Protective role of membrane tumour necrosis factor in the host’s resistance to mycobacterial infection

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Introduction

Protective immunity to *Mycobacterium tuberculosis* infection, both in humans and experimental animals, is regulated by T cells, macrophages and cytokines, which include interferon-γ (IFN-γ), interleukin-12 (IL-12) and tumour necrosis factor (TNF). IFN-γ derived from T and natural killer (NK) cells has been shown to be essential, as mice with a disruption of the IFN-γ system are unable to restrict the growth of *M. tuberculosis* and succumb to the infection.

A critical role for TNF in mycobacterial defence is inferred from neutralization and deletion experiments in mice. In addition, the TNF-related cytokine lymphotixin-α, (LTα, previously known as TNF-β), has also been shown to be required to control mycobacterial infection. Secreted TNF and LTα signal both through the p55 and p75 TNF receptors, TNFR1 and TNFR2 respectively, while the cell bound LTαβ heterotrimer recognize the LTβR. TNFRII signalling appears to be critical for the control of mycobacterial infection, while a role of TNFR2 is not excluded. LTβR signalling was similarly found to protect against mycobacterial infection.

TNF is produced by macrophages, but is expressed by a variety of cells and is a major regulator of inflammation...
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and leucocyte trafficking. Although a key role of TNF in controlling intracellular bacterial infections is uncontested, the function of membrane TNF, which is subsequently cleaved by the metalloproteinase-disintegrin TACE (TNF-α converting enzyme) into the secreted trimERIC TNF is, in mycobacterial host resistance, less clear.

Several biological functions of membrane TNF have been described, such as cytotoxicity, polyclonal activation of B cells, and induction of IL-10 by monocytes and intracellular adhesion molecule-1 expression on endothelial cells. The transgenic expression of membrane TNF demonstrated an in vivo role including the control of BCG and Mycobacterium infection but this approach is imperfect as the transgenic expression of high levels membrane TNF is artificial and may cause altered immune responses and non-physiological pathology. As an example the spontaneous development of arthritis in transgenic mice expressing mutant membrane TNF was directly attributed to the deregulation of membrane TNF production.

Two novel mutants have been generated where the endogenous TNF was either replaced by a Δ1-9,K11E TNF allele or Δ1-12 allele, which represent a major advance allowing interesting insights in the role of membrane TNF in lymphoid structure development and inflammation. We and others demonstrated that the Δ1-9,K11E mutant has enhanced resistance to Mycobacterium bovis and Listeria monocytogenes infection. Δ1-12 TNF mutant is resistant to low-dose L. monocytogenes infection, but no data are available on mycobacterial infections.

Here, we demonstrated that the Δ1-12 TNF mutant is partially resistant to infection with 50% of mice that succumb to the non-virulent vaccine strain Mycobacterium bovis BCG, while 100% of TNF-deficient mice succumb to infection. TNFm/m mice were able to recruit and activate macrophages and T cells, and generate mycobactericidal granuloma in response to M. bovis BCG infection. However, resistance to the virulent M. tuberculosis strain was less pronounced, suggesting substantial differences between this and the Δ1-9,K11E mouse mutant.

Methods

Animals

Adult, 8- to 10-week-old female mice of the wild type (WT) strain C57BL/6, homozygous Δ1-12 TNF mutant (TNFΔ1-12), TNFR1Δ/Δ, TNFR2Δ/Δ and TNFΔ−/− mice, all on a C57BL/6 genetic background were bred and maintained under specific pathogen-free conditions. TNFm/m mice were crossed on TNFR1Δ/Δ mice or TNFR2Δ/Δ mice in order to study the signalling of membrane TNF. The genotypes of the mouse populations were confirmed by polymerase chain reaction (PCR) analysis of tail biopsies. PCR primers: Δ1-12 TNF mutant – 5'-CTCTCTTGATGCAGCAGT-3', 5'-TCCTGAGTGAAGCTCT-3', and 5'-AGAAATCTCAG ACAAATCTCTG-3'; TNFR2 – 5'-CCTCTCATGCTGT CCGGAAT-3', 5'-AGCTCCAGGCACAGGGCGG-3', 5'-CGGTCTCTTGTCTCAACAG-3' and 5'-ATCTCCTC GCCGTGGGATGC-3'. All protocols employed in this study were approved by the University of Cape Town Research Ethics Committee.

