significantly reduced levels of bioactive TNFα, significantly lower IL-12p40 synthesis and subsequently a reduction in the number of activated antigen presenting cells in WT mice compared to TNFRp75$^{-/-}$ mice. Treatment of WT dendritic cells with anti-TNFRp75 altered their phenotype to resemble that of TNFRp75$^{-/-}$ dendritic cells thereby directly linking TNFRp75 shedding with bioactive TNFα levels. This was shown be critical to the number of activated antigen presenting cells present and thus to the outcome of disease during an aerosol inhalation *M. tuberculosis*

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infection. It was concluded that TNFRp55 is the predominant signaling pathway involved in protective immunity against aerosol inhalation *M. tuberculosis* infection and that TNFRp75 has a significant regulatory role in down-modulating the immune response, the absence of which provides better protection and improved outcome of disease during an aerosol inhalation *M. tuberculosis* infection.

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Chapter 1:

Literature Review

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Literature Review

LR. 1.1 The TNFα/TNFR Superfamily

The first members of this family which were identified are the transmembrane form of TNFα (Tm-TNFα) and the secreted Lymphotoxin α (LTα) cytokines (Granger and Williams, 1968; Locksley *et al.*, 2001; Orlinick and Chao, 1998; Pennica *et al.*, 1984; Pfeffer, 2003). TNFα can exist in two forms namely Tm-TNFα or soluble TNFα (sTNFα). Tm-TNFα is a 26kDa cell surface transmembrane type II polypeptide which is active in its homotrimeric form and acts as the precursor for sTNFα. Cleavage by the metallo-protease TNF-alpha-converting enzyme (TACE) releases soluble TNFα with a molecular weight of 17kDa (Black *et al.*, 1997; Moss *et al.*, 1997a; Moss *et al.*, 1997b; Smookler *et al.*, 2006). TNFα is produced by a wide variety of immune and non-immune cells alike that include macrophages, T cells, natural killer cells (NK), fibroblasts and smooth muscle cells (Tracey *et al.*, 2008). The core members of the Superfamily are considered to be LTα, LTβ, TNFα and LIGHT and are important for a wide variety of biological activities including the mediation of the host inflammatory response and protective immunity against several different pathogens (Locksley *et al.*, 2001; Naismith and Sprang, 1995; Smith *et al.*, 1994; Ware *et al.*, 1995). LTα and TNFα are biologically active in trimeric form with each cytokine having the ability to signal through more than one receptor. In the homotrimeric form, $LT\alpha_3$ and $TNF\alpha_3$ signal through both TNFRp55 and TNFRp75, while LTα, in association with LTβ, can form the heterotrimers $LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$ and both heterotrimers can bind and signal through the LTβ receptor (Banner *et al.*, 1993; D'Arcy *et al.*, 1993; Eck and Sprang, 1989; Ware *et al.*, 1995).

TmTNFα and sTNFα forms are biologically active and either form can signal through either TNFRp55 or TNFRp75 resulting in nuclear factor κB (NFκB) activation and cytokine production, or apoptosis (Olleros *et al.*, 2002; Olleros *et al.*, 2005; Orlinick and Chao, 1998; Peschon *et al.*, 1998).

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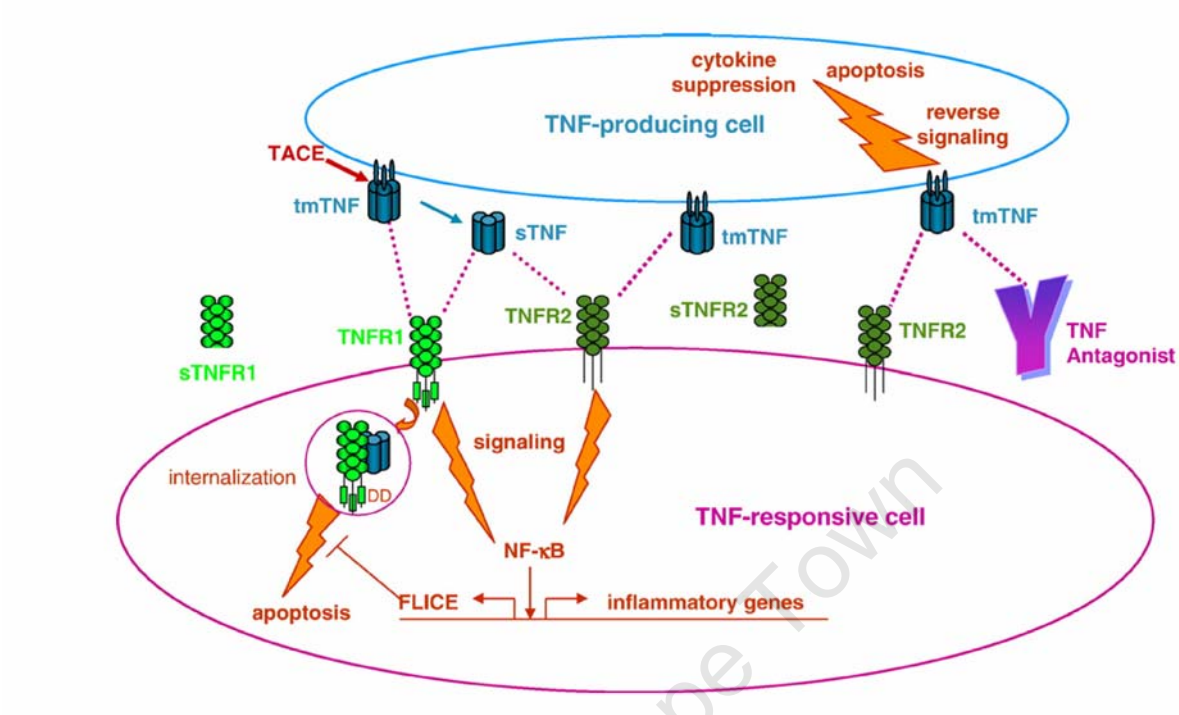

Figure A: Biology of TNFα production, receptor interaction and signalling.
Stimulation of a TNFα-producing cell (top) results in cell surface expression of tmTNFα trimers and enzymatic cleavage by TACE to release sTNFα. Both tmTNFα and sTNFα can bind to cell surface TNFR1 (p55) or TNFR2 (p75) on a TNFα-responsive cell (bottom), initiating signalling pathways that lead to apoptosis or NF-κB activation and inflammatory gene activation. The induction of apoptosis by sTNFα via TNFR1 involves internalization of the ligand–receptor complex and association of death domains (DD) in the cytoplasmic tail of TNFR1 with adapter proteins and is normally blocked by FADD-like IL-1β–converting enzyme (FLICE). Reverse signalling can be initiated by TNFR2 or TNFα antagonist binding to cell surface tmTNFα, resulting in cytokine suppression or apoptosis. Soluble TNF receptors (sTNFR1 and sTNFR2) can be released from a TNFα-responsive cell following enzymatic cleavage (Tracey *et al.*, 2008).

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Both receptors are membrane glycoproteins which can be cleaved by TACE to release soluble receptor forms (Eissner *et al.*, 2004; Rossol *et al.*, 2007). Similarly to TNFα, soluble TNFRp55 and particularly TNFRp75 binds to TNFα and regulates its activity both *in vivo* (Pinckard *et al.*, 1997) and *in vitro* (Balcewicz-Sablinska *et al.*, 1998). The outcome of TNFα signaling through TNFRp55 or TNFRp75 is distinct and is directly due to structural differences in the cytoplasmic tail (Chan *et al.*, 2000; Grell *et al.*, 1995). In addition, TmTNFα can act as a receptor for soluble TNF receptors or membrane TNF receptors through cell to cell contact resulting in reverse signaling through the cytoplasmic tail of TmTNFα which can trigger cell apoptosis, cytokine

suppression or cell activation (Domonkos *et al.*, 2001; Eissner *et al.*, 2000; Eissner *et al.*, 2004; Harashima *et al.*, 2001; Tanabe *et al.*, 1998). The biology of TNF production, signaling and receptor interactions is summarized in Figure A above (Tracey *et al.*, 2008).

LR. 1.2 The Role of TNFα/ TNFR Signaling in Tuberculosis

LR. 1.2.1 Global *M. tuberculosis* Health Risk

Tuberculosis is the leading cause of death from a single infectious agent (Graham and Clark-Curtiss, 1999) resulting in 9.2 million new infections in 2006, (WHO, 2008) and two million deaths every year (WHO, 2008; Park et al., 2003). It is estimated that about a third of the world population is latently infected with *M. tuberculosis* and of these approximately 10% will develop active disease. In individuals that become co-infected with human immunodeficiency virus (HIV) this risk increases to an 8 - 10% chance of reactivation per year (Sherman *et al.*, 2001). In a large majority of cases, tuberculosis is caused by reactivation of a past infection (Cunningham and Spreadbury, 1998; Scanga *et al.*, 1999) rather than re-infection and is ascribed to the ability of the bacteria to lie dormant in response to a strong immune response and then to reactivate under conditions of immune depravation. This, together with the observation that dormant bacteria are resistant to normal antimycobacterial drugs (Wayne, 1994; Wayne and Hayes, 1996; Wayne and Sramek, 1994) are influencing factors in the World Health Organization (WHO, 2008) prescribed DOTS 6-month treatment program for *M. tuberculosis* infection (Boon *et al.*, 2001; Cunningham and Spreadbury, 1998). The emergence of multidrug-resistant (MDR) and the more recently described extensively drug-resistant (XDR) strains of *M. tuberculosis* arose as a result of improper use of antibiotics in chemotherapy and poor compliance to treatment regimens contributing significantly to increasing challenges for control programs (WHO, 2008). The treatment of MDR strains requires a lengthy and costly treatment program with second-line drugs, while the treatment of XDR strains are severely challenging. The elimination of *M. tuberculosis* as a global health problem requires new emphasis on the proper control/management of tuberculosis (WHO, 2008).

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LR. 1.2.2 The *M. tuberculosis* Complex

Mycobacteria are rod shaped bacteria which have a lipid rich cell wall that is acid-fast and are therefore termed acid-fast bacilli (AFB) (Glickman and Jacobs, 2001). There are more than seventy species of mycobacteria, of which *M. tuberculosis* and *Mycobacteria leprae* (*M. leprae*) are two major pathogens. The remainder of the mycobacterial family is composed of environmental organisms and, although not usually pathogenic, can cause opportunistic infections in immuno-compromised individuals. Some species are host specific such as *Mycobacterium africanum* (*M. africanum*) and *Mycobacterium cantetti* (*M. cantetti*) which only infect humans whereas *Mycobacterium microti* (*M. microti*) only infects rodents. In contrast other species such as *Mycobacterium bovis* (*M. bovis*) and *M. tuberculosis* are able to infect a number of different host species (Collins and Grange, 1996; Grange, 1996a, b).

M. tuberculosis is a slow growing mycobacterium that is transmissible by the inhalation of droplet nuclei containing one to three bacilli incased in mucus and capable of floating in air (Graham and Clark-Curtiss, 1999). Droplets entering the alveoli in the lungs are taken up by alveolar macrophages which are limited in their ability to kill bacteria. Bacilli recognition occurs via different receptors including DC Sign (Barreiro *et al.*, 2006; Tailleux *et al.*, 2005; Torrelles *et al.*, 2008), Mannose receptor (Ezekowitz *et al.*, 1981; Maeda *et al.*, 2003) and complement receptor 3 (Cr3) on the macrophage. Mycobacterial recognition induces phagocytosis and activates macrophages to produce TNFα. Once internalized, the bacteria are localized to the phagosome (Kaufmann, 2001; Russell, 2001) where replication occurs until the host is able to mount an effective immune response (Cunningham and Spreadbury, 1998) that halts disease progression and confine bacilli to newly formed granulomas (Cunningham and Spreadbury, 1998; Mayuri *et al.*, 2002; Russell, 2001) to prevent bacterial dissemination (Russell, 2001).

LR. 1.2.3 Host immune response to infection

M. tuberculosis is an intracellular pathogen which requires a strong cellular adaptive T_H1 immune response for host protection. Successful control of disease relies not only

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on a complex but organized series of interactions between mycobacteria, antigen presenting cells (APC's) and lymphocytes, but also on the co-ordinated production of chemokines and cytokines. Cytokines are transient short-lived protein hormones, which are released in rapid bursts (Abbas, 1997) usually transmitting their signals over a short range (Male, 1996) and function to regulate the immune and inflammatory responses of the host to microbial invasion (Abbas, 1997; Male, 1996). Cytokines bind to specific receptors on the target cells, many being considered to be pleiotropic. Although redundancy has been illustrated for many cytokines, others cannot be functionally replaced and the absence of any one of these can be lethal during pathogen challenge. For example, a deficiency in TNFα has profound effects on protective immunity against *M. tuberculosis* (Bean *et al.*, 1999; Eriks and Emerson, 1997; Kaneko *et al.*, 1999; Kindler *et al.*, 1989; Roach *et al.*, 2002) even though the homologue, lymphotoxin α (LT α) is able to signal through the same TNFα receptors (Schluter and Deckert, 2000).

Macrophages are the primary cells targeted by *M. tuberculosis* during infection. Bacilli make use of various cell surface receptors to enter the macrophage which include the mannose receptor (Ezekowitz *et al.*, 1981; Maeda *et al.*, 2003), DC sign (Barreiro *et al.*, 2006; Tailleux *et al.*, 2005; Torrelles *et al.*, 2008), complement receptor and Fc receptors (Glickman and Jacobs, 2001; Hope *et al.*, 2004). Upon uptake of *M. tuberculosis,* alveolar macrophages are induced to produce T_H1 cytokines such as TNFα and interleukin-12 (IL-12). TNFα is important for cell recruitment and initiation and maintenance of granulomas that restricts bacilli replication and prevent bacterial dissemination (Bean *et al.*, 1999; Flynn *et al.*, 1995; Jacobs *et al.*, 2000c; Senaldi *et al.*, 1996). Although TNFα is essential for the control of mycobacterial infections, excessive TNFα also has deleterious effects and is considered to play a significant role in destruction of lung tissue by severe inflammation (Moreira *et al.*, 1997; Zganiacz *et al.*, 2004) and aid in bacterial replication in alveolar macrophages (Engele *et al.*, 2002). Macrophages constitute a key component of granulomas. Granulomas are metabolically active structures which form in response to various microbial and parasitic infections

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including schistosomal and mycobacterial infection (Kindler *et al.*, 1989; Silva *et al.*, 2000; Warren *et al.*, 1967). It includes aggregates of macrophage and lymphocyte cells. Where, in humans, macrophages form a central core while the lymphocytes are located on the periphery surrounding the macrophages, in mice the structure differs slightly; the two regions are less well defined with the lymphocytes being interspersed between the macrophages as well as forming part of the outer layer (Bean *et al.*, 1999; Gonzalez-Juarrero *et al.*, 2001; Saunders *et al.*, 1999; Silva *et al.*, 2000). Granuloma formation and maintenance is essential to the host for protective immunity against *M. tuberculosis* as illustrated in gene targeted mice which are defective for granuloma formation. Such animals are unable to control bacilli replication, resulting in bacterial dissemination and premature death after infection with either virulent or avirulent mycobacterial species (Bean *et al.*, 1999; Jacobs *et al.*, 2000b; Kaneko *et al.*, 1999; Kindler *et al.*, 1989; Roach *et al.*, 2002).

Dendritic cells (DC's) act as professional APC's critical for the establishment of an effective adaptive immune response and are instrumental in the development of either T_H1 or T_H2 immune responses (Bottomly, 1999). DC's are derived from progenitor cells in the bone marrow from where they migrate into peripheral tissue and are present in an immature state at high numbers in non-lymphoidal primary sites of infection such as the lung. These immature DC's act as sentinels for foreign antigens and microbial pathogens, thus forming an integral part of the innate immune system (Reis e Sousa *et al.*, 1993). After antigen uptake DC maturation is initiated in peripheral tissues followed by migration to lymphoid organs such as draining lymph nodes (DLN) where maturation is completed (Austyn, 1996; Banchereau and Steinman, 1998; Gonzalez-Juarrero *et al.*, 2001; Guermonprez *et al.*, 2002; Marino *et al.*, 2004; Mellman and Steinman, 2001; van Haarst *et al.*, 1994). DC's are thus ideally suited to act as an important interplay between innate and adaptive immunity. Briefly, its role in innate immunity is to act as sentinels for foreign antigens and microbial pathogens (Reis e Sousa *et al.*, 1993), which subsequent to antigen uptake migrate to the draining lymph nodes where mature DC's present antigen to naïve T cells resulting in the promotion of appropriate adaptive immune responses (Bottomly, 1999; Wolf *et al.*, 2008). Immature

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DC's express high levels of adhesion molecules subsequent to antigen uptake which enables these cells to migrate to draining lymph nodes (Banchereau and Steinman, 1998; Gonzalez-Juarrero *et al.*, 2001). Mature dendritic cells are characterized by high levels of major histicompatibility complex (MHC) I and MHCII expression, crucial for antigen presentation to T cells (Flynn and Chan, 2001; Giacomini *et al.*, 2001; Mellman and Steinman, 2001) as well as costimulatory molecules such as CD80, CD86 (Collins *et al.*, 2005) and CD40.

$CD4^+$ T cell activation relies on antigen processing and presentation by DC's in the DLN and not the lung (Wolf *et al.*, 2008). Resting DC's express low levels of cytokines and chemokines that upon maturation induce enhanced secretion of the chemokine CCL18 (Adema *et al.*, 1997), which specifically attracts naïve T cells to the DLN and stimulates IL-12 synthesis (Cooper and Khader, 2007; Cooper *et al.*, 1997; Giacomini *et al.*, 2001; Khader *et al.*, 2006). IL-12, in conjunction with memory T cell/ NK cell derived interferon γ (IFNγ) (Cooper and Khader, 2007; Cooper *et al.*, 1997; de Jong *et al.*, 1997; Flynn and Chan, 2001), is important for T_H1 polarization and T cell activation. In addition, TNFα dependent T_H1 polarization has been shown to be critically dependent on APC-derived IL-12 production (Becher *et al.,* 1999). The early establishment of a T_H1 environment in the DLN during early infection is critical to the protective immune response against *M. tuberculosis* infection. A depletion of $CD11c^+$ DC's delays the $CD4^+$ T cell response and results in the impaired control of bacilli replication and leads to poor outcome of disease (Tian *et al.*, 2005). The early induction of IL-12, once immature DC's become infected, is therefore a critical requirement of DC migration to DLN's. This was well illustrated in studies where mice deficient for IL-12p40 were defective for DC migration and were unable to activate naïve T cells in DLN (Khader *et al.*, 2006).

Sustained T_H1 mediated responses to foreign antigens could potentially cause inflammatory tissue damage and as a result mucosal sites such as the lung, which are under constant barrage of foreign Ag's, could skew towards a T_H2 response. Pulmonary DC's were found to produce large amounts of interleukin (IL-6) and failed to produce

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the T_H1 polarizing IL-12 (Dodge *et al.*, 2003). Dodge *et al.*, (2003) went on to demonstrate that IL-6 negatively regulates IL-12 production and proposed that the T_H2 cytokine environment observed in the lung is due to DC mediated inhibition of T_H1 responses via IL-6 production. High concentrations of IL-6 stimulate macrophage proliferation within the lung (Yu *et al.*, 2005).

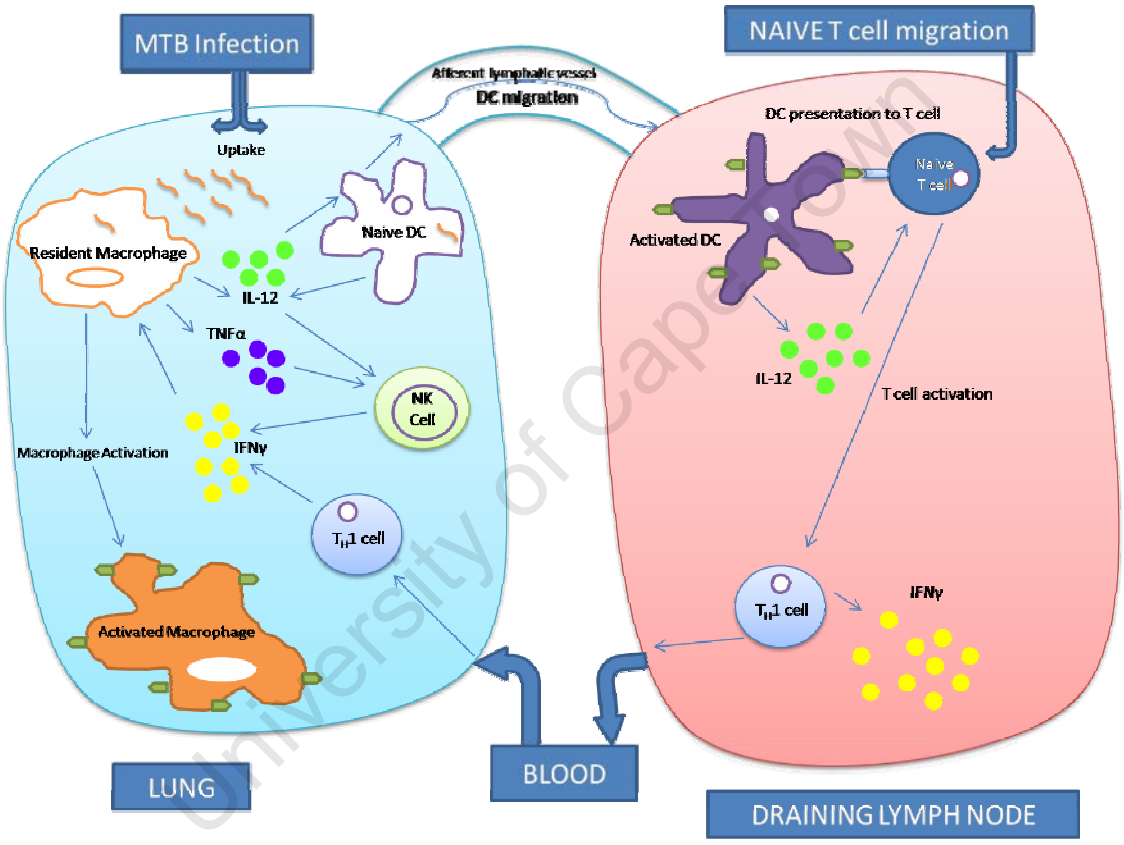

Figure B: Summary of Host Immune Response to Infection. The two compartments of lung and lymph node are represented with all the relevant cell types and cytokine production. Macrophage and DC uptake, cytokine production and activation are represented, as well as DC trafficking, presentation and T cell activation (Adapted from Marino *et al.*, 2004).

Newly activated $CD4^+$ and $CD8^+$ T cells produce additional IFNγ which activates macrophages allowing them to produce TNFα and IL-12 more efficiently (Cooper and Khader, 2007; Cooper *et al.*, 1997). IFNγ and TNFα together induce the increased production of reactive nitrogen intermediates (RNI) (Chan *et al.*, 1992) in recruited,

activated macrophages by inducible nitrous oxide synthase (iNOS). Such macrophages, which are highly active and capable of effectively killing *M. tuberculosis* bacteria, are recruited to the site of infection along with T_H cells by means of chemokines produced by activated T cells (Flynn *et al.*, 1995; Kindler *et al.*, 1989; Roitt, 1988). Chemokines are small molecular mass peptide hormones (Male, 1996) of 8 to 10 kD, all of which have similar structures and contain two internal disulphide loops (Abbas, 1997). They are important contributors to innate immune function and can be divided into two groups; the α chemokine family which is characterized by two cysteins separated by an intervening residue (CXC), and the β chemokine family which have two cysteines adjacent to each other (CC). Both groups are chemotactic agents responsible for the recruitment of lymphocytes and activated macrophages characteristic of granuloma formation (Abbas, 1997; Male, 1996).

M. tuberculosis infection induces pro-apoptotic cytokines such as TNFα which modulates apoptosis by caspase activation and NO synthesis (Gil *et al.*, 2003; Perskvist *et al.*, 2002). Apoptosis along with $CD8^+$ T_H cells is important for releasing *M. tuberculosis* bacilli from poorly activated alveolar macrophages which are inefficient at killing bacilli. Subsequent uptake of released bacilli by newly recruited, activated macrophages results in improved killing and bacilli control (Kaufmann and Flesch, 1988; Keane *et al.*, 2000). Nonetheless acquired immune responses do not completely eradicate bacilli; instead environmental conditions induces *M. tuberculosis* to enter into a latent state (Cunningham and Spreadbury, 1998; Mayuri *et al.*, 2002).

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LR. 1.2.4 The Role Of Tumor Necrosis Factor Alpha In Mycobacterial Infections

The role of TNFα in protective immunity against *M. tuberculosis* infection has been extensively investigated using mice deficient for TNFα signaling (Bean *et al.*, 1999; Kaneko *et al.*, 1999; Roach *et al.*, 2002; Zganiacz *et al.*, 2004) or by neutralization studies (Eriks and Emerson, 1997; Kindler *et al.*, 1989). The recent generation of mouse strains expressing only the membrane form of TNFα has allowed for the investigation of the respective roles of soluble TNFα and membrane bound TNFα in

host immune function during mycobacterial challenge (Olleros *et al.*, 2002; Olleros *et al.*, 2005; Probert *et al.*, 1996; Saunders *et al.*, 2005).

TNFα is essential for host survival after intravenous infection by virulent Kurono mycobacteria (Kaneko *et al.*, 1999) as well as avirulent *M. bovis* BCG (Jacobs *et al.*, 2000b; Jacobs *et al.*, 2000c; Kaneko *et al.*, 1999). TNFα$^{-/-}$ mice are similarly unable to survive a low dose aerosol *M. tuberculosis* infection (Bean *et al.*, 1999). Thus during virulent or non-virulent mycobacterial challenge, death is coincidental with an inability to control bacilli replication and dissemination. TNFα deficient mice are able to achieve a partial but delayed control of bacilli in the liver and spleen but not the lungs subsequent to a *M. bovis* BCG infection (Jacobs *et al.*, 2000b), while such control appears to be dependent on strain virulence as it is absent during *M. tuberculosis* infection (Bean *et al.*, 1999). In contrast, TNFα is not required for host survival or control of bacilli load in a non-pathogenic intravenous *Mycobacterium smegmatis* (*M. smegmatis*) infection (Roach *et al.*, 2002). Although a delay in bacilli clearance was observed, this was compensated for by an enhanced T_H1 response (Roach *et al.*, 2002) accompanied by higher $CD4^+$ T cell total recruitment and high levels of RNI's stimulated by T cell derived IFNγ. Nonetheless the enhanced T_H1 immune response was not sufficient to rescue mice from aerosol *M. tuberculosis* infection (Roach *et al.*, 2002). A subsequent study demonstrated that the increased T_H1 response is uncontrolled during *M. tuberculosis* infection and causes tissue destruction due to an overproduction of IL-12 and IFNγ as a result of the increased frequency of antigen-specific $CD4^+$- and $CD8^+$ T cells present in the lung (Zganiacz *et al.*, 2004). The study further showed that depleting $CD4^+$- and $CD8^+$ T cells decreased IFNγ and necrosis levels and increased the survival time of TNFα deficient mice (Zganiacz *et al.*, 2004).

The inability of TNFα deficient mice to clear bacilli is associated with an unregulated chemokine response required for cellular recruitment and granuloma formation. In response to a non-pathogenic *M. smegmatis* infection, mRNA levels of chemokines are delayed over the first two weeks but appear similar to WT control from day 21 onwards

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(Roach *et al.*, 2002). In contrast, a virulent *M. tuberculosis* infection is characterized by a delay in chemokine mRNA induction within the first 2 weeks which is subsequently significantly increased compared to wild type mice (Roach *et al.*, 2002). Chemokine upregulation in late infection is also apparent on day 56 in an *M. bovis* BCG infection model (Jacobs *et al.*, 2000b; Jacobs *et al.*, 2000c).

Total cellular responses and granuloma numbers and sizes differ markedly in the absence of TNFα. In response to an aerosol inhalation *M. tuberculosis* infection in wild type mice, large granulomas consisting of macrophages, lymphocytes and very few neutrophils are evident on day 28 with no necrosis visible. In contrast, infected TNFα deficient mice have an unregulated cellular infiltration with an unstructured granuloma response. In addition, the composition of infiltrating cells differed markedly from that of wild type mice; where granulomas of wild type mice consisted largely of macrophages and lymphocytes with only a very few neutrophils, cellular infiltrates in TNFα deficient mice were predominantly composed of neutrophils, with only a few macrophages and lymphocytes with visible necrosis in the central areas of the infiltrates (Bean *et al.*, 1999). Necrosis was also observed after intravenous infection with Kurono and *M. tuberculosis* infection. A further difference observed between wild type and TNFα deficient mice was the absence of epitheloid macrophages in the mutant strain (Kaneko *et al.*, 1999; Roach *et al.*, 2002). A similar disruption in granuloma formation was also evident subsequent to intravenous BCG infection (Jacobs *et al.*, 2000b; Jacobs *et al.*, 2000c; Kindler *et al.*, 1989). Fully developed granulomas displayed regression in response to TNFα depletion by monoclonal antibody (mAb) injection indicating that TNFα is not only required for initial granuloma development but also for the maintenance of granuloma structure (Kindler *et al.*, 1989).

LR. 1.2.5. Tumor Necrosis Factor Receptors and Mycobacterial infections

The role of TNFRp55 signaling in the host protective immune response to mycobacterial infections has been addressed in various mycobacterial infection models including intravenous *M. tuberculosis* infection (Flynn *et al.*, 1995) and intraperitoneal

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BCG infection (Senaldi *et al.*, 1996). TNFRp55 deficient mice are unable to control bacilli replication and dissemination, resulting in premature death subsequent to intravenous *M. tuberculosis* infection (Flynn *et al.*, 1995). The death of TNFRp55 deficient mice during intravenous *M. avian* challenge was characterized by excessive T cell infiltration and was associated with the IL-12 dependent disintegration of granulomatous lesions (Ehlers *et al.*, 2000). Granuloma formation in TNFRp55 deficient mice, in response to *M. bovis* BCG infection, was similar to that in TNFα deficient mice in that fewer, smaller granulomas were formed which were disorganized, lacked the normal defined structure characteristic of those formed by wild type mice, and had few activated, epitheloid macrophages present (Kindler *et al.*, 1989; Senaldi *et al.*, 1996). Furthermore, treatment with anti-TNFα mAb resulted in the regression of fully established granulomas in both TNFα and TNFRp55 deficient mice (Kindler *et al.*, 1989; Senaldi *et al.*, 1996). The similarity in phenotype between TNFα and TNFRp55 deficient mice suggests that TNFRp55 signaling is the major conduit during TNFα-mediation of immune function against mycobacterial infections (Kindler *et al.*, 1989; Senaldi *et al.*, 1996) and early *in vivo* studies showed that TNFRp75 has a minimal role in host protective immunity against non-virulent mycobacteria (Jacobs *et al.*, 2000a; Jacobs *et al.*, 2000b; Jacobs *et al.*, 2000c). Subsequent studies have suggested that TNFRp75 may indeed have an immune function (Balcewicz-Sablinska *et al.*, 1998; Corti *et al.*, 1999; Kim and Teh, 2001; Mukhopadhyay *et al.*, 2001). The role of TNFRp75 in mycobacterial infections has not been well studied and whether or not it promotes or inhibits the protective immune response is still contentious. Two opposing theories for the role of TNFRp75 have emerged: 1) soluble TNFRp75 may form a target for mycobacterial evasion of the host immune system (Balcewicz-Sablinska *et al.*, 1998; Corti *et al.*, 1999); 2) it may lower the threshold of T cell activation thereby boosting the immune response (Kim and Teh, 2001). *In vitro* macrophage studies showed that *M. tuberculosis* bacilli are able to evade alveolar macrophage apoptosis by TNFRp75 shedding and supports the first hypothesis. In this study it was demonstrated that TNFRp75 forms a complex with soluble TNFα thus reducing the TNFα bioactivity and the subsequent TNFα mediated macrophage

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apoptosis (Balcewicz-Sablinska *et al.*, 1998). An *in vitro* study by Kim and Teh (2001) supports the second hypothesis where the authors demonstrated that TNFRp75 deficient $CD8^+$ T cells required higher levels of T cell receptor agonist for T cell activation and proliferation to occur thus suggesting that TNFRp75 could act as an important costimulatory molecule. *In vivo*, TNFRp75 cell surface expression is upregulated in response to intraperitoneal *M. avium* infection and the level of soluble TNFRp75 is increased (Corti *et al.*, 1999). In addition, mice treated with anti-TNFRp75 mAb prior to and subsequent to infection with *M. avium* showed a significantly lower bacilli load by day 21 post-infection (Corti *et al.*, 1999). This finding tends to support the first theory that TNFRp75 is targeted by mycobacteria as an evasion mechanism. It does not however exclude the possibility that TNFRp75 has a dual role to play and thus may also assist in protective immunity against mycobacteria.

LR. 1.3. The Role of TNFα/TNFR's in Other Diseases

Deregulation of TNFα and its receptors has been shown to play a role in many diseases including among others *African trypanosomiasis,* malaria, inflammatory diseases and *Orthopoxviruses.* Shedding of soluble TNFRp75 during peak parasitemia stages was shown to occur as a protective host response during *African trypanosomiasis* (Magez *et al.,* 2004). Inflammatory cytokines and in particular, TNFα, are elevated and associated with cerebral malaria, anaemia and death from *Plasmodium falciparum* infection (Grau *et al.*, 1989; Shaffer *et al.*, 1991). It has also been demonstrated that TNFRp75 (Lucas *et al.*, 1997a; Lucas *et al.*, 1997b), and more specifically TNFRp75 expression on vascular cells (Stoelcker *et al.*, 2002), is critical for the development of experimental cerebral malaria in mice. Excessive TNFα or deregulation of TNFα has been implicated in the development of inflammatory diseases that include Korean Behcet's Disease, psoriasis, psoriasis arthritis, rheumatoid arthritis, inflammatory bowel disease and Crohn's disease. The prognosis of many of these inflammatory diseases can be greatly improved by treatment with anti-TNFα therapy (Antoni and Manger, 2002; Bouma *et al.*, 1996; Elliott *et al.*, 1993; Gottlieb *et al.*, 2005; Leonardi *et al.*, 2003; Sfikakis *et al.*, 2001; Wang *et al.*, 2006). TNFα, and TNFα signaling through both TNFRp55 and TNFRp75 is important for host protection against *vaccinia virus* and *ectromelia virus*

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infection (Chan *et al.*, 2003; Ruby *et al.*, 1997; Sambhi *et al.*, 1991) with TNFα and TNFRp55/75 deficient mice showing an increase in viral replication.

In summary the role of TNFα during *M. tuberculosis* infection has been studied in depth, but the importance of its signaling though TNFRp55 and TNFRp75 during a natural *M. tuberculosis* infection remains to be elucidated.

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Aims and Objectives

The major aims of this project were 1) to understand the roles of TNFRp55 and TNFRp75 in protective immunity against *M. tuberculosis* in an aerosol inhalation infection model and 2) to explore the mechanism behind increased resistance of TNFRp75$^{-/-}$ mice to *M. tuberculosis* infection.

The objectives were to:

- Define mortality during both acute and chronic *M. tuberculosis* infection in WT, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$-, and TNFRp55/75$^{-/-}$ mice.

- Assess bacterial replication in the lung, liver and spleen in response to *M. tuberculosis* infection in WT, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$-, and TNFRp55/75$^{-/-}$ mice.

- Compare pulmonary macropathology and cytokine production as well as histological examinations of cellular infiltration, granuloma formation and bacilli loads in WT, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$-, and TNFRp55/75$^{-/-}$ mice during an *M. tuberculosis* infection as an indication of disease progression.

- Assess and compare cellular recruitment and activational status in WT, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$-, and TNFRp55/75$^{-/-}$ mice during an *M. tuberculosis* infection.

- Determine the role of soluble TNFR levels both *in vivo* and in *in vitro* macrophage and dendritic cell cultures on bioactive TNFα levels and the relevance in terms of the mechanism behind the advantage conveyed to mice with a deficiency for TNFRp75.

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Chapter 2:

Materials and Methods

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Materials and Methods

MM. 2.1 Mouse Strains

The following mice were used for the experiments: C57BL/6 mice were used as control animals, TNFRp55$^{-/-}$ mice were obtained from The Jackson Laboratory (Pfeffer *et al*., 1993), TNFRp75$^{-/-}$mice were obtained from The Jackson Laboratory (Erickson *et al*., 1994) and TNFRp55/75$^{-/-}$ mice were obtained by mating TNFRp55$^{-/-}$ mice with TNFRp75$^{-/-}$ mice. TNFRp55$^{-/-}$- and TNFRp75$^{-/-}$ mice were backcrossed for more than 10 generations on a C57BL/6 background. Mice were bred and housed in IVC's under specific pathogen free (SPF) conditions in the Animal Unit, University of Cape Town. All experiments were approved by the Research Ethics Committee of Cape Town.

MM. 2.2 Genotyping

Genotypes of mutant strains were confirmed by polymerase chain reaction (PCR) analysis of tail biopsies. All mice were genotyped for the presence of both TNFRp55 and TNFRp75. Primers were purchased from The Department of Medical Biochemistry, University of Cape Town.

TNFRp75 specific primers were as follows:

5' CCT CTC ATG CTG TCC CGG AAT 3'
5' ABC TCC AGG CAC AAG GGC GGG 3'
5' CGG TTC TTT TTG TCA AGA C 3'
5' ATC CTC GCC GTC GGG CAT GC 3'

TNFRp55 specific primers were as follows:

5' TGT GAA AAG GGC ACC TTT ACG GC 3'
5' GGC TGC AGT CCA CGC ACT GG 3'
5' ATT CGC CAA TGA CAA GAC GCT GG 3'

Amplification of TNFRp55 and TNFRp75 genes were performed by PCR using the PTC-200 Peltier Thermal Cycler (BioRad,) according to the conditions set out in tables 1 and 2 respectively. $MgCl_2$, PCR buffer and *Taq* polymerase were purchased

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from Vector Laboratories, (UK) and the dNTP stocks were obtained from Promega, Madison, United states.

Table 1: Amplification Conditions for TNFRp55 Gene

	Temperature °C	Time (minutes)
1. Initial denaturation	94	11
2. Denaturation	94	1
3. Primer Annealing	55	1
4. Extension	72	2
5. Final Extension	72	2
Steps 2 –4 repeated 35 x		

Table 2: Amplification Conditions for TNFRp75 Gene

	Temperature °C	Time (minutes)
1. Initial denaturation	94	11
2. Denaturation	94	1
3. Primer Annealing	58	1
4. Extension	72	2
5. Final Extension	72	5
Steps 2 –4 repeated 35 x		

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Amplification reaction products were electrophoresed on 1.6% agarose gels (Appendix A) with Ethidium Bromide at 60 V for 90 minutes using 1x Tris borate ethylene diamino tetra acetic acid (TBE) (Appendix A) as the electrophoresis buffer. A 1Kb DNA ladder (Promega, Madison, United States) was used as a molecular weight marker to determine amplification product size.

MM. 2.3 Mycobacteria

Mycobacterium tuberculosis H37Rv was obtained from the Trudeau Mycobacterial Culture Collection and grown at 37°C until mid log phase in Difco Middlesbrook 7H9 medium (Becton, Dickinson and Company, Le Pont de Claix, France) containing 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton, Dickinson and Company, France). Mycobacterial cultures were aliquoted and stored at –70°C. The mycobacterial concentration of frozen aliquots was

determined by 10-fold serial dilution on Difco Middlebrook 7H10 agar (Appendix A) (Becton, Dickinson and Company, France).

MM. 2.4 Aerosol Inhalation Infection

To prepare an inoculum for infection an aliquot of *M. tuberculosis* was thawed and aspirated 30x through a 29½ G needle (B. Braun, Melsungen, Germany) to reduce clumping. The inoculum was prepared at a final concentration of 2 x 10^6 colony forming units per ml (cfu/ml) in a total volume of 6 ml sterile saline (Appendix A). The inoculum concentration was confirmed by 10-fold serial dilution on Difco Middlebrook 7H10 agar plates (Becton, Dickinson and Company, France).

Aerosol inhalation infection was performed using a Glas-Col Inhalation Exposure System Model A4224 (Glas-Col, USA) under biosafety level 3 conditions using the following program and instrument settings.

Preheat cycle	15 minutes (900 seconds)
Nebulising cycle	40 minutes (2400 seconds)
Cloud decay cycle	40 minutes (2400 seconds)
Decontamination cycle	15 minutes (900 seconds)
Main air flow	60 cubic feet per hour
Compressed air flow	10 cubic feet per hour

The pulmonary infection dose was calculated after euthanasia of 10 mice on day 1 after infection. Whole lungs were removed and homogenized in 2ml sterile Phosphate-buffered saline (PBS)/0.04% Tween 80 (v/v) and titrated in duplicate on Difco Middlesbrook 7H10 agar (Becton, Dickinson and Company, France).

MM. 2.5 Bacterial Burden Assessment

Mice were killed at defined time points and mycobacterial burdens in the lungs, liver and spleen obtained by colony forming enumeration. Briefly, organs were homogenized in 1-2ml of Homogenization Buffer (Appendix A) and tissue homogenates plated in 10-fold serial dilution in duplicate on Difco Middlesbrook 7H10 agar (Becton, Dickinson and Company, France). Mycobacterial cultures were semi-sealed in plastic bags and incubated for 18-21 days at 37^{O}C after which mycobacterial colonies were counted and final organ burdens calculated.

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MM. 2.6 Enzyme-linked Immunosorbant assays (ELISA)

ELISA's were carried out on lung samples and bone marrow and dendritic cell supernatants. Lung samples were obtained by the removal of lungs on day 30 post-infection. Homogenisation was carried out in sterile saline plus 0.04% Tween 80. Homogenates were subsequently centrifuged twice at 1500rpm for 10 minutes at 4^{o}C and the supernatant aliquoted and stored at –80^{o}C. All ELISA kits were purchased from R&D Systems, Germany except TNFα which was purchased from BD Pharminogen, Sandiego.

Cytokine and soluble receptor sample concentrations were assessed by ELISA as described by the manufacturer. Briefly, 96-well Maxisorb microtiter plates (Nalge Nunc International, Naperville, IL, USA) were coated with 2μg/ml primary capture antibody (diluted in 1x PBS, Appendix A) with the exception of IFNγ which was coated at 42μg/ml and TNFα which was coated at 1μg/ml (diluted in coating buffer Appendix, A). All ELISA's were incubated for 16-18 hours at room temperature (RT) except TNFα which was incubated for 3 hours at 37^{O}C. Plates were washed 4x with Washing Buffer (Appendix A) and non-specific binding sites saturated by adding 200μl Blocking Buffer 1 (Appendix A) and incubating for 1 hour at room temperature with the exception of TNFα which was incubated at 4^{O}C for 12 hours (Blocking Buffer 2 Appendix A). After washing 4x with washing buffer recombinant mouse standards were added in 2 fold serial dilutions (diluted in Reagent Diluent, Appendix A) except for TNFα ELISA's which were added in 3 fold serial dilutions (diluted in Dilution Buffer, Appendix A) with a concentration range of 2000pg/ml – 0.96pg/ml for IFNγ, TNFRp55 and TNFRp75; 1000pg/ml – 0.49pg/ml for IL-6, 60 000pg/ml – 29.29pg/ml IL-12p40 and 100ng/ml- 0.001ng/ml for TNFα. Samples were added at 3 fold dilutions, and samples and standards were incubated at room temperature (RT) for 2 hours except for TNFα ELISA's which were incubated at 37^{O}C for 6 hours. Samples were washed 4x with washing buffer, the appropriate biotinylated secondary antibody added at the recommended concentration and incubated at RT for 2 hours with the exception of the TNFα ELISA which was incubated for 12 hours at 4^{O}C. Samples were washed 4x with washing buffer and incubated with Streptavidin conjugated to Alkaline phosphatase for 20 minutes (R & D Systems, Minneapolis, USA) (1/1000 dilution in Reagent

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Diluent) except TNFα ELISA's which were incubated with Alkaline phosphatase-labled goat anti-rabbit (BD Pharmingen, Sandiego) for 90 minutes (1 in 10 000 dilution in Dilution Buffer). After washing 4x with Washing Buffer, samples were incubated with p-nitrophenyl phosphate substrate solution (Boehringher Mannheim, Germany) (1mg/ml in Substrate Buffer, Appendix A), the reaction was read at 405nm using a VERSAmax Tunable Microplate Reader (Molecular Devices Corporation, California, United States) and data was analysed using SoftMax Pro Version 4.3 Software (Molecular Devices Corporation, California, United States).

MM. 2.7 Bioactive TNFα Assay

Bioactive TNFα was determined using the TNFα sensitive fibroblast cell line WEHI (Walter and Eliza Hall Institute) 164, clone 13. An aliquot of WEHI cells were thawed at 37^0C and cultured in 90mm tissue culture petridish (Bibby Sterilin, UK) containing 10 ml WEHI Complete medium (Appendix A) until confluent. Cells were harvested in trypsin/EDTA (Appendix A) and seeded at 2 x 10^4 cells/ well in 96 well tissue culture plate (Nunc, Denmark). The cells were allowed to adhere overnight by incubating at 37 ºC with 5% CO_2. TNFα standards (recombinant mouse TNF, Cat # 19321T, BD Pharmingen, San Diego) were prepared in 2 fold serial dilution at a concentration range of 8pg/ml – 0.06pg/ml and together with samples was added to WEHI cells and incubated at 37 ºC with 5% CO_2 for 18 hours. 50µl of (2mg/ml) MTT (Cat # M2128, Sigma, Germany) was added to the samples and incubated at 37 ºC for 2 hrs after which the supernatant was discarded by aspiration. 50 µl of WEHI detection solution (Appendix A) was added to each well and the samples read at 570nm with reference filter at 690 nm using a VERSAmax Tunable Microplate Reader (Molecular Devices Corporation, California, USA). Data was analysed using SoftMax Pro Version 4.3 Software (Molecular Devices Corporation, California, USA).

MM. 2.9 Macroscopic Lung Pathology

Mice infected with *M. tuberculosis* by aerosol inhalation at a dose of 50-100 CFU/lung, were euthanized by cervical dislocation and whole lungs recovered after 28 days and fixed in 10% formalin. Photographic images were captured with a

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Nikon 4500 digital camera and analysed using Nikon View 5 software (Jacobs *et al*., 2000).

MM. 2.10 Histology

Sections of lung were recovered from euthenised mice at defined time points and immersed in a volume of 10% formalin (Appendix A) at least 10x that of the tissue.

MM. 2.10.1 Paraffin Wax Embedding

Tissues were dehydrated in an automated tissue processor (LEICA TP1020, Wetzlar, Germany) and embedded in wax (LEICAEG1140C, Wetzlar, Germany) as indicated below:

70% alcohol	30 minutes
96% alcohol (x2)	45 minutes
100% alcohol (4x)	45 minutes
xylol (2x)	60 minutes
Wax (55^{o}C to 60^{o}C) (2x)	45 minutes with vacuum

A microtome (Leica RM-2125RT or SM 2000R, Heidelberger, Strasse) was used to cut tissue sections of 2µm. Sections were floated onto glass slides and fixed by overnight incubation at 37^{o}C. Wax was removed from tissue sections prior to staining by incubation at 60^{o}C for 2 – 18 hours. Tissues were rehydrated for staining as follows:

Xylol	3 minutes
Xylol (2x)	1 minute
Absolute Alcohol (2x)	1minute
96% Alcohol (2x)	1 minute
70% Alcohol	1 minute
Water	1 minute

MM. 2.10.2 Haemotoxylin and Eosin staining (H&E)

Rehydrated tissue was immersed in Haematoxylin for 8 minutes and rinsed in water after which they were immersed in 1% acid alcohol and rinsed under running water for 30 minutes. Tissues were subsequently counterstained by immersion in 1%

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Eosin for 2 minutes, given a rapid wash in water and dehydrated by a 10 second sequential immersion in the following chemicals: 70% alcohol, 96% alcohol and xylol. Sections were mounted using Entellen.

MM. 2.10.3 Ziehl-Neelson Staining (ZN)

Rehydrated tissues were covered in filtered Carbol Fuchsin (Appendix A) solution and flamed. Tissues were cooled for 5 minutes and the flaming and cooling steps were repeated before rinsing briefly with water. The slides were immersed in 1% acid alcohol for 30 seconds to remove excess stain and rinsed in water prior to staining with 25% H_2SO_4 for 20 minutes, rinsing under running water and staining with Loeffler' Methylene Blue (Appendix A) for 1 minute. Tissues were rinsed briefly in water and dehydrated by a 10 second immersion in the following chemicals: 70% alcohol, 96% alcohol and xylol. Sections were mounted using Entellen (Merck, Germany).

MM. 2.11 Macrophage and Dendritic Cell Culture

Femurs were collected from 6 to 8 week old mice of each strain subsequent to sacrifice by cervical dislocation. The femurs were rinsed in 70% ethanol and placed in 5ml ice-cold complete bone marrow derived macrophage (BMDM) medium (Appendix A) or complete dendritic cell medium (DCM) (Appendix A). The epiphysis of the femurs were removed with a sterile scalpel blade and the bone flushed with appropriate complete medium. Harvested cells were centrifuged at 405 RCF and resuspended in 1ml complete macrophage medium for macrophage culture or 1ml complete dendritic cell medium for dendritic cell culture, counted and seeded at 2 x 10^6 cells in 90mm Sterilin bacteriological plates (Bibby Sterilin, UK) containing 10ml of the appropriate complete medium.

Macrophage cultures were incubated at 37^{o}C with 5% CO_2 until adherence (day 4) and fresh medium added. Cells were subsequently cultured until day 10 with medium changes every 3-4 days. Dendritic cell cultures were incubated with 5% CO_2 and 10ml fresh medium was added on day 3. On day 6 and 8 half the supernatant was removed from each plate, centrifuged at 405 RCF, resuspended in 10 ml medium and added back to each plate. On day 10 macrophage and dendritic

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cells were harvested by adding 1xPBS/0.02% EDTA/4mg/ml lidocain hydrochloride monohydrate (Sigma-Aldrich, L5647-15G) and incubating cultures at 37^0C for 5 minutes. After washing cultures with PBS/EDTA/Lidocain, the cell suspension was centrifuged at 405 RCF, counted and reseeded as follows: 2 x 10^6 cells per 10 ml complete medium in 90mm Sterilin Bacteriological plates (Bibby Sterilin, UK)); 5 x 10^5 cells per well in 24 well tissue culture plates (Corning, New York, USA) or at 2 x 10^5 cells per well in 96 well tissue culture plate (Nunclon, Denmark) and incubated overnight at 37^oC with 5% CO_2 to allow cell adherence.

MM. 2.12 Macrophage/Dendritic Cell Stimulation

Macrophages and dendritic cells were seeded at 5x10^5 cells per well in 48-well plates to obtain culture supernatants for cytokine and soluble TNF receptor analysis by ELISA, or at 2x10^6 cells in 90mm sterilin plates (Bibby Sterilin, UK) for analysis of activation by flow cytommetry. Culture supernatants were aspirated and cells incubated with either medium alone or with *M. tuberculosis* H37Rv at a MOI of 5 :1 with or without anti-TNFRp75 monoclonal antibody (2µg/ml, Clone Number: TR75-32). Cells were stimulated for either 4 or 24 hours at 37^OC with 5% CO_2. Cells were harvested, counted and 1x10^6 cells analysed by flow cytommetry. Supernatants were collected from the 48 well plates at the indicated time points.

MM. 2.13 Whole Lung Harvesting and Tissue Preparation

Mice were euthanised with a lethal dose of general anaesthetic (Appendix A) and exanguinated via the femoral vein. The thoracic cavity was opened to expose the heart and lungs and the pulmonary vasculature perfused with 5ml PBS containing 20U/ml heparin (Bodene (PTY) Limited, RSA). Lungs were removed and dissected into smaller pieces (2-3mm) using a scalpel blade and lung pieces were incubated with rotation for 90 minutes at 37^oC in 10ml DMEM medium supplemented with 50 U/ml Collagenase 1(Worthington Biomedical Corporation, Lakewood, NJ) and 13 U/ml DNase 1 (Boehringer Mannheim, West Germany). Digested lung pieces were passed through a 70µm cell strainer prior to centrifugation at 405 RCF at 4^oC for 10 minutes. Cells were washed with Complete Restimulation Medium (appendix A) and the red blood cells lysed in 5ml/lung Red Blood Cell Lysing Buffer (Appendix A). Cells were left on ice for 1 minute and 5ml Complete Restimulation Medium

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added prior to centrifugation at 405 RCF at 4°C for 10 minutes. Cells were then either washed in PBS (1X) or in Complete Restimulation Medium, or counted for cell staining or restimulation assays. Single cell suspensions of lymph nodes were prepared by passage through 70µm cell strainers prior to centrifugation at 4°C for 10 minutes. Similarly, cells were then either washed in PBS (1X) or in Complete Restimulation Medium for cell staining or restimulation.