Mycobacteria

M. tuberculosis H37Rv (Trudeau Mycobacterial Culture Collection; Trudeau Institute, Saranac Lake, NY) and M. bovis Bacille Calmette–Guérin (BCG) (Prof. G. Marchal, Pasteur Institute, Paris) were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI), supplemented with 10% oleic acid albumin dextrose catalase (OADC) (State Vaccine Institute, Cape Town, South Africa) and 0-5% glycerol (Merck, Darmstadt, Germany) in 5% CO2 at 37° and frozen in aliquots. Prior to use, an aliquot was thawed, briefly vortexed, diluted in sterile saline and clumping disrupted by aspirating through a 29-gauge needle (Omnican®; Braun, Melsungen AG, Germany) 20 times.

Mycobacterial infection

WT, TNFm/m and TNF-deficient mice were injected intravenously (i.v.) at an infective dose of 2 x 106 BCG. Groups consisted of 10 mice and the experiments were repeated twice. Mice were killed at 2, 4 and 8 weeks after infection. For the aerosol infection mice were infected with 30 viable CFU of H37Rv via the aerosol route using an inhalation exposure system (Glas-Col, Terre Haute, IN).

Lipopolysaccharide (LPS)/d-galactosamine (d-Gal)-induced septic shock

LPS (Escherichia coli, serotype O111:B4; Sigma, St Louis, MO) with d-Gal (Sigma) was injected intraperitoneally (i.p.) to induce systemic TNF and endotoxic shock as described previously. Briefly, mice were injected i.p. with 10 µg of LPS in combination with 20 mg d-Gal. Mice were monitored for 24 hr and the number of moribund mice was recorded.

Histology and immunohistochemistry

Lungs were removed and weighed. The organs were fixed in 4% buffered formalin, or frozen on dry ice and maintained at −80°C. Two to 5 µm-thick sections of the lungs were cut and stained with either haematoxylin and eosin or Ziehl–Neelsen for acid-fast bacilli. Formalin-fixed, paraffin-embedded sections were de-paraffinized and re-hydrated through decreasing concentrations of alcohol as described. Sections were stained with a rabbit
antimouse antibody specific for inducible nitric oxide synthase (iNOS; obtained from J. Pfleischifter, University of Frankfurt, Germany). Sections were then washed in phosphate-buffered saline (PBS) and incubated for 30 min at room temperature with biotinylated rat anti-rabbit serum followed by Vectastain ABC system (Vector Laboratories Inc., Burlingame, CA) and 3,3′-diaminobenzidine (DAB) substrate. Finally, sections were mounted in Immunomount (Shandon, Pittsburgh, PA).

**Colony-forming units (CFU)**

Bacterial loads in the lung, liver and spleen of infected mice were evaluated at 2, 4 and 8 weeks post-infection. Organs were weighed and defined aliquots were homogenized in saline containing 0·04% Tween-80. Tenfold serial dilutions of organ homogenates were plated in duplicates onto Middlebrook 7H10 agar plates containing 10% OADC (Difco). Plates were incubated at 37°C for 19–21 days and colonies counted. Data are expressed as mean CFU per organ.

**Bronchoalveolar lavage**

Under anaesthesia a 20-gauge catheter (Introcan, B. Braun, Melsungen AG, Germany) was inserted into the exposed trachea. The lungs were lavaged with two volumes of 300 µl PBS. Lavage fluid was centrifuged at 405 g for 5 min, the supernantant was removed, aliquoted and stored at −80°C for cytokine and chemokine analysis. Lungs were subsequently lavaged with four volumes of 800 µl PBS. To remove traces of red blood cells, pooled samples were incubated in 1 ml red cell lysis buffer for 5 min, washed twice with 5 ml PBS. Samples were centrifuged at 405 g for 5 min and the cells were used for flow cytometric analysis. Procedures were performed under sterile conditions and samples kept on ice for the duration of the experiment.