MM. 2.14 Lung and Lymph Node Restimulation

$2.2x10^6$ cells per well were seeded in a 96 well round bottom nunc (Nunclon, Denmark) plate. Cells were stimulated with ESAT 6 (10µgml), *M. tuberculosis* at an MOI of 5:1 or anti-CD3/anti-CD28 diluted in Complete Restimulation Medium (Appendix A) at 37°C for a total of 6 hours with medium as a control. After the first 2 hours Brefaldin A (Epicenter Biotechnologies, USA) was added to a final concentration of 5µg/ml. For anti-CD3/anti-CD28 restimulation wells were coated overnight with anti-CD3/anti-CD28 (5µg/ml in a volume of 50µl), and the antibody cocktail aspirated off prior to the addition of cells. After 6 hour stimulation cells were centrifuged for 5 minutes at 514 RCF, resuspended in 220µl of Facs Buffer (Appendix A) and replated into 96 well v-bottom sterilin plates (Bibby Sterilin, UK) for analysis by flow cytommetry.

MM. 2.15 Fluorescently Activated Cell Sorting (FACS)

Bone marrow derived macrophages, dendritic cells, single cell suspensions of lungs or lymph nodes were labled in Sterilin 96 well plates (Bibby Sterilin, UK) with antibodies as indicated in Table 3 below. Samples were analysed on a FACS Calibur (Beckton Dickinson) flow cytometer using Cell Quest software (Beckton Dickinson). Briefly 1 x 10^6 bone marrow derived macrophages or dendritic and 2 $x10^6$ lung cells were incubated with 25µl FACS blocking solution (Appendix A) for 20 minutes on ice, washed twice with 250µl FACS buffer (Appendix A) and centrifuged at 4°C for 5 minutes at 514 RCF. Cells were incubated on ice for 20 minutes in 25µl of specific antibody (2µg/ml) or relevant anti-IgG (2µg/ml) control antibody. Cells were subsequently washed twice with 250µl FACS buffer, centrifuged at 4°C for 5 minutes at 514 RCF and resuspended in 250µl FACS fixation buffer (Appendix A). Cells were then either transferred into 5ml Falcon

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tubes (Gilson Inc., USA) containing a further 250µl FACS fixation buffer for analysis or stored overnight at 4^0C.

For intracellular staining, cells were centrifuged at 514 RCF for 5 minutes, resuspended in 250µl saponin buffer and incubated on ice for 20 minutes, after which cells were centrifuged for 5 minutes at 514 RCF and incubated on ice for 30 minutes in 25µl of antibody diluted in Saponin Buffer (Appendix A) at a final concentration of 2.5µg/ml. 250µl Saponin Buffer was then added, cells were centrifuged for 5 minutes 514 RCF, resuspended in 250µl of FACS fixation buffer and transferred to 5ml Falcon tubes containing a further 250µl FACS fixation buffer for analysis.

Table 3: Antibodies used in Flow Cytommetry

Antibody	Clone	Source
FITC Rat IgG 2a	R35-95	Pharmingen
PE Rat IgG 2a	R35-95	Pharmingen
APC Rat IgG 2a	R4005	Pharmingen
PE Rat IgG 2b	R35-95	Pharmingen
FITC Hamster IgG	G70-204	Pharmingen
PE Hamster IgG	G70-204	Pharmingen
PE Rat Anti-Mouse CD120b	HN102	Pharmingen
PE Anti-Mouse CD44 (Pgp-1)	IM7	Pharmingen
PE Rat Anti-Mouse I-A/I-E	M5/114.15.2	Pharmingen
PE Hamster Anti-Mouse CD80	16-10A1	Pharmingen
PE Rat Anti-Mouse CD86	GL1	Pharmingen
PE Anti-Mouse CD8a (Ly-2)	53-6.7	Pharmingen
FITC Anti-Mouse CD8a (Ly-2)	53-6.7	Pharmingen
FITC Hamster Anti-Mouse CD11c	HL3	Pharmingen
FITC Rat Anti-Mouse IFNγ	XMG1.2	Pharmingen
APC rat Anti-Mouse CD11b	M1/70	Pharmingen
APC Rat Anti-Mouse CD4	RM4-5	Pharmingen
Rat Anti-Mouse CD16/CD32	2.4G2	Pharmingen
Anti-Mouse CD3e	145-2c11	Pharmingen
Anti-Mouse CD28	37.51	Pharmingen

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MM. 2.16 Statistical Analysis

The data are expressed as the mean ± SEM. Statistical analysis was performed by ANOVA using GraphPad Prism software (version 4.01). For mortality studies, analysis was performed using the log-rank test. For all tests, a p-value of < 0.05 was considered significant.

Chapter 3 Results:

Phenotypic Characterization of *Mycobacterium tuberculosis* infection in TNFR Deficient Mice

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Results: Phenotypic Characterization of *Mycobacterium tuberculosis* infection in TNFR Deficient Mice

3.1 Mice Genotyping

Genotypes of mice were confirmed by PCR analysis. Lanes 1-9 represent the DNA amplification results of WT-, TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice using TNFRp55 specific primers whereas lanes 10-18 represent the DNA amplification results of WT-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice using TNFRp75$^{-/-}$ specific primers. In lanes 1-3 a 470bp amplification product corresponds to the WT TNFRp55 allele, while the 300bp amplification product in lanes 4-9 shows the presence of the inserted neo cassette and disruption of TNFRp55 gene in the TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice strains. A faint non-specific amplification product (< 2%) was also evident in lanes 4-9. The presence of the TNFRp75 allele was evident in the WT mice, corresponding to an amplification product of 200bp. Disruption of the TNFRp75 gene was confirmed by the presence of the 400bp amplification product in TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice. The presence of the TNFRp55 allele in the TNFRp75$^{-/-}$ mice as well as the TNFRp75 allele in the TNFRp55$^{-/-}$ mice was confirmed (data not shown). It is clear from these results that the respective strains were homozygous for the corresponding alleles and confirm the correct genotypes of the animals used in this study.

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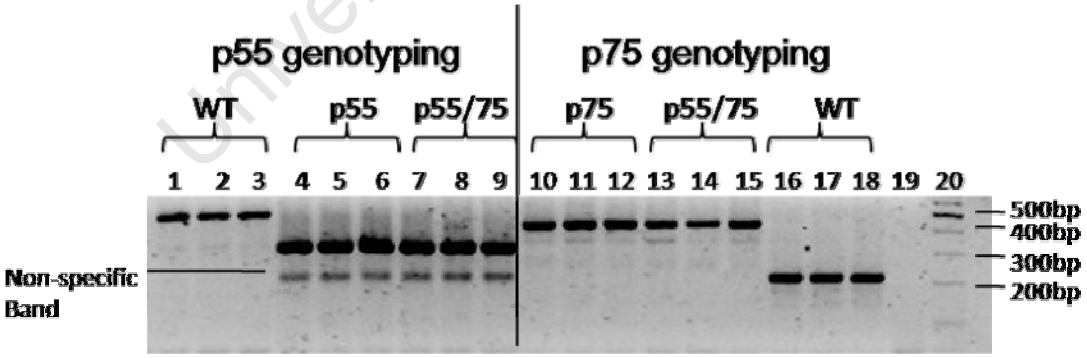

Figure 3.1: Genotyping of WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice. Genotyping of mutant mouse strains was carried out by PCR analysis of tail biopsies. The results indicate the presence of the 470bp band in WT mice (lane 1-3) and 300bp band in TNFRp55$^{-/-}$- (lane 4-6) and TNFRp55/75$^{-/-}$ (lane 7-9) mice using TNFRp55 primers. Genotyping using TNFRp75 primers showed the 200bp band indicative of WT mice (lane 16-18) and 400bp band in TNFRp75$^{-/-}$ mice (lane 10-12) and TNFRp55/75$^{-/-}$ mice (lane 13-15) thus confirming the genotypes of mutant mice.

3.2 TNFRp75$^{-/-}$ Mice Resist Acute *M. tuberculosis* Infection.

TNFα is a crucial cytokine involved in protective immunity against *M. tuberculosis* infection. In mice deficient for TNFα, death is coincidental with an inability to control mycobacterial replication and dissemination (Roach *et al*., 2002; Jacobs *et al*., 2000; Bean *et al*., 1999). Signaling through TNFRp55, the primary conduit of TNFα signaling, is important for protective immunity against *M. tuberculosis* infection after intravenous infection (Flynn *et al*., 1995) but a specific role for TNFRp75 in immune function remains unclear. In this study we employed a natural infection approach using aerosol inhalation at a defined *M. tuberculosis* dose range to investigate and compare the function of TNFRp55 and TNFRp75 in active disease.

WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were infected with *M. tuberculosis* by aerosol inhalation at a dose of 50-100cfu/lung and changes in body mass and mortality were recorded as indicators of active disease progression. Cachexia (wasting) is closely associated with active disease progression in patients suffering from tuberculosis and therefore weight loss, in conjunction with mortality act as key indicators to monitor active tuberculosis disease progression in the mouse model. We found that TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were highly susceptible to infection with weight loss first measured in TNFRp55/75$^{-/-}$ mice at day 21 post infection (Figure 3.2 B). The onset of weight loss in TNFRp55$^{-/-}$ mice was delayed and first observed at 28 days post infection. Nonetheless both strains lost more than 30 percent of their initial body weight and eventually succumbed to infection (Figure 3.2 A). In contrast, TNFRp75$^{-/-}$ mice resisted *M. tuberculosis* infection with no progression to active disease during acute phase. TNFRp75$^{-/-}$ mice maintained a constant weight for the duration of the experiment (Figure 3.2 B) with no mortality being observed, similar to WT control mice (Figure 3.2 A).

Thus we have shown that in a natural aerosol inhalation infection, TNFRp55 signaling remains the critical conduit of TNFα signaling, while TNFRp75 plays a minor role.

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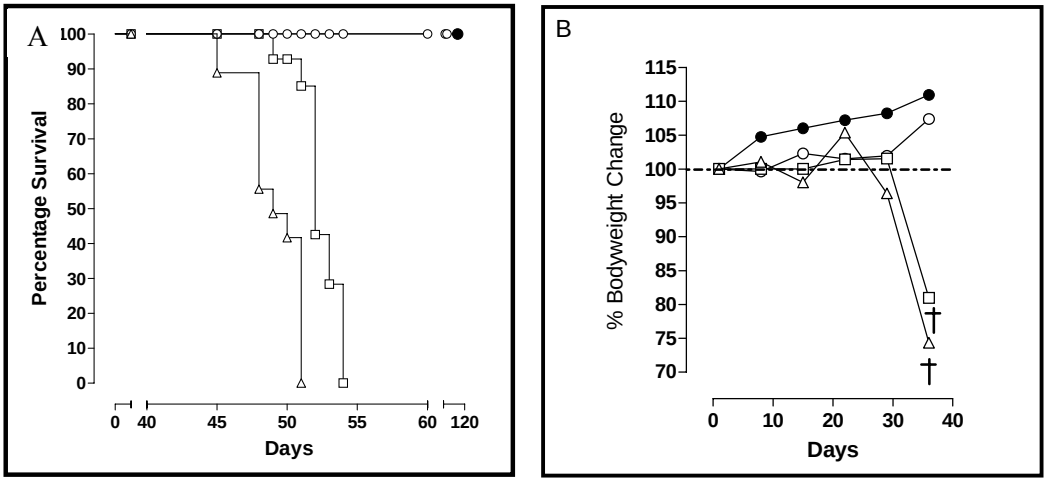

Figure 3.2: Survival of *M. tuberculosis* Infected WT-, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice. WT- (closed circle), TNFRp75$^{-/-}$ (open circle), TNFRp55$^{-/-}$ (open square) and TNFRp55/75$^{-/-}$ (open triangle) mice were infected with 50-100 CFU *M. tuberculosis*. Mice were monitored for survival (A) and body weight changes were measured (B). Briefly, data showed that TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice succumb during the acute stage of infection (A) associated with a significant decrease in body weight (B), while WT and TNFRp75$^{-/-}$ mice survive the duration of the experiment (A) with no weight loss (B). (n= 10 mice/strain, the experiment is one representative of three similar experiments).

3.3 Increased Control of *M. tuberculosis* Replication in TNFRp75$^{-/-}$ Mice

Disease progression in tuberculosis is associated with replication of the organism at the primary pulmonary site of infection. The inability to control pulmonary infection leads to the dissemination of disease to local draining lymph nodes and ultimately to extra-pulmonary sites such as the liver and spleen (Glickman and Jacobs, 2001). The importance of TNFα mediated suppression of mycobacterial replication and prevention of bacilli dissemination was evident in reported studies (Roach *et al*., 2002; Jacobs *et al*., 2001; Bean *et al*., 2000). We therefore asked whether TNFRp55 and TNFRp75 mediated immune function is required to control bacilli replication during acute *M. tuberculosis* infection. WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were infected with *M. tuberculosis* via aerosol inhalation at a dose of 50-100cfu/lung and bacilli burden determined by colony enumeration assays at defined time points.

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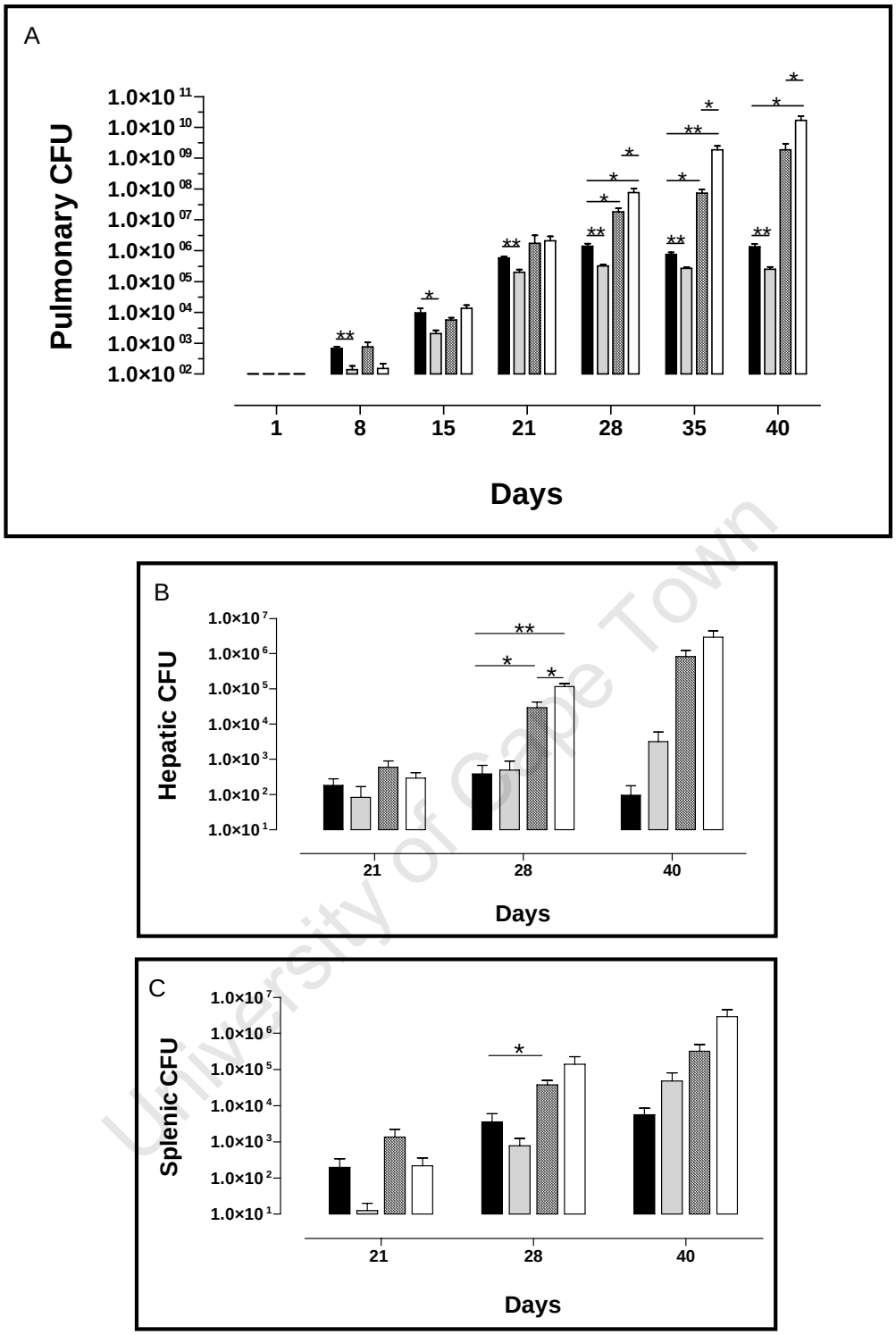

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Figure 3.3: Mycobacterial Replication is Reduced in TNFRp75$^{-/-}$ Mice During Acute *M. tuberculosis* Infection. WT- (solid bars) TNFRp55$^{-/-}$- (hatched bars), TNFRp75$^{-/-}$- (grey bars) and TNFRp55/75$^{-/-}$ (open bars) mice were infected by aerosol inhalation with 50-100 CFU *M. tuberculosis*. Bacilli burden was determined at the indicated time points by colony enumeration assay in the lungs (A), liver (B) and spleen (C). Briefly, the data indicates an increased control of bacilli replication in the lungs (A) and a transient protection in the spleen (C) of TNFRp75$^{-/-}$ mice compared to WT mice. A complete lack of control of infection in TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice in the lung (A), liver (B) and spleen (C) was noted. The data points are the mean ± SEM of the colony-forming units of 4 mice per time point and are representative of one of three similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA.

The pulmonary bacilli burden in WT mice increased $4\log_{10}$ from 10^2 cfu at the onset of infection to approximately $1x10^6$ cfu at day 21 with subsequent immune control maintaining the bacilli burden constant for the duration of the experiment (Figure 3.3 A). Similarly TNFRp75$^{-/-}$ mice controlled bacilli replication 21 days after infection. However, comparative bacilli burdens in TNFRp75$^{-/-}$ mice were significantly lower ($p<0.01$) than those in WT mice, noticeable as early as day 8, and remained lower for the entire duration of the experiment. In contrast to WT- and TNFRp75$^{-/-}$ mice, TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were unable to control bacilli growth with bacilli burdens increasing exponentially beyond day 21 to reach a maximum of $5x10^9$ cfu/lung and $1x10^{10}$ cfu/lung respectively by day 40 (Figure 3.3A). Comparative bacilli burden in TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were significantly higher than WT mice at day 28 ($p<0.05$), day 35 ($p<0.05$) and day 40 ($p<0.05$) post-infection. Pulmonary bacilli burden data obtained by colony enumeration was confirmed by histological examination. Here Ziehl-Neelson (ZN) staining of lung sections 35 days post infection showed large numbers of bacilli present in TNFRp55$^{-/-}$ mice (Figure 3.4 C) and even higher levels of bacilli in TNFRp55/75$^{-/-}$ mice (Figure 3.4 D). In contrast Ziehl-Neelson stained lung sections of WT (Figure 3.4 A) and TNFRp75$^{-/-}$ (Figure 3.4 B) mice had visibly reduced bacilli levels present.

We further assessed the association of TNFα receptor mediated signaling and generation of protective immunity at secondary sites of infection through the enumeration of bacilli burdens in the liver and spleen of WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice (Figure 3.3 B and 3.3 C). In both the livers (Figure 3.3 B) and the spleens (Figure 3.3 B) of TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice, bacilli levels were significantly higher than WT mice 28 days after infection, and higher but not significantly so at day 40. Interestingly, the enhanced protective immunity observed in the lungs of TNFRp75$^{-/-}$ mice was not maintained at the secondary sites of infection. Instead no protection was observed in the liver and only transient protection was seen in the spleen.

Therefore our studies clearly illustrated that the lack of signaling through TNFRp55 results in a loss of control over bacilli replication at the primary site of infection

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resulting in increased dissemination to secondary sites. In contrast, TNFRp75 appears to promote bacilli replication and its absence in TNFRp75$^{-/-}$ mice improved protection.

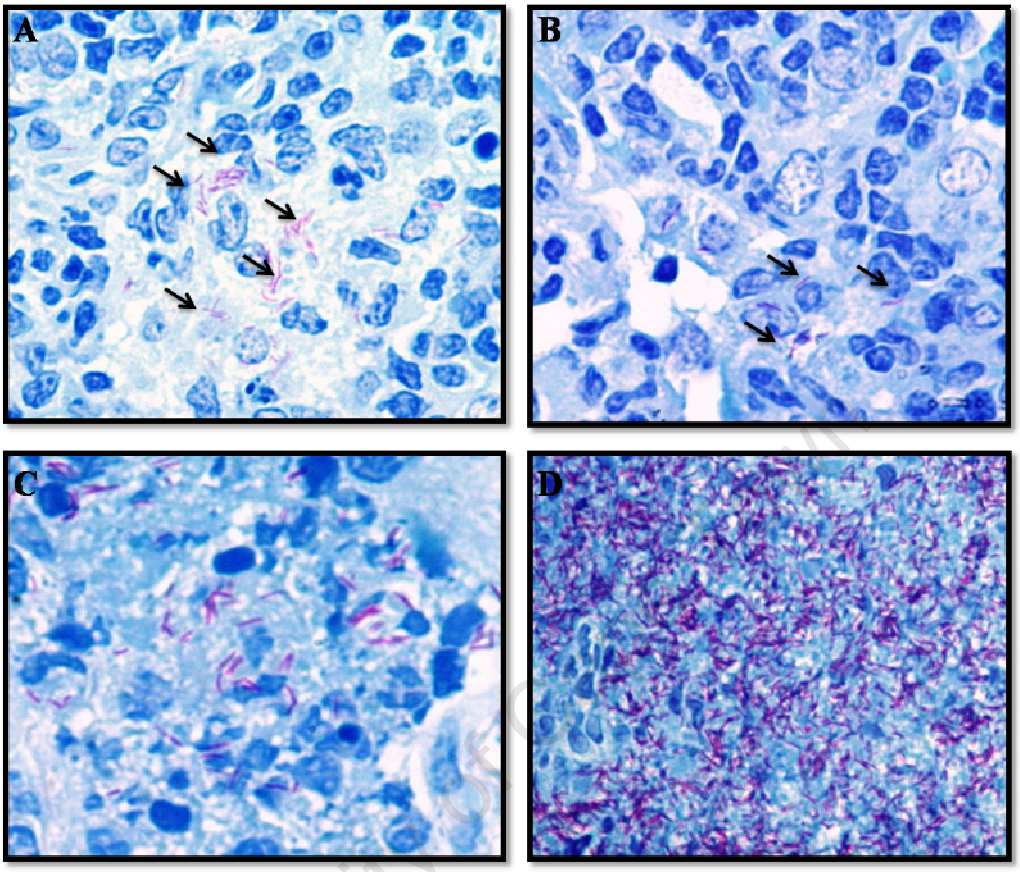

Figure 3.4: Bacilli Replication is Controlled in *M. tuberculosis* Infected WT- and TNFRp75$^{-/-}$- but not TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice. Mice were infected at 50-100 CFU *M. tuberculosis* and bacilli burden assessed by Ziehl-Neilson staining. Briefly the data shows fewer bacilli present in TNFRp75$^{-/-}$ mice (B) compared to WT mice (A), while the number of bacilli present in TNFRp55$^{-/-}$ mice (C) is higher than WT mice but still lower than the number of bacilli in TNFRp55/75$^{-/-}$ mice (D). (Magnification A-C 1000x magnification in D 400x)

3.4 Granuloma Formation is Dependent on TNFRp55 but Independent of TNFRp75 Signaling During Acute *M. tuberculosis* Infection.

Organized cellular recruitment and granuloma formation is crucial for an effective immune response to *M. tuberculosis* challenge (Flynn and Chan, 2001). The establishment of granulomas is a complex process requiring activation and recruitment of lymphocytes and monocytes to the site of infection where formation restricts dissemination of bacilli (Roach *et al.*, 2002). The initiation and maintenance of granulomas during *M. tuberculosis* infection is TNFα dependent (Roach *et al.*, 2002; Kindler *et al.*, 1989). Deletion of TNFα results in failure to form proper granulomas (Roach *et al.*, 2002), while neutralization of TNFα induces

disintegration of established granulomas (Mohan *et al.*, 2000; Kindler *et al*., 1989). Thus in this study we investigated the association between TNFRp55 and TNFRp75, and cellular recruitment, and its role in granuloma formation during acute *M. tuberculosis* infection. Lungs of *M. tuberculosis* infected WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were examined for macroscopic pathology and lung weights assessed as a surrogate marker for inflammation. Further, lungs were sectioned and stained with haemotoxylin and eosin (H&E) for histological examination.

Lung weights measured at 21 days post infection were equivalent in all strains (Figure 3.5 A). In the absence of complete TNFR signalling, the lung weights of TNFRp55/75$^{-/-}$ mice increased significantly by 28 days post infection ($p<0.01$) and remained significantly higher for the remainder of the experiment when compared to WT mice. This rapid increase in lung weight was probably indicative of an early, uncontrolled inflammatory response due to the complete absence of TNFR mediated regulatory signalling. Inflammation in TNFRp55$^{-/-}$ mice was delayed compared to that of TNFRp55/75$^{-/-}$ mice, with significantly higher lung weights only first being noted at 42 days post infection compared to the WT control mice ($p<0.01$). In contrast, inflammation in TNFRp75$^{-/-}$ mice was significantly lower than those observed in WT mice from as early as 28 days after infection ($p<0.05$) and remained as such for the duration of the experiment ($p<0.01$).

Pulmonary pathology (Figure 3.5 B) was characterized by small distinct lesions on the pleural lung surface in WT mice. In TNFRp75$^{-/-}$ mice such lesions were noticeably smaller and fewer, in contrast to the enlarged lesions observed in TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice. Comparatively, the severity of pathology was distinctly more pronounced in TNFRp55/75$^{-/-}$ mice than TNFRp55$^{-/-}$ mice (Figure 3.5 B) with an overall improved pathology noted in TNFRp75$^{-/-}$ mice compared to WT mice.

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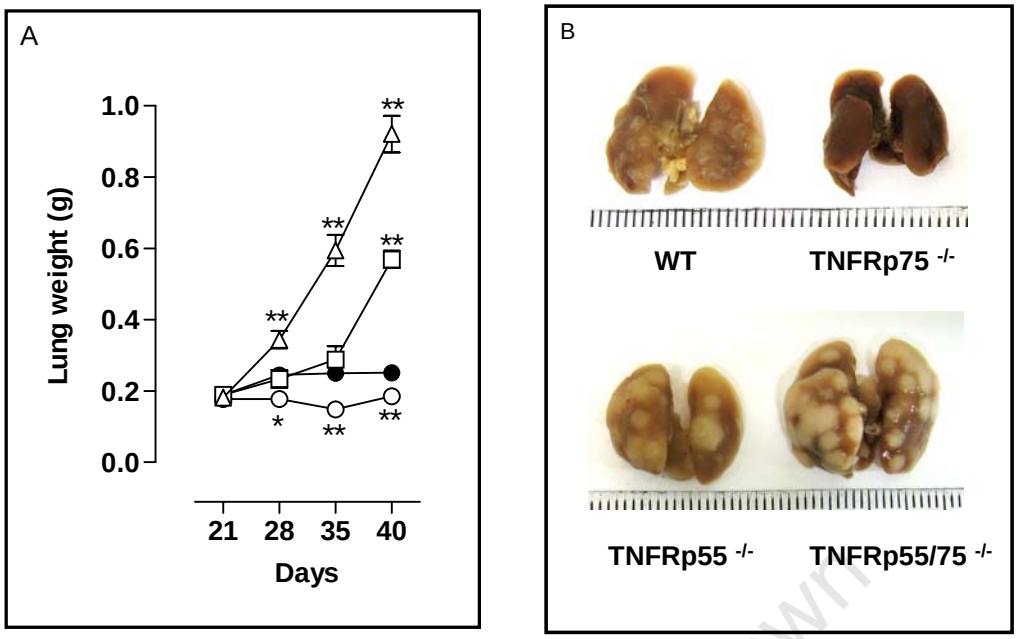

Figure 3.5: TNFRp75$^{-/-}$ Mice Control Acute *M. tuberculosis* Infection Characterised by Reduced Lung Weights and Fewer Lesions on the Pleural Lung Surface. WT- (closed circle), TNFRp75$^{-/-}$- (open circle), TNFRp55$^{-/-}$- (open square) and TNFRp55/75$^{-/-}$ (open triangle) mice were infected by aerosol inhalation with 50-100 CFU *M. tuberculosis*. Mice were killed at the indicated time points, lung weights assessed (A) and pulmonary pathology recorded on day 35 post infection (B). Results show significantly reduced lung weights on day 35 and 40 (A) and fewer smaller lesions on the pleural lung surface on day 35 post infection (B) in TNFRp75$^{-/-}$ mice compared to WT control animals. In contrast, lung weights of TNFRp55$^{-/-}$ mice were increased compared to WT mice but still lower than TNFRp55/75$^{-/-}$ mice (A) with large lesions on the pleural lung surface (B) The data points in A are the mean ± SEM of the lung weights of 4 mice per time point and both A and B are representative of one of three experiments. Significant differences in A (* $p<0.05$, ** $p<0.01$) were determined by ANOVA.

Next we investigated whether specific TNFR signaling influences the establishment of defined granulomas and the maintenance of its structural integrity in response to *M. tuberculosis* infection. Pulmonary morphology of tissue sections in WT-, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were indistinguishable from each other and were characterized by defined alveoli and bronchi without the presence of granulomas 14 days (Figure 3.6 A-D) after infection. By day 28, both WT (Figure 3.6 E) and TNFRp75$^{-/-}$ (Figure 3.6 F) mice developed well defined granuloma structures characterized by epitheloid macrophages interspersed with lymphocytes.

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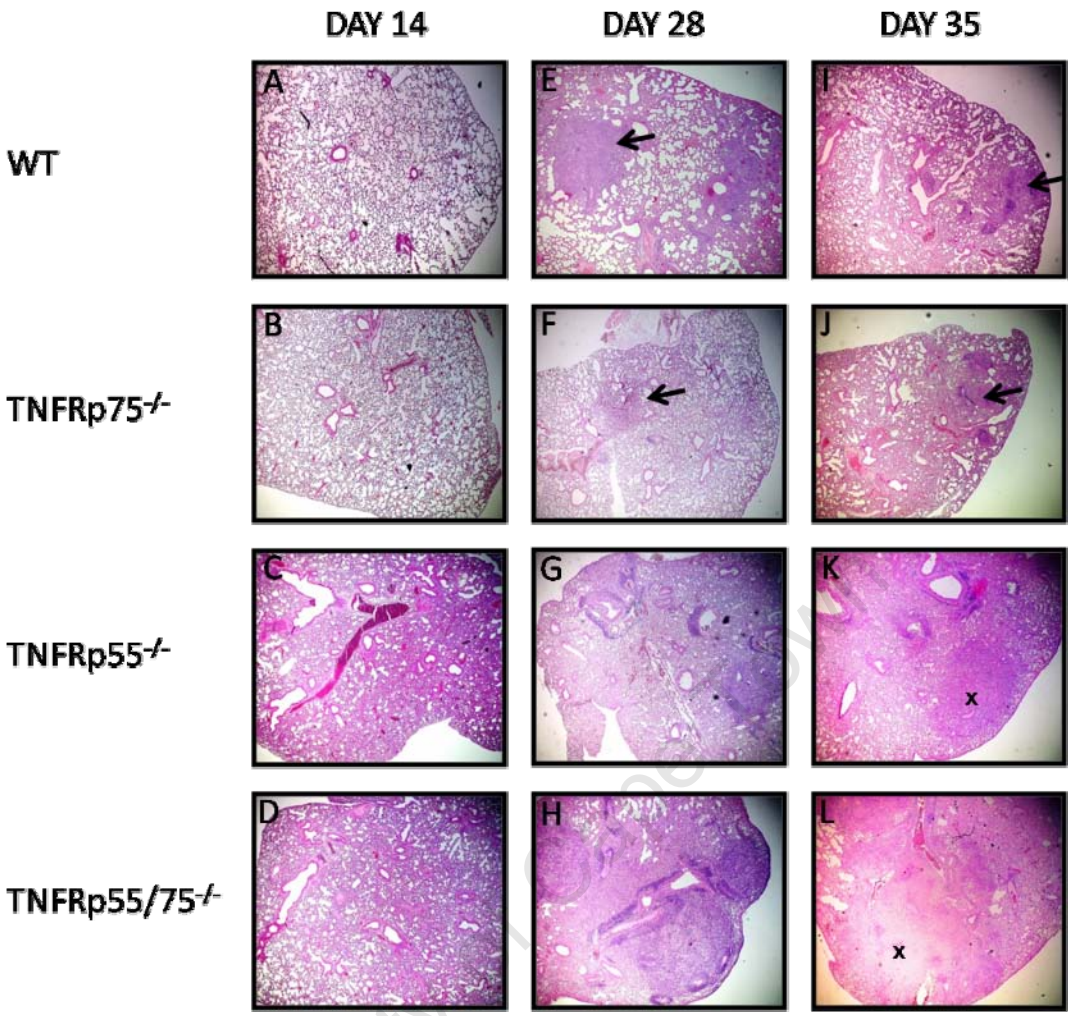

Figure 3.6: Granuloma Formation is TNFRp75 Independent during *M. tuberculosis* Infection. Mice were infected at 50-100 CFU *M. tuberculosis* and killed at the indicated time-points. Lungs were removed fixed in formalin, embedded in wax, sectioned and Haemotoxylin and Eosin (H&E) staining was performed. Briefly the data shows fewer smaller granulomas present in TNFRp75$^{-/-}$ mice (F and J) compared to WT mice, while a complete lack of granuloma formation is noted in TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice (G, K, H and L). Necrosis is clearly visible in both TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice by day 35 post-infection (Indicated by X in K and L). (Magnification 100 x, experiments are representative of one of three similar experiments).

Interestingly, granulomas in TNFRp75$^{-/-}$ mice appear smaller and more compact than those in WT mice. Lung sections of TNFRp55$^{-/-}$- (Figure 3.6 G) and TNFRp55/75$^{-/-}$ (Figure 3.6 H) mice however, were devoid of any defined granuloma structures 28 days postinfection. Instead they were characterized by an unorganized general cellular infiltration which was more severe in TNFRp55/75$^{-/-}$ mice than in TNFRp55$^{-/-}$ mice. Granuloma structures in WT- (Figure 3.6 I) and TNFRp75$^{-/-}$ (Figure 3.6 J) mice became increasingly compact with a more distinct reorganization of cell content as bacilli burdens reached stasis concommitant with control of infection 35 days post infection. In contrast, lung sections from

TNFRp55$^{-/-}$- (Figure 3.6 K) and TNFRp55/75$^{-/-}$ (Figure 3.6 L) mice presented with pathology indicative of uncontrolled cellular infiltration, large areas of necrosis (indicated by X) and few clear alveoli 35 days after infection. The inability of TNFRp55$^{-/-}$ mice to form structured granulomas in response to an aerosol inhalation of *M. tuberculosis* is corroborated by established findings in which TNFRp55$^{-/-}$ mice infected intravenously with *M. tuberculosis* (Flynn *et al*., 1995) or *M. Bovis* BCG (Senaldi *et al.,* 1996) presented with disregulated granuloma structures.

Thus our results show that TNFRp55 signaling is important to control granuloma formation and its absence resulted in greatly increased lung weight, large lesions on the lung pleura and unorganized granuloma structures. In addition we showed that TNFRp75 is not crucial for granuloma formation, in fact in the absence of this receptor we found reduced lung weight, fewer smaller lesions on the lung pleura and bactericidal granulomas that were compact.

3.5 Pulmonary Inflammation is reduced in the Absence of TNFRp75 during Acute *M. tuberculosis* Infection.

Regulated recruitment of effector cells is an essential requirement for effective pathogen inhibition and limitation of cellular damage. Excessive cellular infiltration leads to an exacerbated pro-inflammatory cytokine response with severe consequences to the host such as tissue destruction (Zganiacz *et al.,* 2004). In view of our findings that TNFRp55 and TNFRp75 mediate distinct effects on the inflammatory response during *M. tuberculosis* infection, we next sought to determine their effects on the inflammatory cellular profile during acute *M. tuberculosis* infection. Single cell suspensions were generated from lungs of infected WT, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice at 35 days post-infection and analysed for the presence of CD11b^{+}-, CD11c^{+}-, CD4^{+}- and CD8^{+} cells. We found that complete deletion of TNFR results in uncontrolled cellular infiltration subsequent to *M. tuberculosis* infection; the total number of lung cells in TNFRp55/75 double deficient mice being > 10x higher compared to that of WT mice (Figure 3.7 A). Comparative analysis of the recruitment of specific cell populations showed that CD4^{+} T cells were 10x higher (Figure 3.7 B) and CD8^{+} T cells were 3x higher (Figure 3.7 C) in TNFRp55/75$^{-/-}$ mice compared to wild type

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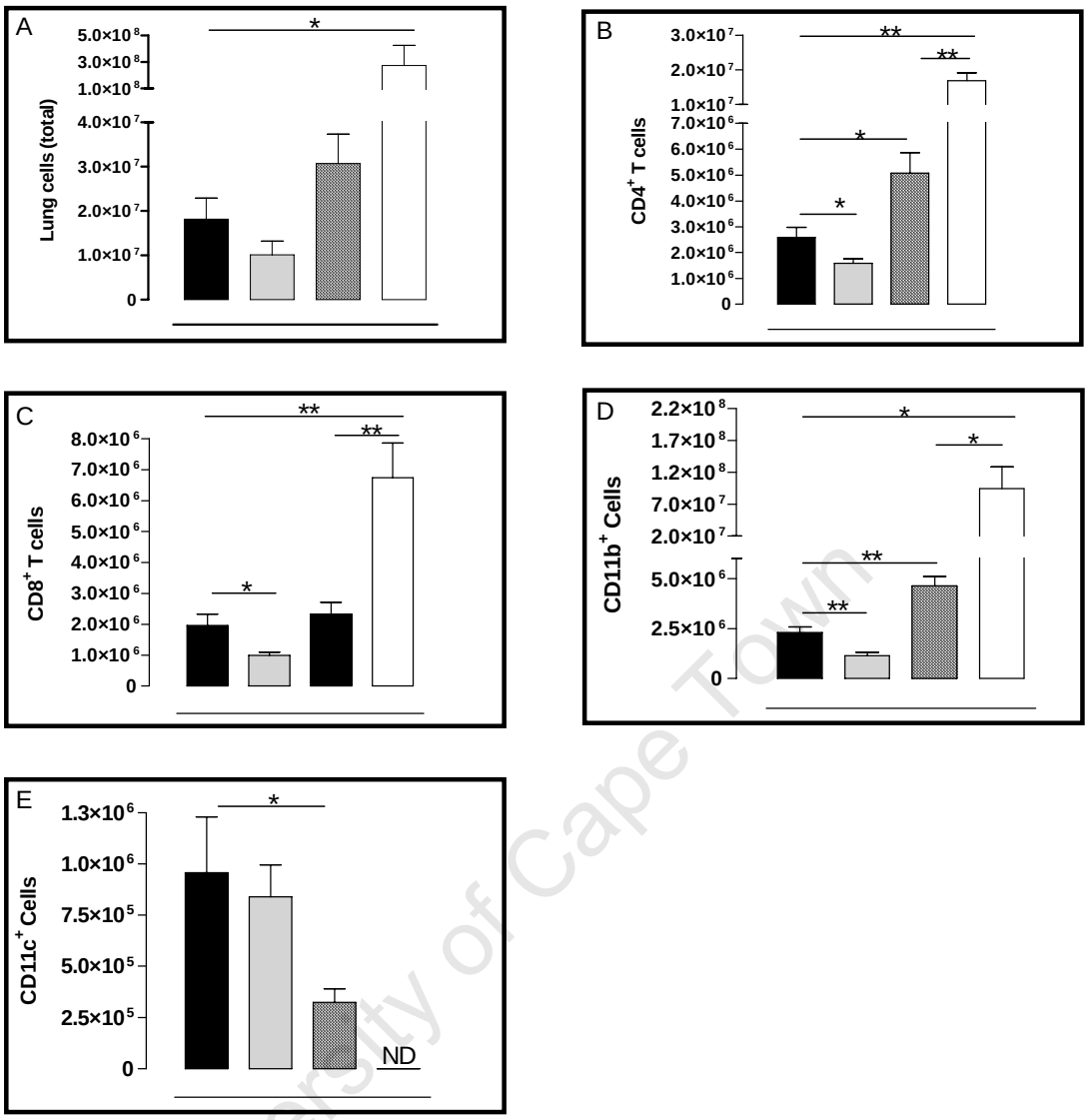

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<u>Figure 3.7:</u> Cellular Recruitment is Reduced in TNFRp75$^{-/-}$ Mice During Acute *M. tuberculosis* Infection. WT (solid bars) TNFRp55$^{-/-}$- (hatched bars), TNFRp75$^{-/-}$- (grey bars) and TNFRp55/75$^{-/-}$ (open bars) mice were infected with 50-100 CFU *M. tuberculosis*. Cells from whole lungs were isolated from *M. tuberculosis* infected mice at 35 days post infection and the cellular recruitment profile analysed by staining with APC conjugated anti- CD4-, CD8-, CD11b- or CD11c monoclonal antibodies. Briefly the data shows a reduction in the total number of pulmonary cells (A) as well as a reduced number of CD4^{+}- (B) and CD8^{+} (C) T cells and CD11b^{+} (D) cells but similar CD11c^{+} (E) cells in TNFRp75$^{-/-}$ mice compared to WT control mice. In contrast TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice have an increased number of total lung cells (A) as well as an increased infiltration of CD4^{+}- (B) and CD11c^{+} cells (D) with an inability to recruit CD11c^{+} cells (E) being noted in TNFRp55$^{-/-}$ mice. The data points are the mean ± SEM of 4 mice per time point and are representative of one of two similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA. (ND= not done).

mice. The difference in recruitment of CD11b^{+} cells was even more impressive with a 50x increase (Figure 3.7 D) present in TNFRp55/75$^{-/-}$ mice compared to wild type mice. The inflammatory response of TNFRp55$^{-/-}$ mice was similarly increased compared to WT mice but showed an overall reduction compared to TNFRp55/75$^{-/-}$ mice with the total number of lung cells in TNFRp55$^{-/-}$ mice being 1.5x higher than

WT but 10x lower than TNFRp55/75$^{-/-}$ mice (Figure 3.7 A). Recruitment of CD4^{+} T cells in TNFRp55$^{-/-}$ mice were 5x lower than TNFRp55/75$^{-/-}$ mice but 2x higher than WT mice (Figure 3.7 B), CD8^{+} T cells in TNFRp55$^{-/-}$ mice were 3.5x lower than TNFRp55/75$^{-/-}$ mice and similar to WT mice (Figure 3.7 C) and CD11b^{+} cells in TNFRp55$^{-/-}$ mice were >20x lower than TNFRp55/75$^{-/-}$ mice and 2x higher than WT mice (Figure 3.7 D). Interesting to note was that TNFRp55 deficiency was associated with a defective ability to recruit mature CD11c^{+} cells to the site of infection, illustrated in Figure 3.6 E which shows that TNFRp55$^{-/-}$ mice have a significantly lower number of CD11c^{+} cells in the lungs at day 35 post-infection ($p<0.05$). In contrast, the more effective immune response of TNFRp75$^{-/-}$ mice was characterized by reduced CD11b^{+} cells ($p<0.01$) (Figure 3.7 D), CD4^{+} T cell ($p<0.05$) (Figure 3.7 B) and CD8^{+} T-cell ($p<0.05$) (Figure 3.7 C) recruitment which was significantly lower compared to WT mice.

Our results clearly show the importance of signaling through TNFRp55 in the regulation of recruitment of effector cells to the site of infection. In contrast, the lack of signaling through TNFRp75 resulted in controlled cellular recruitment.

3.6 Reduced T_H1 Pro-inflammatory and T_H2 Cytokine Levels In TNFRp75$^{-/-}$ Mice During Acute *M. tuberculosis* Infection.

Cytokines are important regulators of cells in the immune system. The profile and kinetics of cytokines produced in response to *M. tuberculosis* are crucial and determine the outcome of disease, where excessive or imbalanced pro-inflammatory cytokine production may contribute to chronic infection and associated pathology that is detrimental to the host (Flynn and Chan, 2001; Flynn *et al.*, 1995; Zganiacz *et al.*, 2004). Recent gene deletion studies which targeted cytokines associated with T_H1 immune function have found several cytokines that are non-redundant for host protection during *M. tuberculosis* infection (Cooper and Khader, 2007; Cooper *et al.*, 1997; Flynn *et al.*, 1995; Khader *et al.*, 2006). Such examples include reports which found that a T_H1 immune response driven by IL-12 production is important for protection against *M. tuberculosis* infection (Cooper and Khader, 2007; Cooper *et al.*, 1997; Flynn *et al.*, 1995). Recognition and phagocytosis of *M. tuberculosis* bacilli by macrophages and dendritic cells leads to IL-12 synthesis and activation of microbiocidal mechanisms that control mycobacterial replication. The importance

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of IL-12 in protective immunity against *M. tuberculosis* was illustrated in IL-12p40$^{-/-}$ mice where the inability to produce IL-12 rendered the host highly susceptible to infection, characterized by increased bacilli burden, high mortality and reduced IFNγ production (Flynn and Chan 2001). Furthermore, neutralization of IL-12 during persistent infection leads to reactivation of *M. tuberculosis* indicating that IL-12 not only initiates but maintains immune function during infection (Feng *et al.*, 2005). Similarly TNFα is non-redundant for favorable outcome of disease (Florido and Appelberg, 2007; Flynn *et al.*, 1995; Kaneko *et al.*, 1999; Lin *et al.*, 2007) despite its reported dual role of promoting protective immune function, while excessive expression may have pathological consequences (Zganiacz *et al.*, 2004). Other studies have also reported a role for TNFα in promoting pathogen persistence (Engele *et al.* 2002). Nonetheless, it remains an absolute requirement for the control of acute *M. tuberculosis* infection as demonstrated in TNFα$^{-/-}$ mice infected with *M. tuberculosis,* which succumb rapidly with an excessive bacilli burden and poor granuloma formation (Roach *et al.,* 2002, Bean *et al.,* 1999). Also, IFNγ is crucial to the outcome of an *M. tuberculosis* infection with mice deficient for this gene being highly susceptible (Flynn *et al.*, 1993; Mitsuyama *et al.*, 2003). In these mice NOS_2 expression is low, macrophage activation is defective and granulomas which form are poorly structured and rapidly become necrotic (Flynn *et al.*, 1993). Furthermore, Saunders *el. al.,* (2000) demonstrated that IL-6 was important during the early innate immune response. IL-6$^{-/-}$ mice developed increased susceptibility to low dose aerosol *M. tuberculosis* infection with high bacilli burdens accompanied by reduced IFNγ production during early acute infection. Mice which survived the early acute phase of infection were able to control the disease and survived the duration of the experiment, indicating that IL-6 is important during early immune responses but may be replaced during chronic infection.

Thus, to understand the mechanism associated with TNFR mediated regulation of immune function we initially investigated pulmonary levels of the pro-inflammatory cytokines IL-12, IL-6 and TNFα as well as IFNγ in *M. tuberculosis* infected mice over 5 weeks. TNFα levels in WT mice remained relatively constant for the duration of the experiment within a narrow margin of between 1.02-1.46ng/ml during acute *M. tuberculosis* infection (Figure 3.8 A) suggesting that peak concentrations had

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been reached prior to day 14. In contrast, both the TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice had significantly lower TNFα levels during early infection ($p<0.05$) (D14, D21) with peak concentrations only being measured at end stages of infection in these mice. The absence of TNFRp55 clearly contributed to an early delay in TNFα production that resulted in an uncontrolled 10 fold induction during end stage disease in TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice. TNFα profile in TNFRp75$^{-/-}$ mice was similar to WT mice and may have peaked prior to day 14. Interestingly

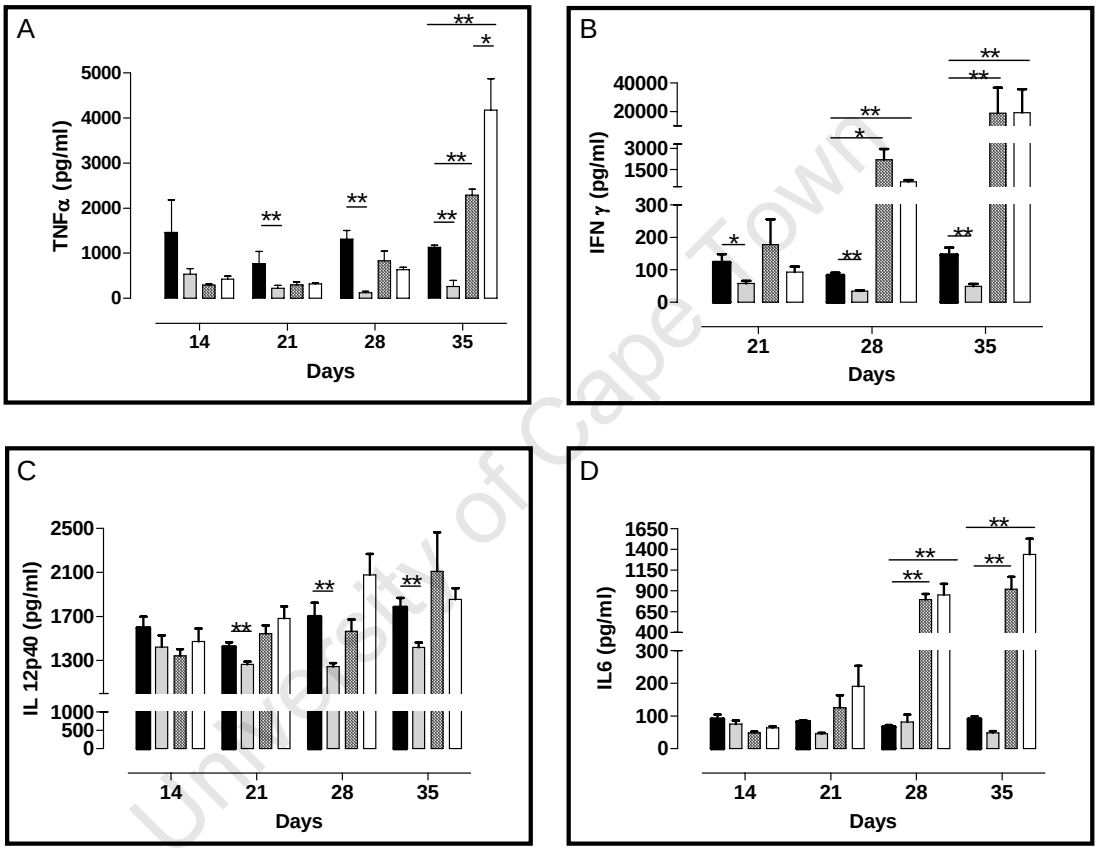

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Figure 3.8: Pulmonary Cytokine Production in WT-, TNFRp75$^{-/-}$-, TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ Mice During Acute *M. tuberculosis* Infection. WT- (solid bars) TNFRp55$^{-/-}$- (hatched bars), TNFRp75$^{-/-}$- (grey bars) and TNFRp55/75$^{-/-}$ mice (open bars) were infected by aerosol inhalation with 50-100 CFU *M. tuberculosis*. Mice were killed at the indicated time points and lung cytokine levels assessed in the supernatants from homogenized whole lungs by ELISA. Briefly, data shows reduced expression of TNFα, IFNγ and IL-12p40 but similar IL-6 levels in TNFRp75$^{-/-}$ mice compared to WT control animals at all time points. In contrast TNFRp55$^{-/-}$and TNFRp55/75$^{-/-}$ mice show delayed and excessive TNFα, IFNγ and IL-6 expression but similar IL-12p40 levels compared to WT mice. The data points are the mean ± SEM of the cytokine values of 4 mice per time point and are representative of one of three similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA.

however, TNFα and IL-12 levels were significantly lower in TNFRp75$^{-/-}$ mice at each time point that was tested ($p<0.05$), suggesting that TNFRp75 may play a

regulating role in the TNFα and IL-12 response during *M. tuberculosis* infection. Similar to TNFα, IFNγ production in WT mice remained constant and within a narrow margin of between 83.1-145.8pg/ml for the duration of the infection (Figure 3.8 B). Both the TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice had a delay in IFNγ production and an uncontrolled increase in IFNγ during end stage infection of approximately 10x on day 35. In contrast, IFNγ production in TNFRp75$^{-/-}$ mice remained constant suggesting that maximum IFNγ production may have been reached earlier than 21 days post infection. However, similar to TNFα production, the IFNγ levels were significantly lower ($p<0.05$) in TNFRp75$^{-/-}$ mice at all time points, suggesting that TNFRp75 may also regulate IFNγ production. No significant differences were detected in IL-12p40 production between WT-, TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice during acute *M. tuberculosis* infection (Figure 3.8 C). In contrast, the inability to signal through TNFRp75 resulted in significantly lower IL-12p40 production on days 21, 28 and 35 post-infection ($p<0.01$). The results indicated that IL-6 levels in WT mice remained constant and within a narrow margin of between 66.26-91.17png/ml during acute *M. tuberculosis* infection (Figure 3.8 D). However, both TNFRp55$^{-/-}$- and TNFRp55/75$^{-/-}$ mice showed an uncontrolled increase in IL-6. Interestingly the production of IL-6 in TNFRp75$^{-/-}$ mice was similar to that of wild type mice at all time points.