**Cytological analysis**

Teflon coated, eight-well (6 mm) multi-well slides (Highveld Biological, Lyndhurst, South Africa) were used for cytopsins. A 100 µl of a 2·5 × 10⁵ cells/ml cell suspension was pipetted into each well and the liquid was drained from the well by attached Whatman filter paper. The slide was left to air dry overnight at room temperature. The cells were stained with RapiDiff staining kit, after removal of the filter paper, for differential cell counting. Unstained slides were stored at −20°C, wrapped in aluminium foil. Cells were stained by immersing the slides three times (±5 s) in the following solutions: RapiDiff fixative, RapiDiff solution 1 (eosin in phosphate buffer) RapiDiff solution 2 (haematoxylin) and a final rinse in distilled H₂O. Slides were left to dry for 1–2 hr at room temperature and mounted with entellan (Merck). Routinely, 100 cells per cytopsin, in duplicate, per mouse were differentiated in a random fashion.

**Flow cytometry**

Cells (1 × 10⁶) were incubated with 25 µl fluorescence-activated cell sorting (FACS) blocking solution for 30 min after which they were washed with 1 ml FACS buffer and centrifuged at 405 g for 5 min. Cells were incubated with 2 µg/ml anti-CD3 phycocerythrin (PE; clone 145-2C11) and either 2 µg/ml anti-CD4 fluoroscein isothiocyanate (FITC; clone HI29-19) or 2 µg/ml anti-CD8 FITC (clone 53-6-7) in a total volume of 50 µl for 30 min. Cells were washed with 1 ml FACS buffer, centrifuged at 405 g for 5 min and resuspended in FACS fixation buffer. Samples were kept on ice for the duration of the labelling procedure. Fluorescently labelled antibodies were purchased from BD Pharmingen (San Diego, CA). Samples were analysed on a FACSCalibur (Beckton Dickinson, San Jose, CA) flowcytometer using Cell Quest software.

**Primary bone-marrow-derived macrophage (BMDM) cultures and infection**

Mice 6–8 weeks old were killed and the femurs and tibia removed aseptically as described before by Müller et al. The bone marrows were flushed out with ice-cold DMEM (Dulbecco’s modified Eagle’s minimal essential medium with 4500 mg/l glucose, l-glutamine and 25 mS HEPES without sodium bicarbonate) using a syringe fitted with a 29-gauge needle. The cells eluted were resuspended in DMEM supplemented with 30% L929 conditioned medium and 20% horse serum and plated at a concentration of 2 × 10⁶ cells/10 ml in Sterilin 90-mm bacteriological plates. On day 10, cells were pooled, pelleted by centrifugation and plated at 3 × 10⁵ cells/well in a 48-well plate (Costar; Corning Incorporated, Corning, NY). Cells were stimulated with 10 µg/ml LPS (Escherichia coli, serotype 0111:B4; Sigma-Aldrich, St Louis, MO) and 100 U/ml rIFN-γ (BD Pharmingen) for 72 hr. In the infection study, the cells were prestimulated with 100 U/ml rIFN-γ for 16 hr and infected with M. bovis BCG (multiplicity of infection = 1:2) and the supernatants collected after 54 hr.

**Enzyme-linked immunosorbent assay (ELISA) assay**

TNF, IFN-γ and IL-12, IL-4, IL-5, monocyte chemoattractant protein-1 (MCP-1) and RANTES (regulated on activation, normal, T-cell expressed, and secreted) in supernatant of bronchoalveolar lavage fluid (BALF) were measured by ELISA with a sensitivity of <15 pg/ml (R&D Systems, Abingdon, UK and BD Pharmingen, San Diego, CA).
Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by the use of the Student’s t-test and ANOVA with a level of significance of $P < 0.05$.

Results

Absence of secreted TNF in TNF$^{tm/tm}$ mice

To confirm that TNF$^{tm/tm}$ knock-in mice do not secrete TNF, mice were injected i.p. with 100 μg LPS and blood was collected after 90 min and serum analysed by ELISA. In contrast to WT mice that displayed significant serum TNF levels, TNF was undetectable in the sera of both TNF$^{-/-}$ and TNF$^{tm/tm}$ mice (Fig. 1a). In addition, we showed that d-Gal sensitized WT mice were susceptible to LPS and succumbed from TNF-induced endotoxic shock after 24 hr as shown previously$^{32}$ whereas TNF$^{tm/tm}$ mice survived the endotoxic shock as did the TNF$^{-/-}$ mice (Table 1).