Our results therefore illustrated the importance of signaling though TNFRp55 for the control of pro-inflammatory cytokine production. The absence of TNFRp55 resulted in delayed but excessive pro-inflammatory cytokine production. In contrast, deleting the TNFRp75 receptor resulted in reduced but regulated pro-inflammatory levels.

3.7 Increased Survival of TNFRp75$^{-/-}$ Mice During Chronic *M. tuberculosis* Infection.

In view of the increased protection of TNFRp75$^{-/-}$ mice observed during acute *M. tuberculosis* infection, we assessed whether disease outcome was influenced during chronic infection. WT- and TNFRp75$^{-/-}$ mice were infected with *M. tuberculosis* by aerosol inhalation at a dose of 50-100cfu/lung and mortality recorded. Figure 3.9 shows that the enhanced immune response observed in TNFRp75$^{-/-}$ mice during acute *M. tuberculosis* infection correlated with a significantly reduced mortality rate

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in TNFRp75$^{-/-}$ mice during chronic infection ($p< 0.0005$). The calculated median survival time of TNFRp75$^{-/-}$ mice was 407 days compared to 229 days in WT mice. The presence of TNFRp75 therefore contributed significantly to host mortality during *M. tuberculosis* infection.

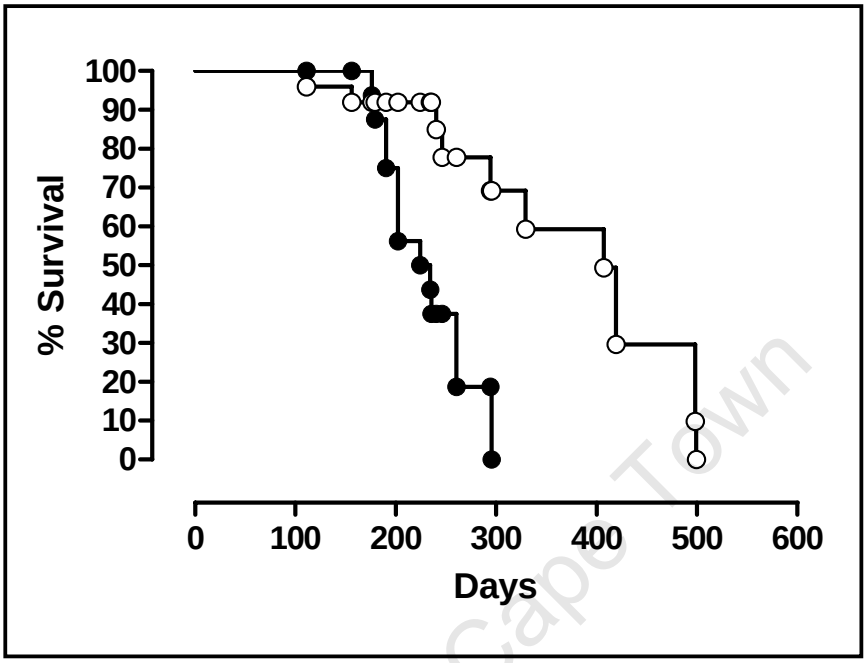

Figure 3.9: Increased Survival of TNFRp75$^{-/-}$ During Chronic *M. tuberculosis* Infection. WT- (closed circles) and TNFRp75$^{-/-}$ mice (open circles) were infected by aerosol inhalation with 50-100 CFU *M. tuberculosis* and monitored for survival. The data clearly shows a prolonged survival of approximately 200 days of TNFRp75$^{-/-}$ mice compared to WT mice (n=10 mice/strain. Experiment is representative of 4 similar experiments, $p< 0.0005$). Statistical analysis was done using the Logrank Test.

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3.8 Enhanced Protective Immunity Observed in TNFRp75$^{-/-}$ Mice During Acute *M. tuberculosis* Infection Is Maintained During Chronic Infection.

We next addressed whether increased survival in TNFRp75$^{-/-}$ mice correlated with improved control of bacilli replication by measuring both pulmonary and extra-pulmonary mycobacterial burdens. Wild type, TNFRp55$^{-/-}$-, TNFRp75$^{-/-}$- and TNFRp55/75$^{-/-}$ mice were infected with *M. tuberculosis* via aerosol inhalation at a dose of 50-100cfu/lung and organ bacilli levels measured after 2, 4.5 and 7.5 months. The pulmonary bacilli burdens determined in WT mice between 2 and 7.5 months remained relatively constant ($2x10^6$ – $3x10^6$ cfu). The pulmonary bacilli burdens in TNFRp75$^{-/-}$ mice were significantly reduced at all the time points indicating that increased control of bacilli replication noted during acute infection, compared to WT mice, extended into the chronic phase. The significantly lower

bacilli burdens ($p< 0.05$) in TNFRp75$^{-/-}$ mice which was established during acute infection remained at > 1 $\log_{10}$ at 2 and 4.5 months (Fig 3.10A). Pulmonary bacilli burden data, obtained by enumeration was confirmed by histological examination. Here Ziehl-Niehlson (ZN) staining of lung sections at 7.5 months post-infection showed more bacilli present in the lungs of WT mice (Figure 3.10 B) compared to TNFRp75$^{-/-}$ mice (Figure 3.10 C).

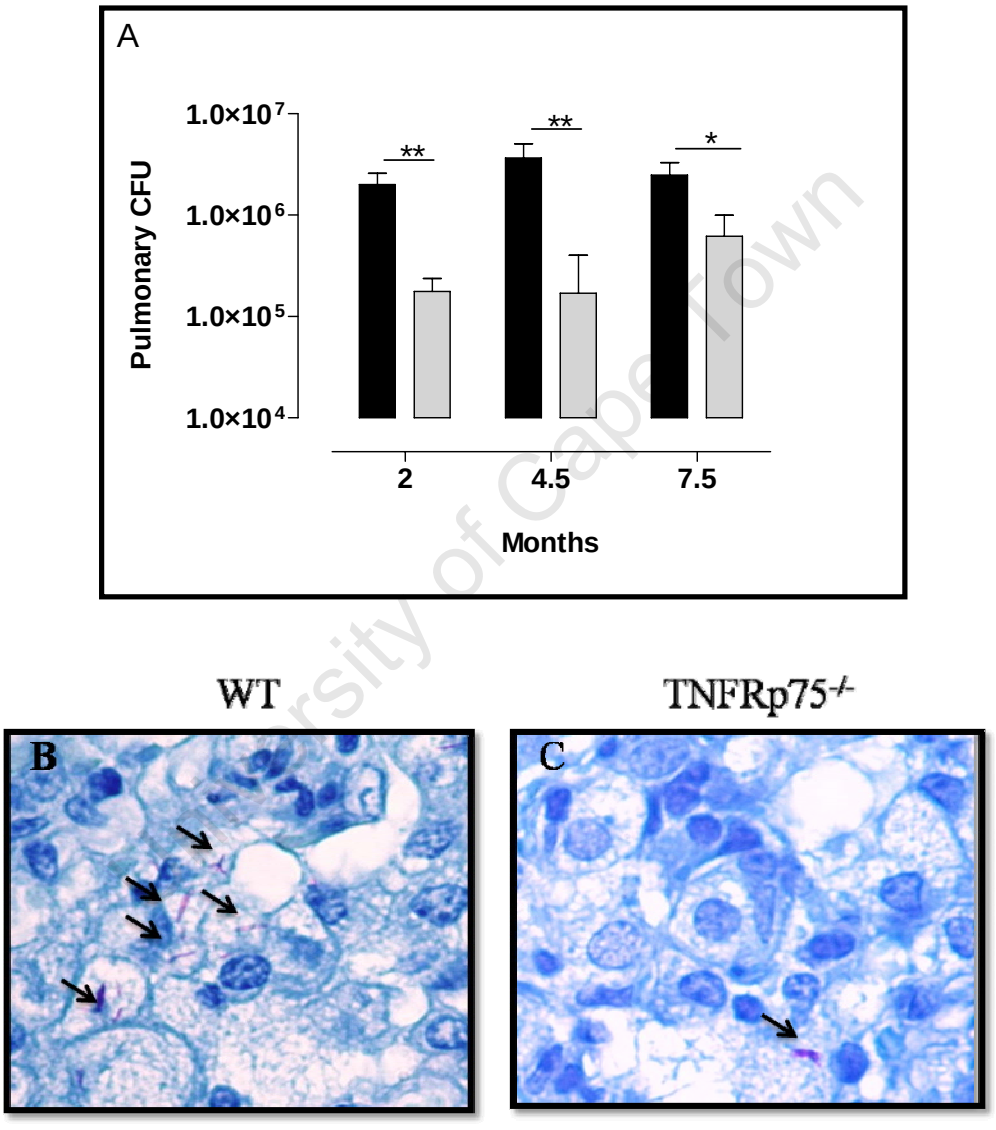

Figure 3.10: Enhanced Protection in TNFRp75$^{-/-}$ Mice Compared to WT Mice During Chronic *M. tuberculosis* Infection. WT (solid bars) and TNFRp75$^{-/-}$ mice (grey bars) were infected by aerosol inhalation with 50-100 CFU *M. tuberculosis*. Bacilli burdens were determined at the indicated time points by colony enumeration assay in the lungs (A) and visualized by histological staining of lung sections by ZN staining (B, C magnification 1000x). CFU data shows an increased control of bacilli replication in the lung (A) of TNFRp75$^{-/-}$ mice compared to WT mice. CFU data is supported by histological staining in which fewer bacilli are observed in TNFRp75$^{-/-}$ mice at 7.5 months (C) compared to WT mice (B). The data points in A are the mean ± SEM of the colony-forming units of 4 mice per time point and B and C are representative of one of three similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA.

In WT mice the bacilli burdens at the terminal stages of infection did not approach those observed at the terminal stages of TNFRp55$^{-/-}$- or TNFRp55/75$^{-/-}$ mice (approximately 1x10^{9} at 5 weeks). We therefore hypothesised that mortality was thus not a direct result of a lack of control over bacilli replication but that it could be due to tissue damage caused by long term exposure to an increased pro-inflammatory immune response. We therefore assessed pulmonary pathology, in particular granuloma structures in WT- and TNFRp75$^{-/-}$ mice at 4.5 and 7.5 months post *M. tuberculosis* infection.

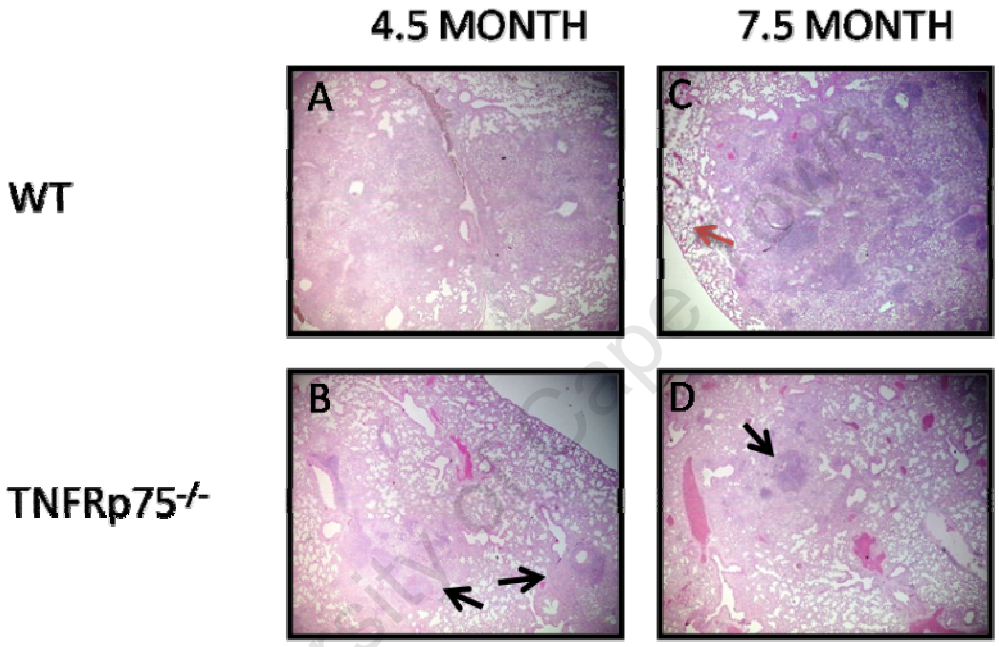

Figure 3.11: Structured Granuloma Formation is Maintained in TNFRp75$^{-/-}$ mice During Chronic *M. tuberculosis* Infection. Mice were infected at 50-100 CFU *M. tuberculosis* and killed at the indicated time-points. Lungs were removed fixed in formalin, embedded in wax, sectioned and H&E staining was performed. The staining shows a degeneration of the structured granuloma formation with massive cellular infiltration in WT mice (A and C). The red arrow points to the small amount of clear alveolar space remaining for normal breathing at 7.5 months in the WT strain. In contrast the structure of granulomas remains intact in TNFRp75$^{-/-}$ mice (black arrows in B and D) (magnification 400x, experiments are representative of one of two similar experiments).

The results show that in wild type mice at 4.5 (Figure 3.11 A) and more so at 7.5 months (Figure 3.11B) post infection, the granuloma's observed during acute infection are no longer compactly structured and defined; but started to resemble the unorganised general infiltration observed in TNFRp55$^{-/-}$ mice. In fact the remaining amount of clear alveolar space (indicated by the red arrow in Figure 3.10B) at 7.5 months was significantly reduced. In contrast, the granuloma structures in TNFRp75$^{-/-}$ mice remain defined with excellent structural integrity at both 4.5 (Figure 3.11 C) and 7.5 months post infection (Figure 3.11 D). The data therefore

supports a TNFRp75 dependent mechanism associated with increased pulmonary pathology that leads to mortality.

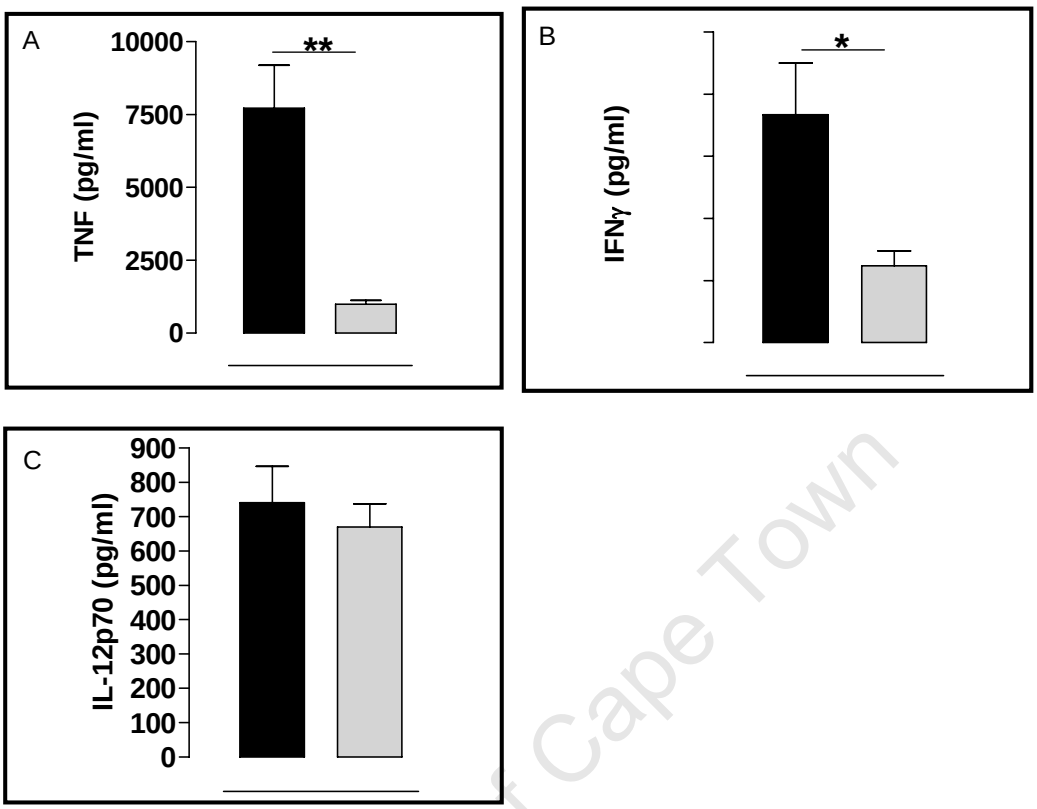

Figure 3.12: Pulmonary Cytokine Production in WT- and TNFRp75$^{-/-}$ Mice During Chronic *M. tuberculosis* Infection. WT- (solid bars) and TNFRp75$^{-/-}$ (grey bars) mice were infected with 50-100 CFU *M. tuberculosis*. Samples were collected at 6 months post infection and analysed for pulmonary cytokine levels in the supernatants from homogenized whole lungs by ELISA. The results clearly show a significant reduction in TNFα and IFNγ but similar IL-12p70 expression in TNFRp75$^{-/-}$ mice compared to WT animals. The data points are the mean ± SEM of the cytokine values of 4 mice per time point and are representative of one of three similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA.

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To further understand the factors associated with increased susceptibility in WT mice we postulated that increased pathology was driven by an increased pro-inflammatory immune response. We therefore measured pulmonary IL-12, TNFα and IFNγ in *M. tuberculosis* infected mice at 6 months post-infection. We found that excessive expression of TNFα (Figure 3.12 A) and IFNγ (Figure 3.12 B), cytokines both known to be pro-inflammatory, was associated with mortality in chronically infected WT mice.

Our data clearly illustrates the advantage conferred to the bacilli in the presence of TNFRp75. Here the absence of this receptor results in a distinctly improved outcome to disease with increased survival and decreased bacilli burdens.

Chapter 4 Results:

Understanding the Mechanism Underlying TNFRp75 Dependent Susceptibility during *Mycobacterium tuberculosis* Infection.

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Results: Understanding the Mechanism Underlying TNFRp75 Dependent Susceptibility during *Mycobacterium tuberculosis* Infection.

The phenotypic characterization showed a reduced bacilli burden associated with fewer, smaller pulmonary lesions and reduced pro-inflammatory cytokine production which demonstrated an enhanced protective immune response in TNFRp75$^{-/-}$ mice during early acute *M. tuberculosis* infection, leading directly to the increased survival of this mouse strain during chronic infection. This increased protection in TNFRp75$^{-/-}$ mice was observed as early as day 8 post-infection thus suggesting a crucial involvement of the innate immune system. Since antigen presenting cells (APC's) form an integral part of the innate immune response, we next investigated the recruitment and activation of APC's in WT and TNFRp75$^{-/-}$ mice subsequent to *M. tuberculosis* infection.

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4.1 Antigen Presenting Cell (APC) Activation

4.1.1 Increased Activation of Antigen Presenting Cells in *M. tuberculosis* Infected TNFRp75$^{-/-}$ Mice.

CD80 and CD86, also known as B7.1 and B7.2 respectively, form part of the B7 ligand family and are only expressed on lymphoid tissue, particularly antigen presenting cells such as macrophages and dendritic cells. Low levels of CD86 expression occurs in resting macrophages and dendritic cells, which upon activation, is upregulated and *de nova* CD80 expression is initiated. Both ligands have an extracellular and a transmembrane domain and it has recently been suggested that they are capable of reverse signalling (Collins *et al*., 2005). Corresponding receptors for CD80 and CD86 are CD28 and CTLA-4, expressed on T and B cells with CD80 having a higher affinity for CTLA-4 whereas CD86 binds more effectively to CD28 (Collins *et al*., 2002). CD80 and CD86 are able to initiate both costimulatory and coinhibitory signals upon binding to their receptors. Interaction with CD28 results in the initiation of a costimulatory signal, while binding to CTLA-4 induces a coinhibitory signal which counteracts costimulatory signals. The CD28-induced costimulatory signal causes T cell activation, expansion, differentiation and survival leading to effective antibody and cellular immune

responses (Collins *et al*., 2005). In dendritic cells reverse signalling occurs on CD80 or CD86 binding of soluble CTLA-4-immunoglobulin chimeric protein (CTLA4.Ig) or soluble CD28--immunoglobulin (CD28.Ig.) During CTLA4.Ig binding, indoleamine 2,3-dioxygenase (IDO) is produced and Tryptophan levels are decreased, leading to an inhibition of T-cell proliferation and cell death (Grohmann *et al.,* 2002). Whereas CD80 or CD86 binding to CD28.Ig induces IL6 synthesis by dentritic cells (Orabona *et al*., 2004).

In view of the enhanced protection afforded in TNFRp75$^{-/-}$ mice during *M. tuberculosis* infection, we next assessed whether TNFRp75 regulates immune function through recruitment and activation of APC's. Here we first infected WT- and TNFRp75$^{-/-}$ mice with *M. tuberculosis* by aerosol inhalation at a dose of 50-100 cfu and evaluated CD11b^{+}- and CD11c^{+} cell presence in the lungs and draining lymph nodes at 21 and 28 days post infection. To assess cellular activation of each population, we measured co-expression of CD80, CD86 and MHCΠ. We found that the total number of both CD11b^{+}- (Figure 4.1 A) and CD11c^{+} (Figure 4.1 B) cells recruited to the lungs in TNFRp75$^{-/-}$ mice was significantly higher ($p< 0.05$) on day 21 post-infection but similar by day 28. Further analysis of recruited cells showed that the number of recruited pulmonary CD11b^{+}- and CD11c^{+} cells expressing CD86 (Figure 4.1 G and 4.1 H respectively) and MHCΠ (Figure 4.1 C and 4.1 D respectively) as well as CD11c^{+} cells expressing CD80 (Figure 4.1 F) were significantly higher in TNFRp75$^{-/-}$ mice compared to WT mice after 21 days. CD11b^{+}CD80^{+} cells were similarly, although not significantly, higher in TNFRp75$^{-/-}$ mice (Figure 4.1 E). With progressive infection, the overall presence of CD11b^{+}CD80^{+}-, CD11b^{+}CD86^{+}- and CD11b^{+}MHCΠ^{+}- as well as CD11c^{+}CD80^{+}-, CD11c^{+}CD86^{+}- and CD11c^{+}MHCΠ^{+} was reduced and similar expression levels were noted in both WT- and TNFRp75$^{-/-}$ mice at 28 days post-infection.

We confirmed our *in vivo* findings in culture stimulation assays where bone marrow derived dendritic cells from WT- and TNFRp75$^{-/-}$ mice were infected with *M. tuberculosis.* Expression of CD80, CD86 and MHCΠ on CD11c^{+} was measured and compared after 4 hours and we found increased numbers of CD11c^{+}MHCΠ^{+}-, CD11c^{+}CD80^{+}- and CD11c^{+}CD86^{+} populations in TNFRp75$^{-/-}$ stimulated cultures.

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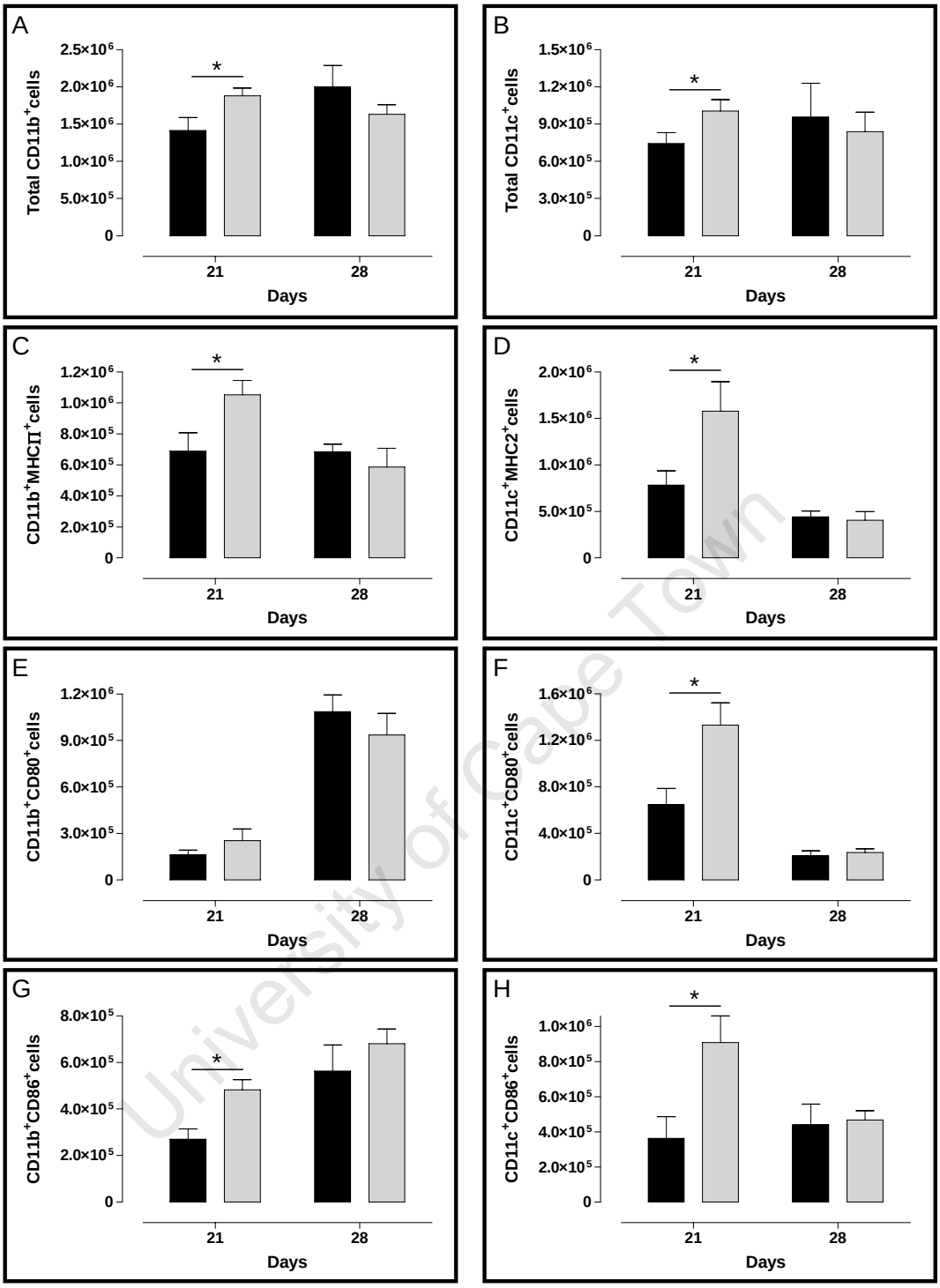

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Figure 4.1: Increased Recruitment of Activated Pulmonary CD11b$^+$- and CD11c$^+$ Cells. WT- (closed bars) and TNFRp75$^{-/-}$ mice (grey bars) were infected at 50-100 CFU with *M. tuberculosis*. Whole lungs were harvested at 21 and 28 days post-infection and CD11b$^+$- (A) and CD11c$^+$ (B) cell numbers and activation was assessed on single cell suspensions generated from whole lungs by staining with anti-CD11b (C, E and G) or anti-CD11c monoclonal antibody (D F and H) and double staining with anti-CD11b or anti-CD11c monoclonal antibody (C) and anti-CD80 (E), anti-CD86 (F) or anti-MHCII (D). The results show an increased recruitment of activated CD11b$^+$- and CD11c$^+$ cells at 21 days post-infection. The data points are the mean ± SEM of 4 mice per time point and are representative of one of two similar experiments. Significant differences (* $p<0.05$) were determined using ANOVA.