We then investigated whether cultured BMDM could secrete TNF in response to LPS or after M. bovis BCG infection. Our results show that TNF was secreted by BMDM from WT mice, but undetectable in culture supernatants of BMDM from TNF$^{tm/tm}$ and TNF$^{-/-}$ mice (Fig. 1b).

Furthermore, membrane expression of TNF was found to be inducible upon stimulation.$^{25}$ Taken together, these observations confirm the absence of secreted TNF in TNF$^{tm/tm}$ mice as previously described.$^{25}$

TNF$^{tm/tm}$ mice are more resistant to M. bovis BCG infection than TNF-deficient mice

To determine whether membrane-bound TNF contributes to protective immunity, a comparative study was performed to determine the susceptibility of TNF$^{tm/tm}$, TNF$^{-/-}$ and WT mice to mycobacterial infection. Groups of 10 mice per strain were i.v. infected with $2 \times 10^6$ CFU of the non-virulent vaccine strain M. bovis BCG and the rate of mortality was recorded. Within 3–4 weeks of infection TNF$^{-/-}$ mice displayed rapid weight loss (Fig. 2a), impeded locomotor activity and succumbed to infection between 5 and 8 weeks (Fig. 2b). Although TNF$^{tm/tm}$ mice manifested weight loss during the early phase of infection, the weights stabilized, and about 50% of the TNF$^{tm/tm}$ mice survived the experiment. All WT mice survived the infection. From these observations, we concluded that membrane-bound TNF confers partial protection to systemic BCG infection in the absence of secreted TNF.

Membrane TNF mice induce partial protective immunity to pulmonary M. bovis BCG infection

We and others have shown previously that the elimination of mycobacteria from the lung is absolutely dependent on TNF, while evidence for bacterial clearance from the liver and spleen in a TNF-dependent and -independent manner after systemic infection was reported.$^{3,4,7}$ The importance of membrane-bound TNF for the control of mycobacterial growth in the lung was therefore

| Table 1. Susceptibility of WT, TNF$^{tm/tm}$ and TNF$^{-/-}$ mice to LPS/ d-Gal |
|-----------------|----------------|
| Strain          | Moribund mice |
| WT              | 5/5            |
| TNF$^{tm/tm}$   | 0/5            |
| TNF$^{-/-}$     | 0/5            |

WT, wild type; TNF, tumour necrosis factor.
Each group comprised 10 mice. Infected i.v. with 2×10^6 BCG bacilli and monitored for 3 months. We then determined the mycobacterial growth and monitored CFU. Survivals of TNF^tm/tm and TNF^+/− mice infected with Mycobacterium bovis BCG. Body weight change (a) and survival (b) of TNF^tm/tm (Δ), TNF-deficient mice (○) and WT mice (●) infected i.v. with 2×10^6 BCG bacilli and monitored for 3 months. Each group comprised 10 mice.

By contrast, bacilli burden was higher at 4 weeks in both TNF^tm/tm mice and TNF^+/− mice (P<0.01) than in WT mice. TNF^+/− mice displayed uncontrolled mycobacterial growth with a significant increment in CFU in the lung at 8 weeks as compared to the 4 week values (P<0.05). In contrast to TNF^+/− mice, TNF^tm/tm mice were able to control infection at 8 weeks. CFU in lungs of TNF^tm/tm at 8 weeks were lower than at 4 weeks (P<0.05). In addition, CFU in the lungs of TNF^tm/tm mice were significantly lower than that of TNF^+/− mice (P<0.05). Therefore, the data demonstrate that the sole presence of membrane-bound TNF is able to control M. bovis BCG infection in 50% of mice.

Membrane TNF allows the expression of pulmonary granuloma

The establishment of granulomas is the manifestation of a vigorous cell-mediated immune response, which is crucial for inhibiting mycobacterial growth. A critical role of TNF for initiating and maintaining the structural integrity of granulomas has been demonstrated. We therefore asked whether membrane TNF is sufficient for granuloma formation upon M. bovis BCG infection.