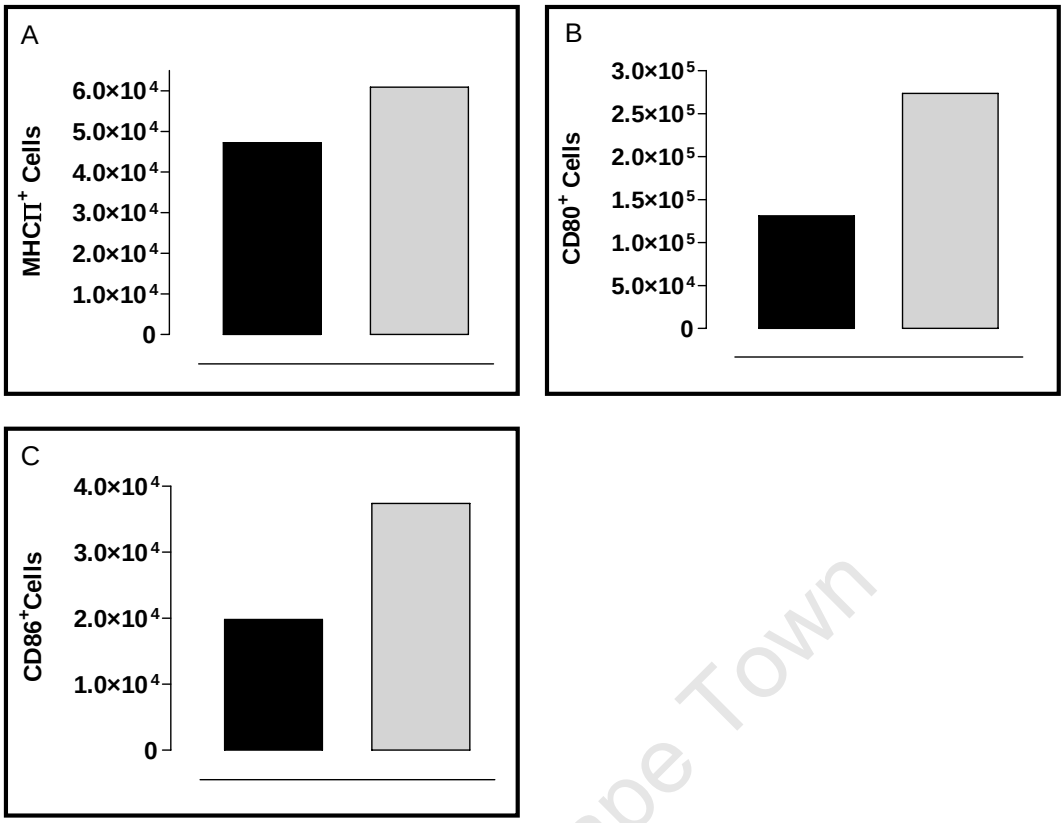

Figure 4.2: Increased Activation of Dendritic Cells *in vitro*. Bone marrow derived dendritic cells were cultured from WT (closed bars) and TNFRp75$^{-/-}$ mice (grey bars) and infected at an MOI of 5:1 with *M. tuberculosis*. Results showed an increase in the number of CD11c^{+}MHCII^{+} (A), CD11c^{+}CD80^{+} (B) and CD11c^{+}CD86^{+} (C) dendritic cells in TNFRp75$^{-/-}$ mice compared to WT control mice. Experiments were preformed in duplicate and are representative of 2 similar expreriments.

Together the data confirms that TNFRp75 regulates CD80, CD86 and MHCII expression during *M. tuberculosis* infection, thereby regulating immune function.

4.1.2 TNFRp75 Deficient Dendritic Cells Show Enhanced *In vitro* IL-12p40 Production.

The early induction of IL-12, once immature DC's become infected, is a critical requirement of DC migration to DLN's. This was supported by studies in which mice deficient for IL-12p40 were defective for DC migration and unable to activate naïve T cells in DLN (Khader *et al*., 2006). The production of IL-12p40 by dendritic cells once in the draining lymph node in conjunction with memory T cell/ NK cell derived IFNγ is crucial for the establishment of a strong T_H1 environment (Flynn and Chan, 2001; de Jong *et al*., 1997; Cooper *et al.,* 1997). Furthermore, Dodge *et al*., (2003) demonstrated that IL-6 negatively regulates IL-12 production

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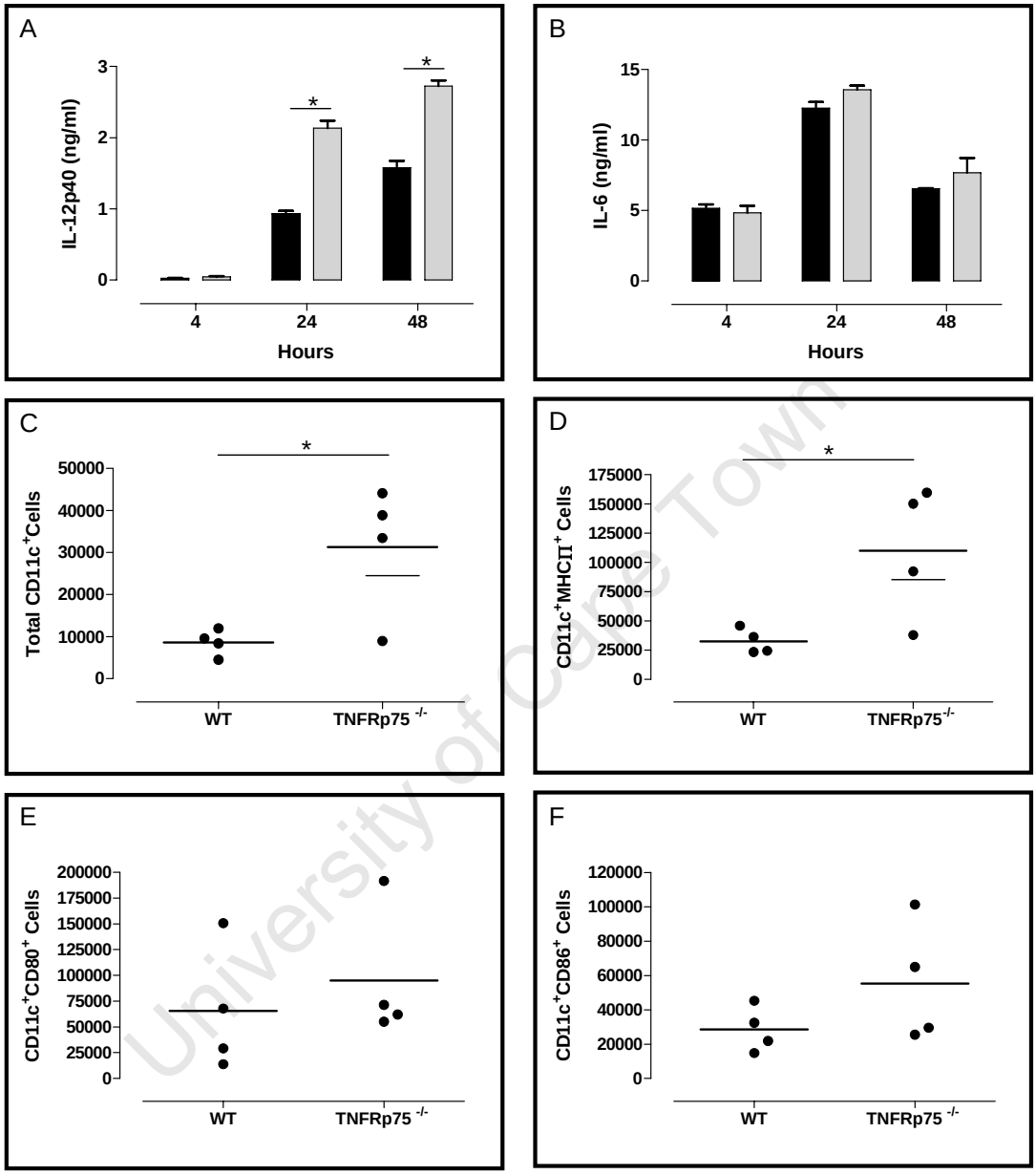
and proposed that the T_H2 cytokine environment observed in the lung is due to DC mediated inhibition of T_H1 responses via IL-6 production.



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<u>Figure 4.3:</u> Increased Dendritic cell IL-12p40 Production is Associated with Increased Numbers of Dendritic Cells in the Lymph Nodes of TNFRp75$^{-/-}$ mice. Bone marrow derived dendritic cells from WT- (closed bars, A and B) and TNFRp75$^{-/-}$ mice (grey bars, A and B) were infected at an MOI of 5:1 with *M. tuberculosis* and IL-12p40 (A) and IL-6 (B) expression was analysed by ELISA. In C –F WT (closed bars) and TNFRp75$^{-/-}$ mice (grey bars) were infected at 50-100 CFU with *M. tuberculosis*. Lymph nodes were harvested at 28 days post-infection and dendritic cell numbers and activation was assessed by staining with anti-CD11c (C) monoclonal antibody and either anti-CD80 (E), anti-CD86 (F) or anti-MHCII (D). The results show an increased expression of IL-12p40 (A) but similar levels of IL-6 (B) in TNFRp75$^{-/-}$ dendritic cells compared to WT dendritic cells which corresponds to a significant increase in CD11c^{+} cells (C) as well as CD11c^{+}MHCII^{+} (D) activated cells in the lymph nodes. The data points on day 28 are the mean ± SEM of 4 mice per group. Significant differences (* $p<0.05$) were determined using ANOVA.

To understand the effect of TNFRp75 on dendritic cell function, we measured the ability of dendritic cells to synthesise IL-12 and IL-6 during *M. tuberculosis* infection. Bone marrow derived dendritic cells from both WT- and TNFRp75$^{-/-}$ mice, exposed to *M. tuberculosis* at a ratio of 5:1 showed a time dependent increase in IL-12p40 synthesis (Figure 4.3A), while maximum IL-6 production was reached after 24 hours. Interestingly we found that IL-12p40 synthesis was significantly higher at 24 and 48 hours ($p< 0.05$) in the supernatants of TNFRp75$^{-/-}$ cultures whereas no differences were observed in IL-6 synthesis.

We next postulated that since dendritic cell-derived IL-12 is important for the migration of dendritic cells to the draining lymph nodes, TNFRp75 dependent regulation of IL-12 synthesis may influence the dendritic cell content of mediastinal lymph nodes during aerosol *M. tuberculosis* infection *in vivo*. Lymph nodes were harvested from WT- and TNFRp75$^{-/-}$ mice on day 21 and 28 post *M. tuberculosis* infection and the number of CD11c^{+} cells analysed by flow cytommetry. Figure 4.3 C shows that indeed the total number of CD11c^{+} dendritic cells was significantly higher in TNFRp75$^{-/-}$ mice compared to WT mice and further revealed that, not only are there more CD11c^{+} cells, but also that the number of activated CD11c^{+} cells expressing CD86 (Figure 4.3 F) was higher and the number of CD11c^{+}MHCII^{+} cells was significantly higher ($p< 0.05$) in the lymph nodes of TNFRp75$^{-/-}$ mice on day 28 post-infection (Figure 4.3 D).

Together this data demonstrates that TNFRp75$^{-/-}$ dendritic cells produce increased levels of IL-12 which is associated with increased migration of activated CD11c^{+} cells to lung draining lymph nodes during *M. tuberculosis* infection.

4.2 T cell Recruitment and Activation

4.2.1 Increased Number of CD4^{+}CD44^{+} Lymph Node Cells in TNFRp75$^{-/-}$ Mice

Dendritic cells are crucial for the initiation of an adaptive T cell response, supported by studies which showed that depletion of CD11c^{+} dendritic cells causes a delay in the initiation of an antigen-specific CD4^{+} T cell response leading to poor control of *M. tuberculosis* replication (Behar *et al.*, 2005). Interactions between CD80 and CD86 ligands on antigen presenting cells, and CD28 receptors on T cells are the major costimulatory signals for the activation of T cells resulting in their expansion,

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differentiation and survival that induces an effective antibody and cellular immune response (Collins *et al*., 2005). In 1995, Engleman *et al* demonstrated that CD86 plays a critical role in dendritic cell dependent induction of primary $CD4^+$ T cell responses to soluble antigens but that CD86 expression in conjunction with additional molecules was required for contribution to the functions of dendritic cells in $CD4^+$ T cell activation. Furthermore, antigen specific IFNγ production is a major indicator of T_H1 cell activation (Becher *et al.,* 1999). In view of our observations that TNFRp75 inhibits recruitment and activation of dendritic cells to the draining lymph nodes during *M. tuberculosis* infection, we asked whether increased recruitment of activated dendritic cells translates into an enhanced T_H1 *M. tuberculosis* specific immune response. Here we measured $CD4^+CD44^+$ expression on isolated cells from the lungs and lymph nodes to assess cellular activation and further evaluated *M. tuberculosis* specific IFNγ production as an indicator of immune function. WT- and $TNFRp75^{-/-}$ mice were infected with *M. tuberculosis* via aerosol inhalation at a dose of 50-100cfu/lung and analysis was performed on lungs and lymph nodes at 21 and 28 days post-infection. We found that recruited $CD4^+$ pulmonary T cells expressing CD44 increased in both WT- and $TNFRp75^{-/-}$ mice with progressive infection from day 21 to day 28 post-infection (Figure 4.4 A). Interestingly the number of $CD4^+CD44^+$ cells was significantly lower in $TNFRp75^{-/-}$

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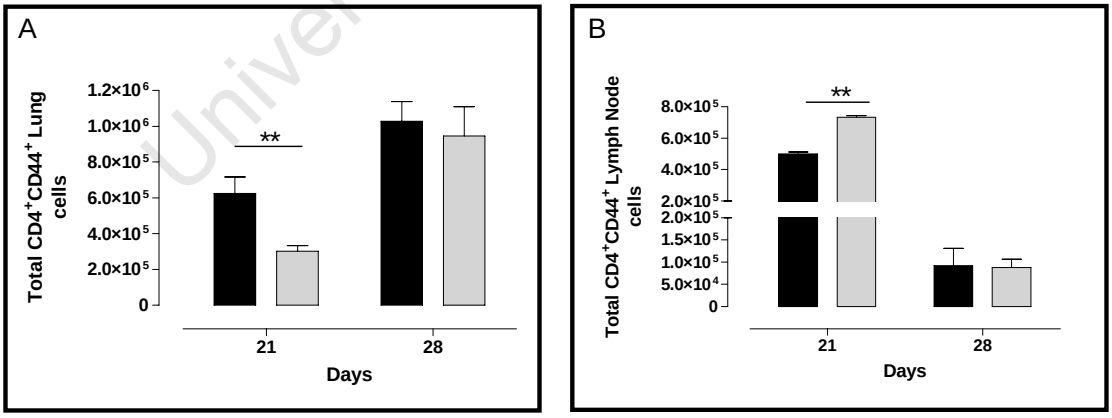

Figure 4.4: $CD4^+CD44^+$ Cells Are Increased in Lymph Nodes After *M. tuberculosis* Infection. Whole lung and lymph node cells were isolated from WT- (closed bars) and $TNFRp75^{-/-}$ mice (grey bars) after 21 and 28 days *M. tuberculosis* infection and co-stained with APC conjugated anti-CD4 monoclonal antibody and PE conjugated anti-CD44 monoclonal antibody. The data indicates a significantly lower number of $CD4^+CD44^+$ T cells in the lungs but a significantly higher recruitment of $CD4^+CD44^+$ T cells in the lymph nodes of $TNFRp75^{-/-}$ mice compared to WT mice at 21 days post infection. The data points are the mean ± SEM of recruited cells of 4 mice per time point and are representative of one of three similar experiments. Significant differences (** $p<0.01$) were determined using ANOVA.

mice at day 21 but similar to WT day 28 post-infection. We further measured $CD4^+CD44^+$ expression in isolated lymph node single cell preparations at 21 and 28 days post-infection and found maximum expression levels at 21 days post-infection in both WT- and TNFRp75$^{-/-}$ mice. Important to note was that the $CD4^+CD44^+$ T cell population present in TNFRp75$^{-/-}$ mice was significantly higher compared to WT animals (Figure 4.4 B). The data thus confirms that the increased number of activated dendritic cells present in the lymph nodes during *M. tuberculosis* infection are indeed able to present antigen and thus activate an increased number of T cells in the lymph nodes.

4.2.2 Pulmonary *M. tuberculosis* specific IFNγ Synthesis is Enhanced in *M. tuberculosis* Infected TNFRp75$^{-/-}$ Mice.

We next asked whether the functional response of recruited T cells was regulated by TNFRp75 in view of the differences in $CD4^+CD44^+$ expression profiles between WT- and TNFRp75$^{-/-}$ mice. Here we measured *M. tuberculosis* specific IFNγ synthesis in response to ESAT6 or H37Rv restimulation as an indication of T cell function.

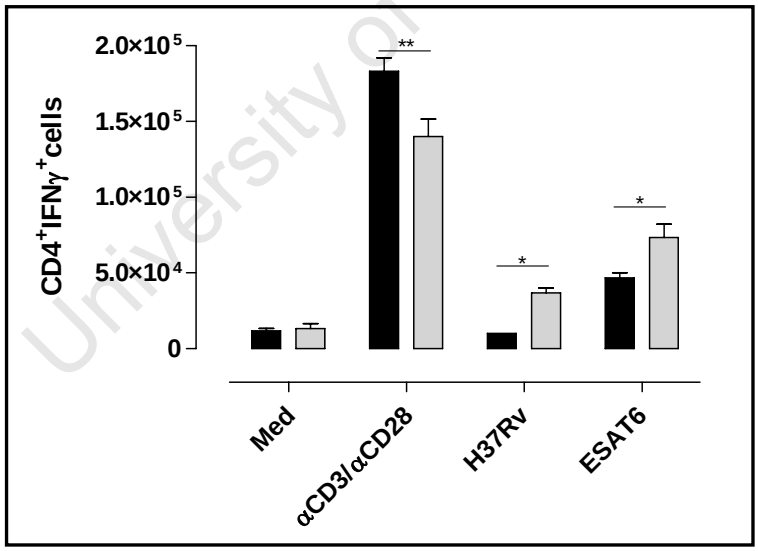

Figure 4.5: Enhanced *M. tuberculosis* Specific IFNγ Synthesis by Pulmonary $CD4^+$ T cells in TNFRp75$^{-/-}$ Mice During *M. tuberculosis* infection. Cells from whole lungs were isolated from *M. tuberculosis* infected WT- (closed bars) and TNFRp75$^{-/-}$ mice (grey bars) at 21 days post-infection and restimulated with non-specific antiCD3/antiCD28 or *M. tuberculosis* specific ESAT6 or H37Rv for 6 hours with Brefaldin. Cells were then co-stained with APC conjugated anti-CD4 monoclonal antibody and FITC conjugated anti-IFNγ monoclonal antibody. The results show significantly lower intracellular IFNγ expression following non-specific antiCD3/antiCD28 restimulation but a significantly higher IFNγ expression after *M. tuberculosis* specific ESAT 6 or H37Rv restimulation in TNFRp75$^{-/-}$ mice compared to WT control mice. The data points are the mean ± SEM of the $CD4^+IFN\gamma^+$ cells of triplicate staining of pooled sample from 4 mice and are representative of one of two similar experiments. Significant differences (*$p<0.05$, ** $p<0.01$) were determined using ANOVA.

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We found similar low levels of IFNγ synthesis in unstimulated $CD4^+$ T cells from both WT- and $TNFRp75^{-/-}$ mice. However, non-specific antiCD3/antiCD28 stimulation induced notable levels of IFNγ synthesis by $CD4^+$ T cells and interestingly, $CD4^+IFN\gamma^+$ cell populations were significantly reduced in the absence of TNFRp75. In contrast, *ex vivo* restimulation with ESAT 6 or live *M. tuberculosis* demonstrated higher $CD4^+IFN\gamma^+$ levels in $TNFRp75^{-/-}$ cultures.

Our data therefore clearly indicates that TNFRp75 contributes to an overall non-specific IFNγ production by $CD4^+$ T cells but inhibits induction of *M. tuberculosis* specific IFNγ synthesis by $CD4^+$ T cells.

4.3 Shedding of TNFRp75 from the Cell Surface is Induced During *M. tuberculosis* Infection.

To understand the mechanism by which TNFRp75 regulates immune function we postulated that recognition of *M. tuberculosis* induces release of membrane bound TNFRp75 which binds and inhibits bioactive TNFα *in vivo*. TNFα/TNFRp75 complexes would render TNFα unavailable for TNFRp55 cell mediated functions and limits APC activation. This theory is supported in part by *in vitro* macrophage culture studies which demonstrated that TNFRp55 and TNFRp75 were shed from cell surfaces into surrounding medium subsequent to *M. tuberculosis* infection in WT mice. Subsequent TNFRp75 complex formation with soluble TNFα, reduced TNFα bioactivity and TNFα mediated macrophage apoptosis (Balcewicz-Sablinska *et al*., 1998). It is further supported by *in vivo* studies, in which TNFRp75 cell surface expression was upregulated in response to intraperitoneal *M. avium* infection and the level of soluble TNFRp75 increased (Corti *et al.,*1999) which has the potential to neutralize TNFα activity.

Thus, to understand the regulatory role of TNFRp75 in immune function during *M. tuberculosis* infection, we first assessed TNFRp75 cell surface expression in culture. Bone marrow derived macrophages from WT mice were generated and infected at an MOI of 10:1 for 4 hours prior to labelling cells for TNFRp75 specific expression and analysis by flow cytommetry. Uninfected cells or cells labelled with an irrelevant monoclonal antibody acted as appropriate controls. The results clearly show TNFRp75 expression on cell surfaces of uninfected macrophages which was

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reduced on exposure to *M. tuberculosis* (Figure 4.6). We postulated that the decrease in TNFRp75 expression was either due to internalisation or release from the cell surface.

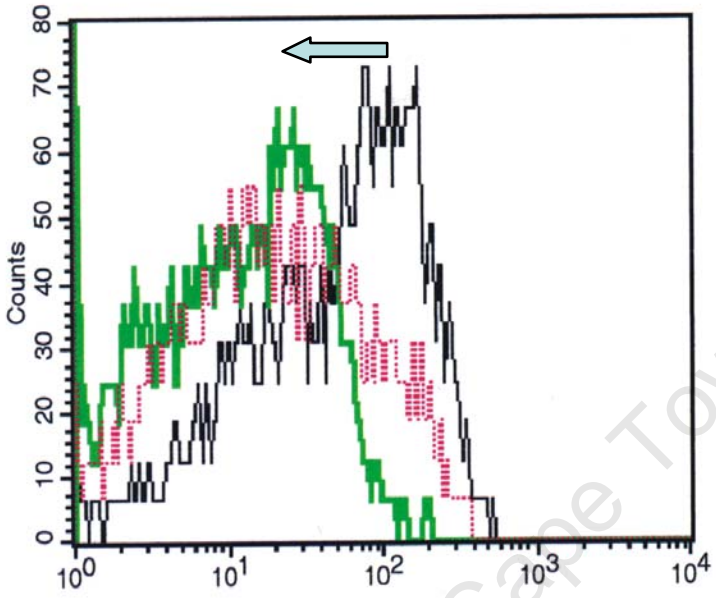

Figure 4.6: TNFRp75 Cell Surface Expression is Reduced During *M. tuberculosis* Infection *In vitro*. Bone marrow derived macrophages from WT mice were infected at an MOI of 10:1 with *M. tuberculosis* with uninfected cells used as a positive control. Surface expression of TNFRp75 was assessed by staining with an anti-TNFRp75 monoclonal antibody. A clear shift is noted in the peak values between *M. tuberculosis* stimulated and unstimulated cells. The data therefore indicates that *M. tuberculosis* infection reduces the surface expression of TNFRp75 similar to the IgG –ve control (experiments were done in triplicate, Green = Isotype control, Black = Medium with anti-TNFRp75, Red = *M. tuberculosis* with anti-TNFRp75).

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Therefore, we next assessed the kinetics of TNFRp75 release in culture supernatants of *M. tuberculosis* infected macrophages and dendritic cells from WT- and TNFRp75$^{-/-}$ mice. We found a time dependent release of TNFRp75 in both WT macrophages (Figure 4.7 A) and dendritic cells (Figure 4.7 B) and confirmed the absence of TNFRp75 in the TNFRp75$^{-/-}$ cell cultures. In addition we compared the kinetics of TNFRp55 release in the culture supernatants of *M. tuberculosis* infected macrophages and dendritic cells of WT- and TNFRp75$^{-/-}$ mice. Previous studies have found that soluble TNFRp55 can inhibit TNFα mediated effects, although to a lesser extent than TNFRp75 (Balcewicz-Sablinska *et al*., 1998). We found that exposure to *M. tuberculosis* induces a linear increase in TNFRp55 release from both macrophages (Figure 4.7 C) and dendritic cells (Figure 4.7 D) over 72 hours in both

WT- and TNFRp75$^{-/-}$ strains. Interestingly, we observed that TNFRp55 release in macrophages but not dendritic cells was significantly reduced in TNFRp75$^{-/-}$ mice compared to WT control mice. This reduction in the release of TNFRp55 in the absence of TNFRp75 expression may contribute to the activation status of APC's, since less soluble TNFRp55 in addition to the lack of TNFRp75 in this mouse strain results in less soluble receptor to complex with bioactive TNFα thus rendering it inactive, with the possibility that bioactive TNFα in this strain would be increased. In addition, less TNFRp55 shedding indirectly implies higher cell surface expression which together with increased availability of bioactive TNFα would facilitate increased TNFα signalling though TNFRp55 for improved APC activation.