Pulmonary pathology in all three mouse strains at 2 weeks post-infection was characterized by areas that had normal alveolar and focal thickened septa, but the alveolar spaces were free from infiltrations (Fig. 4a) and there was no difference between the groups. At 4 weeks post-infection the lungs of WT mice displayed well-defined granulomatous lesions that were characterized by foamy epithelioid-like macrophages with surrounding and interspersed lymphocytes and prominent perivascular lymphocytic infiltration. At 8 weeks WT mice, which control M. bovis BCG infection, showed evidence of resolving granulomatous lesions.

As expected, TNF^+/− mice showed further thickening of septae containing inflammatory cells at 4 weeks, but did not develop granulomatous lesions and only sparse perivascular lymphocytic infiltration. At 8 weeks, a prominent perivascular and peribronchial lymphocyte infiltration was found in TNF^+/− mice. There was, however, no structural organization of the infiltrating lymphocytes and macrophages into a well-structured granuloma, and an increased recruitment of neutrophils was apparent.

In contrast, TNF^tm/tm mice displayed typical, but small granulomas at 4 weeks post-infection, which were less well demarcated than those found in WT mice. Epitheloid macrophages and lymphocytes were distinguishable and perivascular lymphocyte recruitment was evident. These early granulomas developed into enlarged pulmonary lesions at 8 weeks post-infection.

In addition, Ziehl–Neelsen staining revealed that bacilli in both WT and TNF^tm/tm mice are less abundant and predominantly confined to granulomas at 4 weeks and...
8 weeks post-infection, whereas in TNF−/− mice with the absence of properly defined granulomas the bacilli were dispersed in the tissue and predominantly extracellular (Fig. 4b). Therefore, in contrast to complete TNF deficiency the sole presence of membrane-bound TNF allowed a substantial granulomatous response with bactericidal properties.

Membrane TNF allows partial cellular recruitment upon mycobacterial infection

In view of the granulomatous response elicited in TNF−/−mice after M. bovis BCG infection we investigated the cellular recruitment in the lungs. Cells from BAL were obtained at 2 and 4 weeks post-infection. WT mice showed significant recruitment of macrophages, lymphocytes and neutrophils into the BALF 2 and 4 weeks after infection. The total cell number was highest in WT mice, followed by TNF−/− and TNF KO mice, which had significantly reduced total cell recruitment (P < 0.05) at 4 weeks (Fig. 5a). The macrophage recruitment in BALF was significantly reduced in TNF−/− and TNF KO mice (P < 0.05) (Fig. 5b), while the lymphocyte counts (Fig. 5c) showed only a trend of reduction and the neutrophils (Fig. 5d) were elevated, but not different among the experimental groups at 4 weeks.

CD4+ and CD8+ T cells are critical for the generation of cell-mediated immunity. We therefore investigated whether membrane-bound TNF is sufficient to recruit specific T-cell subsets. Cells from BAL at 4 weeks post-infection were analysed by flow cytometry. In the presence of both soluble and secreted TNF (WT mice), effective recruitment of CD3+ CD4+ T cells and CD3+ CD8+ T cells occurred (Fig. 6a). In the absence of TNF, T-cell recruitment was reduced sevenfold (CD3+ CD4+ T cells: 21% versus 3% and CD3+ CD8+ T cells: 6% versus 0.8%). By contrast, the sole expression of membrane-bound TNF was sufficient to allow a significant recruitment of CD4 and CD8 T cells in BALF of TNF−/−mice, which was only 2–3-fold lower than in WT mice (CD3+ CD4+ T cells: 21% versus 8% and CD3+ CD8+ T cells: 6% versus 2%).

In addition, we investigated the activation status of the recruited T cells. We found that >95% of the cells recruited were activated, as defined by the high expression of CD11a and CD44, and there was no difference among the three groups (Fig. 6b).