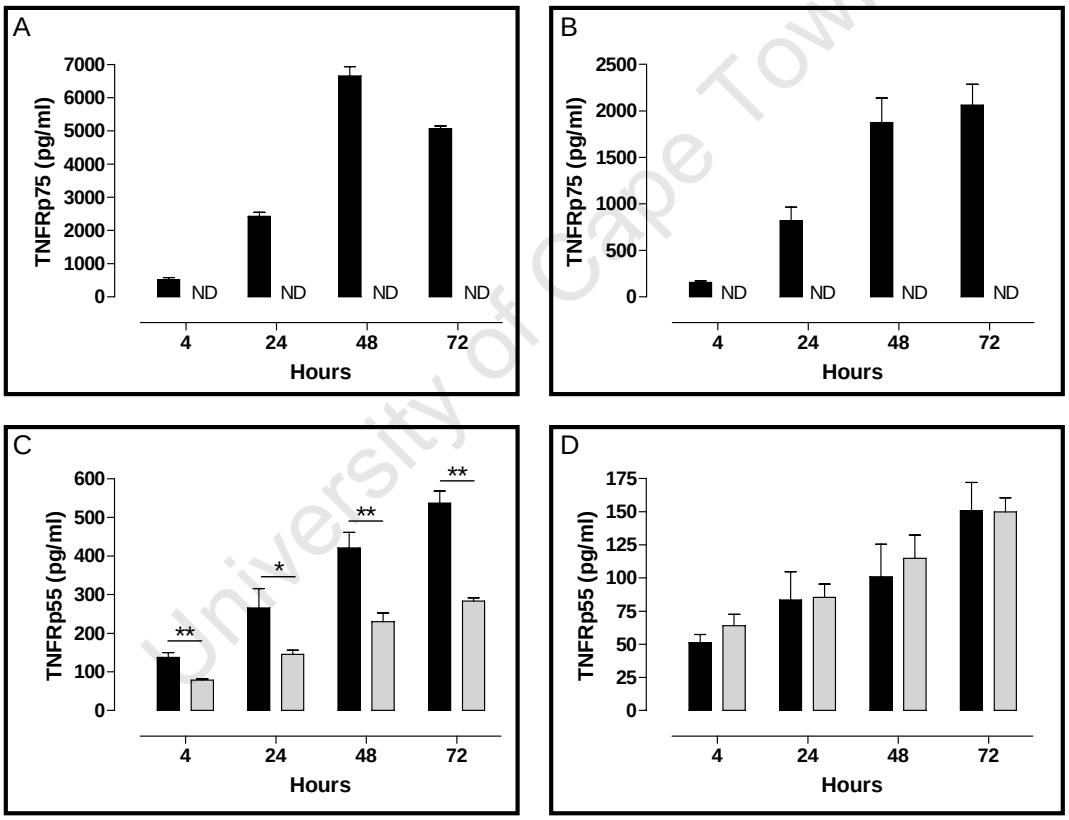

Figure 4.7: TNFRp75 and TNFRp55 Release in *M. tuberculosis* Infected Macrophage and Dendritic Cell Cultures. WT- (closed bars) and TNFRp75$^{-/-}$ (grey bars) bone marrow derived macrophages (A and C) and dendritic cells (B and D) were infected with *M. tuberculosis* at an MOI of 5:1 and the release of TNFRp55 and TNFRp75 into the culture medium was measured by ELISA. The results indicate a time dependent shedding of TNFRp55 and TNFRp75 in both macrophages and dendritic cells with a higher release of both TNFRp55 and TNFRp75 in macrophages compared to dendritic cells. A comparison of soluble TNFRp55 levels in WT- and TNFRp75$^{-/-}$ macrophages and dendritic cells reveals similar TNFRp55 shedding in dendritic cells in both strains (D) but a significantly reduced release of TNFRp55 in TNFRp75$^{-/-}$ macrophages (C). The data points are the mean ± SEM of quadruplicate experiments and are representative of one of two similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA. (ND= not detectable).

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To ascertain whether TNFR release observed in cultures is representative of events observed *in vivo,* we challenged WT-, TNFRp55$^{-/-}$- and TNFRp75$^{-/-}$ mice with *M. tuberculosis* via aerosol inhalation at a dose of 50-100cfu/lung and assessed TNFRp55 and TNFRp75 levels in supernatants of lung homogenates at defined time points. The results clearly showed that TNFRp75 (Figure 4.8 A) and TNFRp55 (Figure 4.8 B) are shed subsequent to *in vivo M. tuberculosis* infection and confirm that TNFRp55 and TNFRp75 were non-detectable in TNFRp55$^{-/-}$- and TNFRp75$^{-/-}$ mice respectively. Interestingly we measured lower TNFRp55 levels in TNFRp75$^{-/-}$ mice compared to WT mice at all time points, confirming observations in cultures that TNFRp75 may regulate TNFRp55 shedding. We further found that TNFRp75 release was significantly lower ($p< 0.01$) in TNFRp55$^{-/-}$ mice compared to WT mice. Thus in addition to the absence of TNFRp75 shedding, the TNFRp75$^{-/-}$ mouse also showed a significantly lower TNFRp55 ($p< 0.01$) shedding in response to *in vivo M. tuberculosis* infection, similar to TNFRp55 shedding observations in *in vitro* macrophage cultures (Figure 4.8 B). Similarly TNFRp55 shedding in TNFRp55$^{-/-}$ mice was absent and these mice displayed significantly lower TNFRp75 release *in vivo* after *M. tuberculosis* infection (Figure 4.8 A).

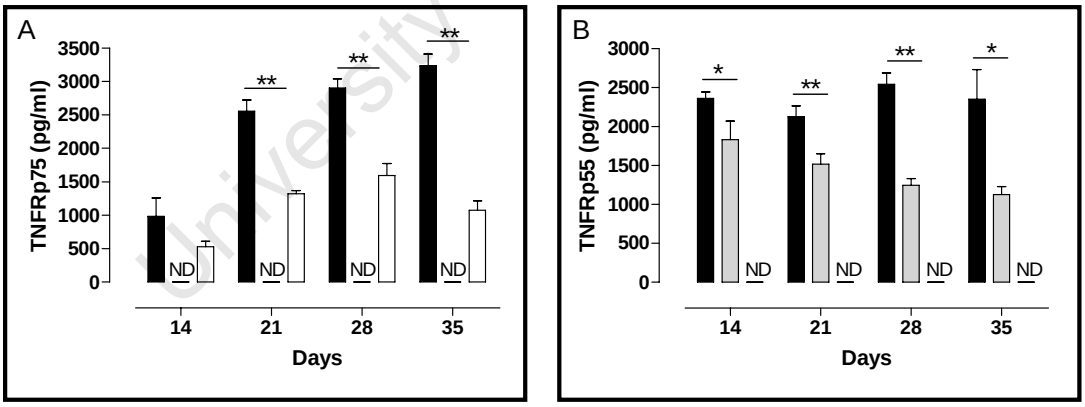

Figure 4.8: TNFRp75 and TNFRp55 Release During *M. tuberculosis* Infection *in vivo.* WT- (closed bars), TNFRp75$^{-/-}$- (grey bars) and TNFRp55$^{-/-}$ mice (open bars) were infected with 50-100 CFU with *M. tuberculosis*. The release of TNFRp55 and TNFRp75 was assessed in lung homogenates by ELISA. The results show a time dependent increase in TNFRp75 shedding with significantly less TNFRp75 release in TNFRp55$^{-/-}$ mice compared to WT mice (A) and a similar lower release of TNFRp55 in TNFRp75$^{-/-}$ mice (B). The data points are the mean ± SEM of 4 mice per time point and are representative of one of two similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA. (ND= not detectable).

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Together this data demonstrates that both TNFRp55 and TNFRp75 are indeed released from the cell surface during host challenge by *M. tuberculosis*. In addition it shows that the amount of soluble receptor available to bind to bioactive TNFα is greatly reduced in both TNFRp55$^{-/-}$- and TNFRp75$^{-/-}$ mice.

4.4 Anti-TNFRp75 Treatment of WT Mice Alters Their Phenotype to Resemble that of TNFRp75$^{-/-}$ Mice.

Since our studies have shown an increased activation of TNFRp75$^{-/-}$ APC's and the ability of TNFRp75 to be shed, we went on to assess the direct role of TNFRp75 in the activation of APC's under controlled *in vitro* conditions. Bone marrow derived dendritic cells from WT- and TNFRp75$^{-/-}$ mice were infected at an MOI of 5:1 with *M. tuberculosis* cultured in the presence or absence of anti-TNFRp75 using medium as a control. Bioactive TNFα, IL-12p40 and MHCΠ expression were used as markers of dendritic cell activation.

We found that upon *M. tuberculosis* infection TNFRp75$^{-/-}$ dendritic cells are more activated than WT dendritic cells. TNFRp75$^{-/-}$ dendritic cells produced significantly higher levels of bioactive TNFα compared to WT dendritic cells after *M. tuberculosis* challenge (Figure 4.9 A). More significantly, treatment of the WT dendritic cell with an anti-TNFRp75 blocking antibody resulted in a bioactive TNFα response similar to that of TNFRp75$^{-/-}$ dendritic cells thus directly associating soluble TNFRp75 with bioactive TNFα production. Similarly, *M. tuberculosis* infection of TNFRp75$^{-/-}$ dendritic cells resulted in an increased expression of IL-12p40 compared to similarly infected WT mice which was partially abrogated upon the treatment of WT dendritic cells with the anti-TNFRp75 blocking antibody thus directly linking TNFRp75 with the regulation of IL-12p40 release. The final aspect of dendritic cell activation which we addressed in this study was MHCΠ expression. The results indicated a significantly higher ($p < 0.05$) expression of MHCΠ by TNFRp75$^{-/-}$ dendritic cells compared to WT dendritic cells. Treatment with the anti-TNFRp75 neutralizing antibody resulted in the MHCΠ expression by WT dendritic cells increasing to a level comparable to that in TNFRp75$^{-/-}$ dendritic cells, thus directly implicating TNFRp75 in dendritic cell expression of MHCΠ.

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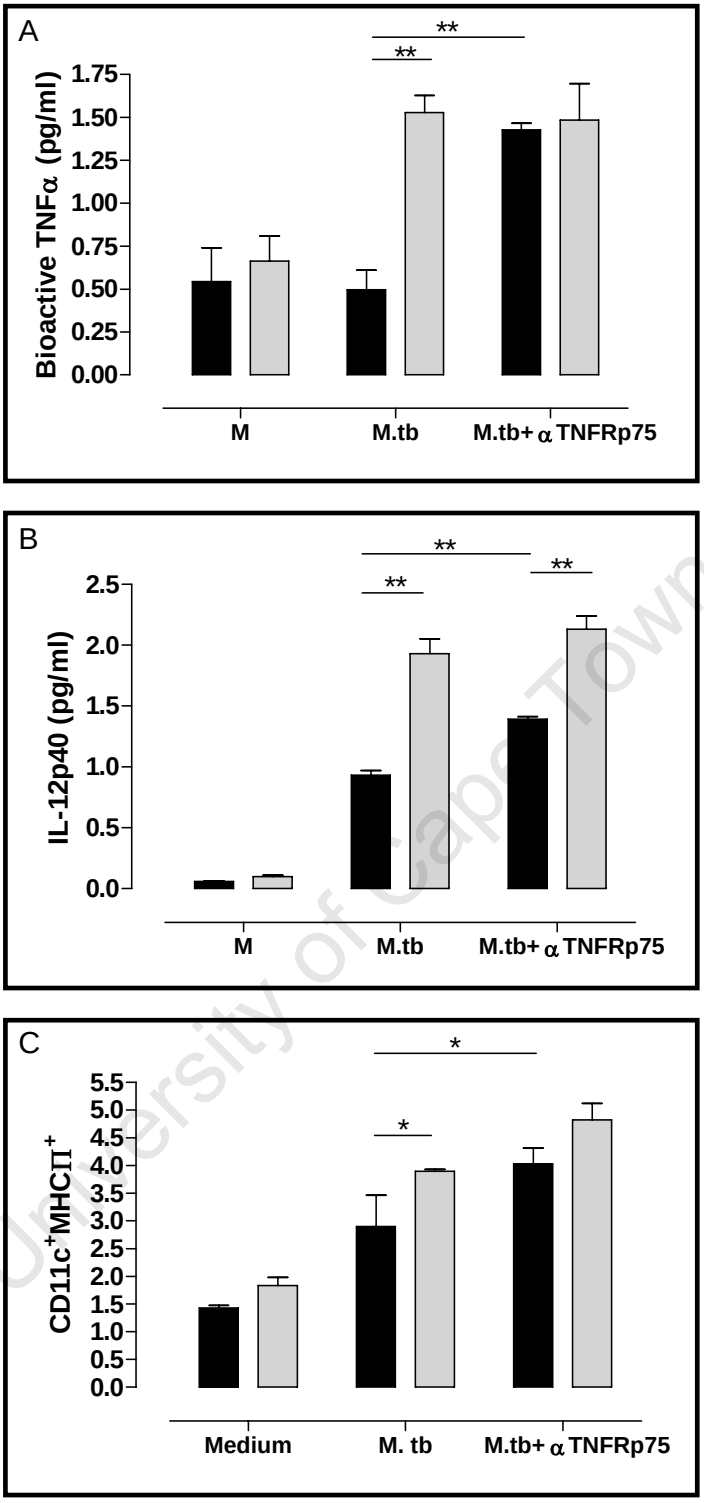

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Figure 4.9: Neutralization of TNFRp75 in WT Dendritic Cells Results in Increased Cellular Activation. Cultured bone-marrow derived dendritic cells from WT- (solid bars) and TNFRp75$^{-/-}$ mice (grey bars) were infected at an MOI of 5:1 with *M. tuberculosis* either in the presence or absence of TNFRp75 and cellular activation status assessed by measuring bioactive TNFα (A), IL-12p40 (B) synthesis and MHCII (C) expression. Briefly the data indicates that anti-TNFRp75 treatment of WT mice increases bioactive TNFα availability and MHCII expression similar to the levels seen in TNFRp75$^{-/-}$ mice, while IL-12p40 is significantly increased although not equivalent to the amount in TNFRp75$^{-/-}$ mice. The data points are the mean ± SEM of experiments done in triplicate and are representative of one of two similar experiments. Significant differences (* $p<0.05$, ** $p<0.01$) were determined using ANOVA.

In summary TNFRp75$^{-/-}$ dendritic cells are more activated than WT dendritic cells following *M. tuberculosis* stimulation. Blocking TNFRp75 in WT mice thus results in an increased activation of WT dendritic cells which is similar to that of TNFRp75$^{-/-}$ dendritic cells. This finding is of significant importance to this study as verification that the increased activation observed in TNFRp75$^{-/-}$ APC's which is the key to increased survival of TNFRp75$^{-/-}$ mice, is in fact a direct result of the lack of the TNFRp75.

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Chapter 5:

Discussion

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Chapter 5: Discussion

The role of TNFα in protective immunity against mycobacteria has been extensively studied. It has previously been shown that TNFα deficiency results in an abhorrent T_H1 immune response resulting in decreased survival (Kaneko *et al*., 1999; Jacobs *et al*., 2000; Roach *et al*., 2002; Bean *et al.,* 1999) and that signaling through TNFRp55 is critical for mediating specific immune protection. TNFRp55 mediated immunity is required for protection against a broader spectrum of intracellular infections that include *Histoplasma capsulatum* (Allendoefer and Deepe Jr, 2000), *Listeria monocytogenes* (Endres *et al.,* 1997), *M. avium* (Corti *et al*., 1999), *M. Bovis* BCG (Jacobs *et al.,* 2000) and *M. tuberculosis* (Flynn *et al.,* 1995; Roach *et al.,* 2002) which was substantiated by challenge studies using TNFRp55$^{-/-}$ mice.

In this study the roles of TNFRp55 and TNFRp75 were investigated using gene deleted mice challenged with *M. tuberculosis* by aerosol inhalation. The findings reported here are novel describing the *in vivo* function of TNFRp75 in immune regulation during tuberculosis. We further defined and report on the importance of TNFRp55 in the host immunity after a natural route of challenge as opposed to previous reports in which animals were infected by intravenous infection. The administration strategy is particularly important in view of reports that mycobacterial virulence and subsequent host immune responses are pertinently influenced by different routes of infection (North, 1995).

The phenotype of TNFRp55$^{-/-}$ mice infected by aerosol inhalation with *M. tuberculosis* was characterized by impaired protective immunity with early mortality associated with an increase in weight loss and significantly higher bacilli burdens than wild type control mice. These observations are supported by previous studies in which intracellular pathogens, particularly intravenous *M. tuberculosis* and *M. Bovis* BCG mycobacterial infections resulted in premature death accompanied by significantly higher bacilli burdens in TNFRp55$^{-/-}$ infected mice (Allendoefer and Deepe Jr, 2000; Endres *et al.,* 1997; Corti *et al*., 1999; Jacobs *et*

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al., 2000; Flynn *et al.,* 1995; Roach *et al.,* 2002) and confirms its fundamental importance in immune function.

The role of TNFRp75 in host immune function during pathogen challenge was largely unexplored. Previous studies reported a protective role for TNFRp75 against *Histoplasma capsulatum* infection, where TNFRp75 deficiency resulted in reduced IFNγ production and premature mortality (Allendoerfer and Deepe Jr., 2000). In contrast, a study by Peschon *et al.,* (1998) demonstrated no biological role for TNFRp75 in host survival subsequent to *Listeria monocytogenes* infection. In this study Peschon *et al.,* (1998) argued that TNFRp75 plays a dominant role in suppressing TNF-mediated inflammatory responses based on the exacerbated pulmonary inflammation as well as increased endotoxin induced serum TNFα levels in TNFRp75$^{-/-}$ mice. However Lucas *et al.,* (1997) reported an inhibiting effect of TNFRp75 during cerebral malaria, where challenge with *Plasmodium falciparum* resulted in enhanced survival of TNFRp75$^{-/-}$ mice compared to either WT or TNFRp55$^{-/-}$ mice. In the current study the role of TNFRp75 was investigated under conditions where TNFRp55 was either expressed or was absent. We found that TNFRp55 is absolutely critical for survival as both TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice succumbed characterized by increased organ burdens and concomitant increased weight loss, a well documented symptom of *M. tuberculosis*. Interestingly, we found a small but repeatable delay in the death of TNFRp55$^{-/-}$ mice compared to TNFRp55/75$^{-/-}$ mice, suggesting a possible degree of protection afforded by TNFRp75. Nonetheless, TNFRp55 mediated immune function was non-redundant and could not be rescued by TNFRp75 expression. In contrast, chronic survival of TNFRp75$^{-/-}$ mice was significantly longer than wild type mice, thus suggesting that TNFRp75 expression inhibits immune function during *M. tuberculosis* challenge, much like the outcome that Lucas *et al.,* (1997) reported for cerebral malaria. Increased survival of TNFRp75$^{-/-}$ was accompanied by significantly lower organ burden, particularly in the lungs during acute and chronic infection. The reduction of bacilli burden as early as 8 days post-infection suggested that TNFRp75 may function during early innate immunity. The lower bacilli load is indicative of a greater control over the *M. tuberculosis* infection in TNFRp75$^{-/-}$ mice compared to wild type mice. TNFRp75 dependent inhibition of the host immune

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system was first reported by Corti *et al*., (1999) using anti-TNFRp75 monoclonal antibodies. In this study Corti *et al.,* (1999) showed an increased TNFRp75 cell surface expression and increased levels of soluble TNFRp75 in response to intraperitoneal *M. avium* infection. In addition, mice treated with anti-TNFRp75 mAb prior to and subsequent to infection with *M. avium* showed a significantly lower bacilli burden by day 21 post-infection (Corti *et al*., 1999). Thus, in their studies these authors clearly illustrated an immune benefit to the host during neutralization of TNFRp75. In addition, an inhibitory role for TNFRp75 in *M. tuberculosis* infection was supported by previous reports of Balcewicz-Sablinska *et al*., (1998) which demonstrated that TNFRp75 shedding is an important *M. tuberculosis* evasion mechanism particularly in virulent strains of *M. tuberculosis*. In these *in vitro* macrophage studies Balcewicz-Sablinska *et al*., (1998) showed that virulent H37Rv *M. tuberculosis* bacilli are able to evade alveolar macrophage apoptosis by TNFRp75 shedding, while less virulent H37Ra *M. tuberculosis* induces less TNFRp75 shedding and is less efficient at evading alveolar macrophage apoptosis. In these studies TNFRp75 shedding was shown to be mediated by IL-10 but the H37Rv virulence factor responsible is undefined. It was further demonstrated that TNFRp75 forms a complex with soluble TNFα thus reducing the TNFα bioactivity and the subsequent TNFα mediated immune responses (Balcewicz-Sablinska *et al*., 1998). Therefore, taken together, these reports and our findings unequivocally support a regulatory role for TNFRp75 in immune function during mycobacterial infection. TNFRp75 mediated regulation of immune function is protective against excessive inflammation and is based on the Kd between soluble TNFRp75 and soluble TNFα. Since affinity plays a crucial role, so too does the concentration of both soluble molecules in the circulation. As the soluble TNFα concentrations drop after acute infection a dissociation of the TNF/TNFRp75 complex would occur thus releasing additional bioactive TNFα. This system not only maintains active circulating bioactive TNFα levels for longer, but also buffers peak TNFα levels during early acute infection. In theory this system affords better protection of the host against damage by its own immune response to a disease. In the case of *M. tuberculosis* infection however, where a rapid inflammatory response is required, buffering of the peak TNFα level during early acute infection results in a delay in the control of bacilli replication. Buffering of

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TNF peak values manifests in early mortality in WT mice in comparison to TNFRp75$^{-/-}$ mice.

To understand how TNFRp75 influences immune regulation during *M. tuberculosis* infection, we investigated and compared several immune parameters in the context of TNFRp75 expression or its absence. Here we noted that cellular recruitment was essential for protective immunity against mycobacterial infections (Roach *et al.,* 2002; Jacobs *et al*., 2000; Bean *et al*., 1999; Kaneko *et al*., 1999; Kindler *et al*., 1989) partly due to its importance in granuloma formation; a critical component in host immunity with studies demonstrating that mice deficient for granuloma formation die prematurely (Ehlers *et al.,* 2000; Kindler *et al*., 1989). TNFα signaling mediates both cellular recruitment and subsequent granuloma formation in response to mycobacterial infections (Bean *et al*., 1999; Kindler *et al*., 1989; Jacobs *et al.,* 2000) where the absence of TNFα resulted in impaired formation of structured granulomas with few epithelial macrophages and significant necrosis (Kaneko *et al.,* 1999; Roach *et al.,* 2002). TNFRp55 signaling in itself has previously been shown, in both *M. tuberculosis* (Islam *et al.,* 2004) and other infection and inflammatory models (MacEwan 2002; Kim *et al*., 2001; Qin *et al.,* 2008) to be important for the activation of nuclear transcription factor NFκB which results in the synthesis of pro-inflammatory cytokines including TNFα. In other words TNFα signaling through TNFRp55 has a feedback loop which promotes efficient TNFα production. Furthermore as described here and previously (Senaldi *et al.,* 1996; Jacobs *et al.,* 2000), TNFRp55 acts as the major conduit of TNFα mediated function and an inability to signal through TNFRp55 yielded granuloma defects comparable to TNFα deficiency. Our analysis of the effects of TNFR function on cellular recruitment during *M. tuberculosis* infection revealed a TNFRp55 dependent regulation of CD11b^{+}-, CD4^{+}- and CD8^{+} cell recruitment as indicated by the increased levels of these cell types during end-stage infection in the absence of TNFRp55. In contrast TNFRp75$^{-/-}$ mice had a reduced presence of CD11b^{+}-, CD4^{+}- and CD8^{+} cells during late acute infection which were associated with a reduced bacilli burden as a consequence of enhanced control at this late (day 35) acute stage infection. TNFRp55 was also critical for the recruitment of CD11c^{+} cells with an absence of TNFRp55 resulting in an inability to recruit these cells. The

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reduction observed in cellular recruitment in TNFRp75$^{-/-}$ mice was possibly due to the increased control of *M. tuberculosis* infection in this strain compared to wild type mice, while excessive cellular infiltration in TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice was indicative of the lack of control established in these strains. Excessive cellular infiltration has previously been shown to lead to overproduction of pro-inflammatory cytokines with severe consequences at a cellular level that resulted in tissue destruction (Zganiacz *et al*., 2004) such as the tissue necrosis observed in the section staining of TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice in our studies. The organization of granulomas in TNFRp75$^{-/-}$ mice resembled the ordered structure characteristic of wild type granulomas, including the presence of macrophages. The fewer, smaller granulomas and reduced cellular recruitment in TNFRp75$^{-/-}$ mice during late acute stage of infection could be attributed to a reduced stimulus resulting from a lower bacilli burden in this strain and does not reflect an inability of these mice to recruit immune cells or form granulomas at the site of infection. This was confirmed by the fact that during early acute phase of infection (up to day 21) TNFRp75$^{-/-}$ mice have an enhanced T_H1 immune response including increased numbers of CD11b^{+} cells. Nonetheless, a study by Kim and Teh (2001) has shown a reduction in T cell proliferation in response to T cell receptor agonist in TNFRp75$^{-/-}$ mice, suggesting that TNFRp75 is a requirement for optimum T-cell responses. Although significant T cell recruitment was observed in TNFRp55$^{-/-}$ and TNFRp55/75$^{-/-}$ mice, structured granulomas were absent and few epitheloid macrophages were noted. In addition, severe necrosis was observed in the absence of TNFRp55 mediated signaling. The lack of classical granuloma formation in TNFRp55$^{-/-}$ mice conforms to previous data (Senaldi *et al.,* 1999) and therefore our findings together with other reported studies indicate that TNFα signaling through TNFRp55 and not TNFRp75 is important for granuloma formation and organized cellular recruitment.