As the T-cell recruitment was reduced in BAL, we asked whether the inflammatory response in the lungs of BCG-infected mice differed in the total or partial absence of TNF. As shown in Fig. 4, TNF−/− mice were able to recruit abundant lymphocytes and macrophages into the lung tissue allowing the formation of granulomas, which were, however, less well developed than in WT mice. We asked whether the partial recruitment of lymphocytes as shown in BALF was sufficient to support macrophage activation. We measured iNOS as an indicator of macrophage activation, semiquantitatively and observed expression in TNF−/−mice, but at a lower level compared with WT mice. However, iNOS expression in

Figure 3. Lung pathology and uncontrolled bacterial growth in TNF−/− mice. TNF−/−mice (△), TNF−/−mice (○) and WT mice (●) were infected i.v. with 2 × 10⁶ BCG M. bovis BCG. (a) Macroscopic lung changes in BCG infected mice at 2, 4 and 8 weeks post-infection. (b) Lung weights of BCG infected mice at 2, 4 and 8 weeks post-infection. (c) Bacterial load (CFU) was determined in lungs after 2, 4 and 8 weeks after BCG infection (2 × 10⁶ BCG i.v.). The results are expressed as the mean ± SD of five mice.
TNF<sup>tm/tm</sup> mice was substantially higher than in TNF<sup>−/−</sup> mice (Fig. 7).

These data suggest that membrane TNF allows substantial recruitment of T cells and the activation of macrophages resulting in mycobactericidal effector mechanisms, which are largely absent with complete TNF deficiency.

Membrane TNF enables cytokine and chemokine secretion

We next investigated the secretion of cytokines and chemokines in BALF as possible factors that influences cellular recruitment and protective immunity. Chemokine expres-
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Figure 5. Cellular recruitment in bronchoalveolar space in the lungs of M. bovis BCG-infected TNF<sup>tm/tm</sup> mice, TNF<sup>−/−</sup> mice and WT mice. BALF was collected from TNF<sup>tm/tm</sup> (line bar), TNF-deficient mice (open bar) and WT mice (black bar) infected i.v. with 2 x 10<sup>6</sup> BCG bacilli at 2, 4 and 8 weeks. Cells (200 cells/mouse) were stained using a Rapidiff staining kit (Clinical Sciences Diagnostics, Southdale, South Africa), the average differential cell count were analysed in triplicate and recorded as previously described.45 Mean values ± SD of total cell counts (a), macrophages (b), lymphocytes (c) and neutrophils (d) are given (n = 5).

Membrane TNF generates partial protective immunity against M. tuberculosis aerosol infection

In order to determine whether the partial protective immune response that was generated after the infection with M. bovis BCG is applicable during a virulent infection, mice were infected with a low dose (10–30 CFU/lung) of M. tuberculosis H37Rv by aerosol inhalation. Body weight ratios were determined for 7 weeks of infection and the groups were scored for mortality. Progressive infection was paralleled by continual lowering of body weight ratios and mortality in TNF<sup>−/−</sup> mice (Fig. 9a,b). In contrast, WT mice and TNF<sup>tm/tm</sup> did not show a loss of body weight during the early course of infection. After 8 weeks of infection, ~80% of TNF<sup>tm/tm</sup> mice were still alive, while all TNF<sup>−/−</sup> mice had succumbed to fatal pulmonary tuberculosis.

We further assessed the mycobacterial burden in the lung of M. tuberculosis-infected mice. While TNF<sup>−/−</sup> mice displayed an uncontrolled infection with significantly increased CFU already at 4 weeks (P < 0.05), the TNF<sup>tm/tm</sup> mice showed at least a partial control of mycobacterial growth, with CFU values significantly less (P < 0.05) than TNF<sup>−/−</sup> mice at 45 days (Fig. 10a). CFU counts in liver and spleen, as a measure of the systemic dissemination of M. tuberculosis, were significantly higher in TNF<sup>tm/tm</sup> and TNF<sup>−/−</sup> mice 45 days post-infection than in WT (Fig. 10a). However, the hepatic CFU in TNF<sup>tm/tm</sup> mice were lower (P < 0.05) than that of TNF<sup>−/−</sup> mice.