In order to understand the immune mechanisms which confer increased protection in TNFRp75$^{-/-}$ mice, we explored the association of TNFRp75 on cellular responses during *M. tuberculosis* infection. It is well documented that macrophages are the primary target cells during *M. tuberculosis* challenge and provide the first line of defense against such intracellular infections (Glickmann and Jacobs, 2001; Hope *et*

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al., 2004). In addition, immature dendritic cells act as sentinels for foreign antigens and microbial pathogens, forming an integral part of the early defense mechanism (Reis e Sousa *et al.*, 1993). They constitute an important interplay between innate and adaptive immunity due to their ability to migrate to the draining lymph node to present phagocytosed antigen to T cells thereby activating specific adaptive immune responses (Bottomly, 1999; Wolf *et al*., 2008). We therefore assessed the number and activation status of these antigen presenting cells in the lungs of *M. tuberculosis* infected animals as well as their ability to become activated after exposure to a foreign antigen/microbe both *in vitro* and *in vivo* to understand their function in protective immune responses of TNFRp75$^{-/-}$ mice. In our model mice presented with an increase in the number of both CD11b^{+} macrophages and CD11c^{+} dendritic cells in the lungs of TNFRp75$^{-/-}$ mice compared to wild type mice at day 21 during early acute infection which is in contrast to day 35 during late acute infection by which time the TNFRp75$^{-/-}$ mice have controlled bacilli replication and the pro-inflammatory immune response has been down-modulated showing reduced numbers of CD11b^{+} cells and CD4^{+}- and CD8^{+} T cells in TNFRp75$^{-/-}$ mice compared to WT mice. In addition, dendritic cells significantly increase IL-12p40 synthesis in the absence of TNFRp75, while IL-6 production remained unaltered. Of more importance, was that an increased number of recruited macrophages and dendritic cells at day 21 during early acute infection, expressed the costimulatory molecule marker CD86 as well as MHCΠ, important for the antigen presentation to T cells. Antigen presentation by MHCΠ to T cells in conjunction with CD80 or CD86 produces either a costimulatory or coinhibitory effect on T cell activation. CD80 or CD86 binding to CD28 induces T cell activation, expansion and survival, while binding to CTLA-4 results in a coinhibitory signal causing an inhibition of T cell activation and cell death. CD80 has a higher affinity for CTLA-4, while CD86 preferentially binds to CD28 with the result that high CD86 expression is regarded as promoter of T cell activation, while high CD80 expression will lead to T cell inhibition (Collins *et al*., 2005). Therefore our data, which shows an increased number of both macrophage and dendritic cells expressing CD86 during early acute infection as well as an increased IL-12p40 expression in TNFRp75$^{-/-}$ derived dendritic cells, would suggest that in this mouse strain there exists a bias towards an

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increase in T cell activation and a T_H1 polarized immune response during the early acute phase of *M. tuberculosis* infection.

In addition to its role in T_H1 polarization, dendritic cell derived IL-12 plays a significant role in the migration of dendritic cells to the draining lymph nodes (Khader *et al*., 2006). Although the total amount of IL-12p40 detected during *in vivo M. tuberculosis* infection was lower in TNFRp75$^{-/-}$ mice, the amount of dendritic cell-derived IL-12p40 was significantly higher as shown in *in vitro* experiments. This apparent discrepancy can be easily explained due to different cell types producing IL-12p40 in varying amounts in response to specific stimuli. In addition *in vivo* cytokine analysis was carried out on lung homogenates and it has been previously reported that pulmonary dendritic cells do not produce large amounts of IL-12 (Dodge., 2003). In our study dendritic cell-derived IL-12p40 was the most important since it has been shown that dendritic cell-derived IL-12p40 is essential for dendritic cell migration into the lung draining lymph nodes where they complete their maturation process (Khader *et al*., 2006).

We next assessed the effect of TNFRp75 on dendritic cell migration by quantification of the number of CD11c$^+$ cells in the lymph nodes of wild type and TNFRp75$^{-/-}$ mice. Our results confirmed an increase in migration of dendritic cells to draining lymph nodes in TNFRp75$^{-/-}$ mice compared to wild type mice. Similar to the lungs, dendritic cells in the lymph nodes were activated and expression of CD86 and MHCII was higher in TNFRp75$^{-/-}$ mice. Since it has been demonstrated that CD4$^+$ T cell activation relies on antigen presentation by dendritic cells in the lymph node and not the lung during a *M. tuberculosis* infection (Wolf *et al*., 2008), and a T_H1 cytokine environment is important for T_H1 polarization and T cell activation (Flynn and Chan, 2001; de Jong *et al*., 1997; Cooper *et al.,* 1997), the increased number of activated dendritic cells together with the observation that TNFRp75$^{-/-}$ derived dendritic cells express more IL-12p40 creates optimal conditions in the lymph node for T cell activation. Thus, to determine whether increased activation of dendritic cells was translated into an increase in T cell activation, we measured and compared CD44 expression on CD4$^+$ T cells in WT and TNFRp75$^{-/-}$ mice during *M. tuberculosis* infection. We confirmed an increased number of activated CD4$^+$CD44$^+$ T cells in the lymph nodes of TNFRp75$^{-/-}$ mice and went on to confirm that these

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newly activated T cells were in fact T_H1 polarized and capable of producing IFNγ once recruited to the lungs. *Ex vivo* restimulation of lung cells from TNFRp75$^{-/-}$ mice demonstrated an increased ability to generate an *M. tuberculosis* specific IFNγ response to ESAT 6 or *M. tuberculosis* H37Rv but did not produce increased IFNγ in response to non-specific CD3/CD28 restimulation compared to wild type mice. Specific and controlled IFNγ synthesis has important consequences in limiting collateral damage in the host's immune response to invading pathogens. Damage includes tissue destruction which occurs in the presence of excessive T_H1 proinflammatory cytokine production and particularly high IFNγ production (Zganiacz *et al.*, 2004). Therefore, collectively our data provides evidence for an environment in TNFRp75$^{-/-}$ mice which is exploited to induce an enhanced T_H1-immune response characterized by increased cellular recruitment, increased antigen presenting potential by macrophages and dendritic cells, increased costimulatory potential by antigen presenting cells and increased specific T cell activation, during early acute infection (up to day 21), which is responsive to stimuli by bacilli, and is down-modulated as soon as the stimulus is removed or reduced. Thus during late acute infection (day 28 and 35) by which stage bacilli replication is under control, a reduced T_H1-immune response is evident. The clear correlation between the early hypo-activated T_H1-immune response and control of bacterial replication in the absence of TNFRp75 emphasizes the need for a strong early pro-inflammatory response to the outcome of disease. It also argues strongly for a regulatory role of TNFRp75 in buffering excessive immune function in order to limit immune-mediated tissue damage during *M. tuberculosis* infection of normal WT mice at the expense of achieving early control of bacilli replication which would ultimately lead to increased host survival.

To gain further insight into the mechanism of TNFRp75 in regulating immune function, our further investigations were guided by *in vitro* macrophage studies of Balcewicz-Sablinska *et al*. (1998) who demonstrated that TNFRp55 and TNFRp75 were released upon *M. tuberculosis* infection. Receptor release resulted in the generation of predominantly soluble TNFRp75 which formed a complex with soluble TNFα thereby reducing TNFα bioactivity and subsequent TNFα mediated macrophage apoptosis. In addition, an *in vivo* study showed an upregulation of

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TNFRp75 cell surface expression and shedding in response to intraperitoneal *M. avium* infection (Corti *et al.,* 1999). Based on the evidence from these studies we hypothesized that upon *M. tuberculosis* infection, TNFRp55 and TNFRp75 would be released via cleavage by metalloproteases allowing the soluble receptors to bind bioactive TNFα thereby reducing the availability of bioactive TNFα for signalling via the membrane-bound receptors. TNFα together with IFNγ are known key requirements for optimal cellular activation (Zganiacz *et al.*, 2004) and therefore reduced availability of bioactive TNFα may negatively affect APC activation.

In our studies, an assessment of TNFRp55 and TNFRp75 shedding both *in vivo* and *in vitro,* confirmed the release of TNFRp75 from cell surfaces and demonstrated increased levels of soluble TNFRp75 and TNFRp55 in supernatants following an *M. tuberculosis* infection in accordance with previous reports (Balcewicz-Sablinska *et al*. 1998; Corti *et al*. 1999). Interestingly the absence of TNFRp75 (in TNFRp75$^{-/-}$ mice) was associated with significantly lower soluble TNFRp55. Reduced release of TNFRp55 together with the absence of TNFRp75 would suggest (i) an increase in TNFRp55 cell surface expression and that (ii) TNFRp75$^{-/-}$ mice will have significantly higher levels of bioactive TNFα based on the premise that TNFα-TNFR complex formation reduces bioactive TNFα availability. Indeed, an *in vitro* analysis of bioactive TNFα showed higher levels in dendritic cell cultures from TNFRp75$^{-/-}$ mice compared to wild type mice. These mice thus show increased levels of bioactive TNFα which potentially can all signal through TNFRp55. TNFRp75$^{-/-}$ mice are therefore characterized by an improved TNF/TNFRp55 signaling which promotes the early T_H1 pro-inflammatory immune response essential for early control of bacilli replication and is associated with reduced pulmonary pathology. In order to demonstrate that TNFRp75 release regulated APC activation through regulating bioactive TNFα levels, we exposed dendritic cells to *M. tuberculosis* in the presence of an anti-TNFRp75 monoclonal antibody. Here we demonstrated that treatment of wild type macrophages or dendritic cells with an anti-TNFRp75 antibody induced a significant increase in bioactive TNFα approximating levels detected for TNFRp75$^{-/-}$ mice cultures. Our observations were supported by Balcewicz-Sablinska *et al*. (1998), in which they did the inverse by using recombinant TNFRp75 treatment to prove that increased levels of soluble

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TNFRp75 resulted in a reduction of TNFα bioactivity. We went on to prove that this increase in TNFα bioactivity resulted in the observed increased activation and MHCΠ expression in dendritic cells. Here dendritic cell cultures were treated with anti-TNFRp75 antibody and the IL-12p40 and MHCΠ expression assessed. The data demonstrated that treatment of wild type dendritic cells with anti-TNFRp75 antibody resulted in an increase in IL-12p40 as well as in increase in the number of dendritic cells expressing MHCΠ to a level comparable to that in TNFRp75$^{-/-}$ mice thus confirming our hypothesis that the increased ability of antigen presenting cells to become activated is due to an increased level of bioactive TNFα caused by a reduction in the total amount of soluble TNF receptor.

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Conclusions

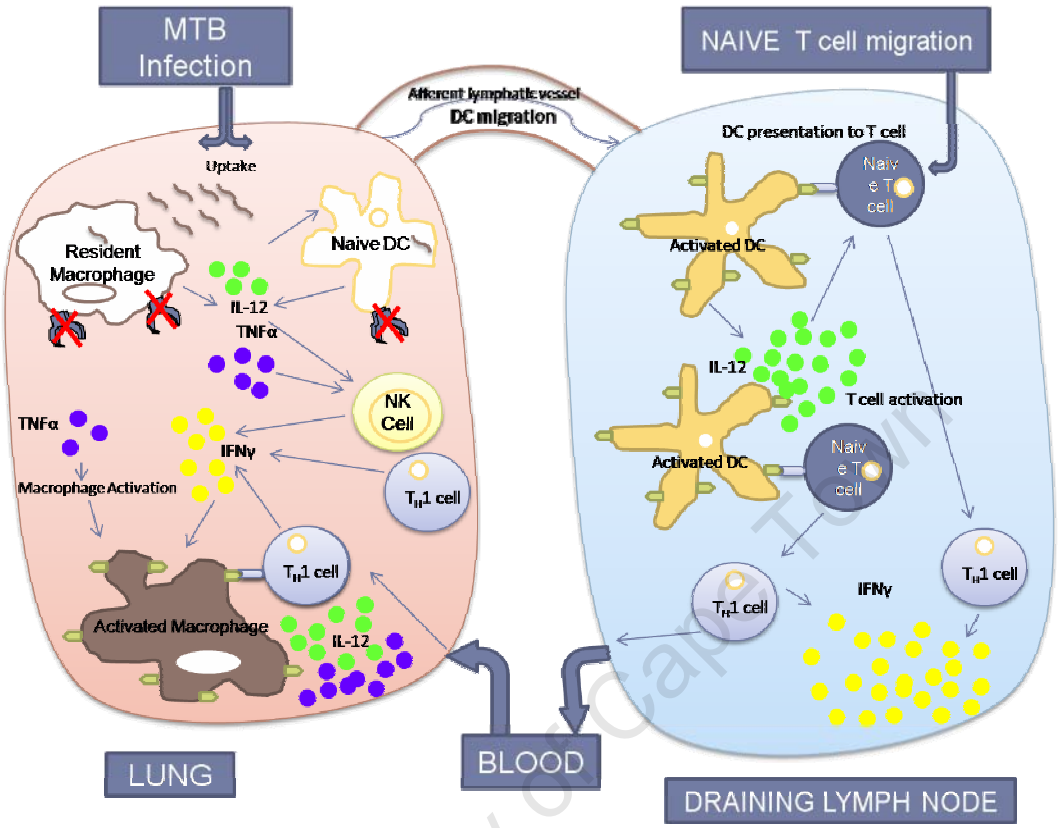

Figure A: Summary of Host Immune Response to During Early Acute Infection in the Absence of TNFRp75. The two compartments of lung and lymph node are represented with all the relevant cell types and cytokine production. Macrophage and DC uptake, cytokine production and activation are represented, as well as DC trafficking, presentation and T cell activation. Briefly the absence of TNFRp75 shedding results in increased amounts of bioactive TNFα which can signal through TNFRp55. The result is an increased number of activated APC's which produce increased levels of TNFα and IL-12. Increased IL-12 results in increased migration of dendritic cells to draining lymph nodes where high IL-12 production creates a T_H1 cytokine environment which together with increased antigen presentation by activated dendritic cells leads to an increased number of antigen-specific T_H1 T cells. These T cells are activated to produce IFN-γ in response to mycobacteria and migrate through the blood back to the site of infection. Antigen specific IFN-γ together with increased bioactive TNFα aids in macrophage activation and bacilli control (Adapted from Marino *et al.*, 2004).

TNFRp75$^{-/-}$ mice present with enhanced protective immunity against an aerosol *M. tuberculosis* infection. During early acute infection an increased T_H1 pro-

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inflammatory immune response is established resulting in early control of bacilli replication which is delayed in WT mice. This enhanced protection results in prolonged survival of the TNFRp75 deficient strain and is associated with reduced bacilli burdens. During late acute infection the increased T_H1 immune response is down-modulated due to bacilli control being established resulting in small but highly structured granulomas and less tissue destruction due to reduced pro-inflammatory cytokines. When the mechanism behind this enhanced protection was analyzed it was determined to be largely associated with antigen presenting cells that had an enhanced activation status and potential to present antigen subsequent to *M. tuberculosis* infection. Increased activation was due to less soluble receptor being present to bind to TNFα, thus resulting in more bioactive TNFα, increased IL-12p40 and increased MHCΠ expression. Increased IL-12p40 allowed for better migration of dendritic cells into the draining lymph nodes, improved antigen presenting potential to T cells and thus increased T cell activation occurring in the lymph node. The increased number of specific IFNγ-producing activated T_H1 cells during adaptive immunity were able to maintain the control of bacilli burdens established during early acute infection by the innate immune response and particularly antigen presenting cells. The comparative analysis of WT verses $TNFRp75^{-/-}$ mice leads to the conclusion that in a normal WT situation, shedding of TNFRp75 results in a buffer for bioactive TNFα peak levels during early acute infection which directly results in a delay in the control of bacilli replication with the outcome of reduced survival time. In summary this study has identified TNFRp75 as a viable host specific target that could potentially be exploited for therapeutic application to improve outcome of disease during *M. tuberculosis* infections.

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Future Studies

1. Anti-TNFRp75 blocking antibodies will be used *in vivo* in WT mice to assess whether the increased protection observed for TNFRp75$^{-/-}$ mice can be emulated in WT mice using anti-TNFRp75 as a "therapeutic".

2. The time period of treatment for which optimum protection is achieved will be determined.

3. Therapeutic treatment of T cell deficient mice will also be done to assess whether such a treatment could be successfully used in immune compromised settings.

4. Treatment therapies which include both anti-TNFRp75 blocking antibodies as well as the conventional TB drugs isoniazid and ripampicin will be investigated in a experimental tuberculosis reactivation model.

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APPENDIX A

All chemical reagents used were of an analytical grade and were purchased from the following companies unless otherwise stated:
Merck Laboratory Supplies, Germany
Sigma-Aldrich, St Louis

General Reagents

Difco Middlebrook 7H10 Agar (M7H10)

19g of M7H10 agar was dissolved in distilled H_2O, 5ml glycerol was added and the volume made up to 900ml with distilled water. The solution was autoclaved at 121^{o}C for 10 minutes and allowed to cool to 55^{o}C before adding 100ml OADC. A volume of 7ml was poured per side of sterile duplicate petridishes (Sterilin). Plates were allowed to set at room temperature before storage at 4^{o}C in the inverted position.

Formalin

100 ml formaldehyde (40% w/v formaldehyde solution) was added to
900 ml PBS (pH 7.4)
Solution was stored in a dark bottle at room temperature

General Anaesthetic

0.25ml Rompun (Bayer Pty Ltd, Germany) and
1ml Brevinase (Centaur Labs, Premier Pharmaceutical Company, Bryanston, South Africa) were added to
11ml of 0.9% Saline solution
The solution was filtered through a 0.45 μm filter (Millipore Corporation, Bedford, USA)

Homogenising Buffer for Organs

0.04% Tween 80 was made up in
1x PBS and autoclaved at 121^{o}C for 30 minutes.
The solution was stored at room temperature

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10x Phosphate Buffered Saline

80g NaCl

2.4g KH_2PO_4

2g KCl and

14.4g $Na_2HPO_4.2H_2O$ were dissolved in

900ml distilled H_2O.

The pH was adjusted to 7.4 using HCL and the volume made up to 1000ml with distilled H_2O prior to sterilization by filtering the solution through a 0.45µm filter (Millipore Corporation, Bedford, USA). The solution was stored at room temperature and diluted to working concentration by making a 1/10 dilution in distilled H_2O.

0.9% Saline Solution

9g NaCl was dissolved in

100ml of distilled water.

The solution was autoclaved at 121^{o}C for 30 minutes and stored at room temperature.

Saline/Tween 80

0.9% NaCl and

0.04% Tween 80 were dissolved in distilled H_2O, autoclaved for 30 minutes and stored at room temperature.

PCR Reagents

Agarose Gel

8g agarose (1.6%)

22.5µl Ethidium Bromide and

25ml 10x TBE buffer (0.5x) were made up to

500ml with distilled water before dissolving in a microwave, allowing it to cool and pouring the gel.

6 x Gel Loading Buffer

0.25% Bromophenol blue,

0.25% Xylene Cyanol and

50% Glucose were dissolved in H_2O.

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10 x Stock TBE

108g Tris (base),

55g Boric acid and

40 ml 0.5 M EDTA were dissolved in 1 litre of water sterilized through a 0.45 μm filter (Millipore Corporation, Bedford, USA) and stored at room temperature.

Tail Lysis Buffer

50mM Tris-HCl, pH 8,

100mM EDTA, pH 8,

100mM NaCl,

1% SDS and

0.5mg/ml Proteinase K were mixed and made up to the required volume with distilled H_2O.

Flow Cytommetry Reagents

10% Sodium azide (NaN_3)

10 g NaN_3 was dissolved in

100ml of distilled H_2O, sterilised by filtration through a 0.45 filter (Millipore Corporation, Bedford, USA) and stored at 4^oC.

FACS buffer

1g Bovine Serum Albumin (Boeringer Mannhaim, Germany) and

0.1g NaN_3 were added to 900ml of 1x PBS. The pH was adjusted to 7.4 and the total volume was made up to 1000ml prior to sterilising by filtering through a 0.45μm filter (Millipore Corporation, Bedford, USA). The solution was stored at 4^oC.

FACS Blocking Buffer

2.5% normal rat serum,

2.5% normal mouse serum and

6.25μg/ml anti-CD32/CD16c (anti-FCγRIII/II) (Clone: 2.4G2, Pharmingen) was made up in FACS buffer.

FACS Fixation Buffer

4g NaOH was dissolved in 100ml 1x PBS,

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20g paraformaldehyde was added and dissolved, prior to adjusting the pH to 7.2 and making the volume up to 1000ml. The solution was filter sterilised through a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored in the dark at 4^{o}C.

Saponin Buffer

0.1% Saponin,
1mM $CaCl_2$,
1mM $MgSO_4$ and
10mM HEPES was diluted in FACS Buffer.
The solution was sterilized through through a 0.45 µm filter (Millipore Corporation, Bedford, USA) and storing at 4^{O}C.

Complete Restimulation Medium

5% FCS,
2mM L-glutamine (Gibco, Invitrogen Corporation, Germany),
50µM 2-Mercaptoethanol,
10U/ml Penicillin (Gibco, Invitrogen Corporation, Germany) and
10µg/ml Streptomycin (Gibco, Invitrogen Corporation, Germany) was diluted in RPMI (Sigma Aldrich, St Louis), filtered through Whatmann filter paper no. 1 and stored at 4^{O}C.

FACS Blocking Solution

0.9375µl αFcγRIII (CD32/CD16c),
3.75µl Normal rat serum (heat inactivated) and
3.75µl Normal mouse serum (heat inactivated) was diluted in FACS buffer to a final volume of 150µl just prior to use.

Red Blood Cell Lysing Buffer

8.29 g NH_4Cl (0.15 M),
1 g $KHCO_3$ (1 mM) and
37.2 mg Na_2EDTA (0.1 M) were dissolved in
800 ml distilled H_2O. The pH was adjusted to 7.2-7.4 with 1 M HCl and the total volume made up to 1 litre with distilled H_2O prior to filter sterilization through a 0.45 µm filter (Millipore Corporation, Bedford, USA) and storing at room temperature.

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ELISA Reagents

Coating Buffer

0.2g NaN_3 was dissolved in
800ml 1x PBS. The pH was adjusted to 7.2 and the volume was made up to 1000ml, filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Dilution Buffer

10g Bovine Serum Albumin (Boeringher Mannheim, Germany) and
0.2g NaN_3 was dissolved in
800ml 1x PBS. The pH was adjusted to 7.4, the volume was made up to 1000ml, filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Reagent Diluent

0.1% BSA and
0.05% Tween 20 were dissolved in saline containing
20mM Trizma base and
150mM NaCl.
The pH was adjusted to between 7.2 and 7.4 and filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Blocking Buffer 1

40g Bovine Serum Albumin (Boeringher Mannheim, Germany) and
0.2g NaN_3 was dissolved in
800ml 1x PBS. The pH was adjusted to 7.4 and filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Blocking Buffer 2

1% BSA and
5% Sucrose were dissolved in 1x PBS with
0.05% NaN_3.
The pH was adjusted to 7.4 and filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

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20x Washing Buffer

20g KCl,

20g KH_2PO_4,

144g NaH_2PO_4 and

800g NaCl were dissolved in 4.5 liters of distilled H_2O.

50ml Tween 20 and

100ml 10% NaN_3 solution were added. The volume was adjusted to 5 liters and filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Substrate Buffer

0.2g NaN_3 and

0.8g $MgCl_2$ were dissolved in 700ml distilled H_2O.

97ml liquefied diethanolamine was added. The pH was adjusted to 9.8, the volume was made up to 1000ml, and filter sterilised using a 0.45µm filter (Millipore Corporation, Bedford, USA) and stored at 4°C.

Histological Reagents

E1 Mayers Haematoxylin

1g haematoxylin was dissolved in

800ml distilled H_2O.

50g aluminium ammonium sulphate was added and dissolved.

0.2g sodium iodate,

1g citric acid and

50g chloral hydrate were added in that order, dissolving each before the addition of the next compound. The volume was made up to 1000ml with distilled H_2O, filtered through Whatmann filter paper (no 1) and stored in the dark at room temperature.

Wegert's Haematoxylin

Solution A

1g haematoxylin was dissolved in

100ml absolute alcohol

Solution B

4ml 30% Aqueous ferric chloride (anhydrate) and

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1ml concentrated HCl were added to
95ml distilled H_2O.
Equal volumes of solution A and B were mixed shortly before use.

Eosin

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

Carbol Fuchsin

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

Loeffers' Methylene Blue

1ml 1% KOH was added to
99ml distilled H_2O and
3ml 0.8% Methylene Blue (in absolute alcohol) was added.
The solution was filtered through Whatmann filter paper no. 1 and stored at room temperature.

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Cell Culture Reagents

WEHI Complete Medium

10% FCS (Gibco, Invitrogen Corporation, Germany),
0.5x Amino acid supplement (Gibco, MEM Amino Acids (50x) without L-glutamine, Invitrogen Corporation, Germany),
10 U/ml Penicillin (Gibco, Invitrogen Corporation, Germany) and
10µg/ml Streptomycin (Gibco, Invitrogen Corporation, Germany) were diluted in RPMI (Sigma Aldrich, St Louis). The pH was adjusted to 7.4 using HCL and the solution was filtered through Whatmann filter paper no. 1 and stored at $4^{O}C$.

WEHI Detection Solution

10ml Propanol,

80µl 5N HCL and

5ml 20% SDS were mixed together immedietly prior to use.

Bone Marrow Derived Macrophage Medium (BMDM)

30% L929 Conditioned medium,

20% Heat inactivated horse serum (Gibco, Invitrogen Corporation, Germany),

10mM L-Glutamine (Gibco, Invitrogen Corporation, Germany),

100U/ml Penicillin (Gibco, Invitrogen Corporation, Germany) and

100µg/ml Streptomycin were diluted in RPMI (Sigma Aldrich, St Louis)

The pH was adjusted to 7.4 using HCL and the solution was filtered through Whatmann filter paper no. 1 and stored at 4^{O}C.

Dendritic Cell Medium (DCM)

50% GM-CSF Conditioned medium,

10% Heat inactivated horse serum (Gibco, Invitrogen Corporation, Germany),

50µM 2-Mercaptoethanol

2mM L-Glutamine (Gibco, Invitrogen Corporation, Germany),

100U/ml Penicillin (Gibco, Invitrogen Corporation, Germany) and

100µg/ml Streptomycin were diluted in RPMI (Sigma Aldrich, St Louis)

The pH was adjusted to 7.4 using HCL and the solution was filtered through Whatmann filter paper no. 1 and stored at 4^{O}C.

Trypsin/EDTA

10x Trypsin/EDTA was diluted 10 fold in 1x PBS

The solution was filtered through Whatmann filter paper no. 1 and stored at 4^{O}C.

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