Lastly, we asked whether the protective effect of membrane TNF is signalled through TNFR1 or TNFR2. Therefore, we generated double transgenic mice by crossing TNF<sup>tm/tm</sup> mice with TNFR1<sup>−/−</sup> or TNFR2<sup>−/−</sup> mice. We further assessed the mycobacterial burden in the lung of M. tuberculosis infected mice. In this experiment, TNF<sup>−/−</sup> mice lost weight rapidly and died within 5 weeks, while TNF<sup>tm/tm</sup> mice were less sensitive, half surviving up to 45 days. TNF<sup>tm/tm</sup> x TNFR2 KO mice seemed essentially...
as sensitive as TNF\(^{-/-}\) mice, while TNF\(^{+/+}\) \times TNFR1 KO mice behaved more like TNF\(^{+/+}\) mice (Fig. 11a). Therefore, signalling of membrane TNF through TNF-R2 likely confers protection. This hypothesis is further corroborated by increased mycobacterial loads in the lung of TNF\(^{+/+}\) \times TNFR2 KO at 6 weeks post-infection, a time point when all TNF\(^{-/-}\) had succumbed, essentially doubled as compared to TNF\(^{+/+}\) or TNF\(^{+/+}\) \times TNFR1 KO mice (Fig. 11b).

Therefore, the sole expression of membrane TNF provided partial protection against H37Rv infection and the data using the double transgenic mice indicate that membrane TNF signalling through TNF-R2 confers the protective effect of membrane-bound TNF.

**Figure 6.** Reduced pulmonary recruitment of CD4 and CD8 T cells in TNF\(^{+/+}\) mice and TNF\(^{-/-}\) mice. BALF was collected from *M. bovis* BCG-infected TNF\(^{+/+}\), TNF\(^{-/-}\) and WT mice at 4 weeks post-infection as described in Methods. Four mice were individually analysed for each mouse strain. (a) Recruitment of CD4 and CD8 T cells in WT, TNF\(^{+/+}\) and TNF\(^{-/-}\) mice. (b) Expression of CD11a and CD44 on CD4 and CD8 cells in WT, TNF\(^{+/+}\) and TNF\(^{-/-}\) mice.
Discussion

Here we demonstrated that the Δ1-12 TNF mutant, which expresses only membrane TNF is partially resistant to infection to the non-virulent vaccine strain *M. bovis* BCG with 50% of mice surviving the experimental period, while all TNF-deficient mice succumb to infection. TNF<sup>tm/tm</sup> mice were able to recruit and activate macrophages and T cells and generate mycobactericidal granuloma in response to BCG infection. However, resistance to the virulent *M. tuberculosis* strain was less pronounced, suggesting substantial differences between this and the Δ1-9,K11E mouse mutant. This TNF mutant differs from the Δ1-9,K11E TNF allele in several aspects – the lymphoid structure especially is abnormal. We and others demonstrated that the Δ1-9,K11E mutant has enhanced resistance to *M. tuberculosis* infection and *Listeria* infection.

A critical role of TNF for the effective control and resolution of mycobacterial infection has been demonstrated previously. Further, TNFR1-mediated signalling is required for generating protective immunity against mycobacterial infection, which is probably more important than TNFR2 signalling. TNF provided by recombinant BCG expressing TNF may reconstitute granuloma formation and host response in TNF<sup>–/–</sup> mice, demonstrating the critical role of TNF and TNFR1 signalling.

The partial protection that is generated in TNF<sup>tm/tm</sup> mice could indicate local cell-to-cell TNF signalling by membrane expressed TNF on T cells or macrophages at the site of infection leading to a partial activation of the immune cells. Several biological functions of membrane TNF have been reported previously and a preferential TNFR2 signalling has been suggested in vitro and in vivo using transgenic mice expressing membrane TNF. The present data therefore suggest a functional role of TNFR2 in host protection. Furthermore, membrane TNF has been shown to be involved in reverse (outside-to-inside) signalling. Upon ligation of its receptor membrane-bound TNF-expressing cells are activated to express E-selectin. Thus, membrane-bound TNF, at least in T cells, might function as a bipolar positive regulator of inflammation either transmitting signals as a ligand to

Figure 7. Reduced expression of iNOS in lungs of BCG-infected TNF<sup>tm/tm</sup> mice and TNF<sup>–/–</sup> mice at 4 weeks post-infection. Lung sections were incubated with iNOS antibody as described. Magnification 100x.

Figure 8. Cytokine and chemokine secretion in BALF of TNF<sup>tm/tm</sup> mice (line bar), TNF<sup>–/–</sup> mice (open bar) and WT mice (black bar). (a) IFN-γ and IL-12 were measured at 28 days, and (b) RANTES was analysed at 28 and 56 days. The results are expressed as the mean ± SD (n = 4).
target cells or receiving signals through membrane TNF itself into T cells.

Although the exact mechanism of how protection is acquired through membrane TNF is unclear, membrane TNF on activated T cells might be sufficient for partial activation of macrophages; this results in the upregulation of iNOS expression, which is crucial for bacterial killing.21,41–43 Membrane TNF expressed on T cells might replace secreted TNF to form granulomas in TNF tm/tm mice. Macrophage and lymphocyte recruitment was significantly reduced in TNF tm/tm mice, with a significant reduction of the CD3+CD4+ and CD3+CD8+ T-cell subsets in the absence of TNF, which might be related to reduced chemokine production in the absence of TNF.14 As the recruitment of the different cell types was partially restored in TNF tm/tm mice, membrane TNF on T cells or other cells might provide a partial signal for chemokine induction and hence increased cell trafficking. The contribution of CD4+ T-cells in cell-mediated immunity against mycobacteria has been investigated by the use of neutralizing antibodies and gene-deficient mice.1 In the absence of CD4+ typical granuloma formation in T cells was largely absent.44

However, in view of the only partial activation and protection, which is likely caused by direct cell–cell contact, secreted TNF and hence distal signalling, appears to be required for a full protective host response. This finding concurs with the recent data demonstrating reduced severity of autoimmune encephalomyelitis, but little effect on inflammation in the spinal cord in a similar genetic mouse model.24

Lastly, the infection studies in double TNF tm/tm × TNFR1 or R2 tm/tm transgenic mice rescues protection in TNF tm/tm × TNFR2 tm/tm mice and comparable protection in TNF tm/tm × TNFR1 tm/tm mice as in TNF tm/tm mice suggest that membrane signalling though TNFR2 likely provides host protection. Our data in this infection model using the ΔI-12 TNF mutant mouse shows a reduced protective immune response during mycobacterial infection when compared to previous findings in which a membrane TNF transgenic mutant or the ΔI-9,K11E mutant were used. Our findings indicate that membrane TNF, in the ΔI-12 TNF mutant may preferentially induce protection through TNFR2. Receptor signalling in membrane TNF transgenic mutant or the ΔI-9,K11E mutant may be mediated through both TNFR1 and TNFR2 different, accounting for the differences observed in the degree of protection afforded by membrane TNF in the different mutant strains.
The results are expressed as the mean ± SD (n = 9–11 mice pooled from two independent experiments). (b) Mycobacterial burden in the lung (CFU) 6 weeks after infection.

### Figure 11. Comparison of resistance to *M. tuberculosis* infection in TNF<sup>tm/tm</sup> and TNF<sup>R1<sup>−/−</sup></sub> or TNF<sup>R2<sup>−/−</sup></sub> × TNF<sup>tm/tm</sup> double transgenic mice. (a) Survival of mice infected by H37Rv i.n. (10–30 CFU per lung, n = 9–11 mice pooled from two independent experiments). (b) Mycobacterial burden in the lung (CFU) 6 weeks after infection. The results are expressed as the mean ± SD (n = 4–6 mice per group of transgenic mice).

The data suggest that sparing membrane expressed TNF in therapeutic interventions neutralizing TNF such as in rheumatoid arthritis might reduce infectious complications. In summary, we show that membrane TNF participates in cell-mediated immunity against mycobacterial infection. The protective effect of membrane-bound TNF is likely mediated through TNFR2 signalling.

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### References

1. Flynn JL, Chan J. Immunology of tuberculosis. *Annu Rev Immunol* 2001; 19:93–129.
2. North RJ, Jung YI. Immunity to tuberculosis. *Annu Rev Immunol* 2004; 22:599–623.
3. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bacterial granulomas during BCG infection. *Cell* 1989; 56:731–40.
4. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotixin. *J Immunol* 1999; 162:3504–11.
5. Flynn JL, Goldstein MM, Chan J et al. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunology* 1995; 2:561–72.
6. Ehlers S, Kutsch S, Ehlers EM, Benini J, Pfeffer K. Lethal granuloma disintegration in mycobacteria-infected TNFRp55–/- mice is dependent on T cells and IL-12. *J Immunol* 2000; 165:483–92.
7. Jacobs M, Marino MW, Brown N, Abel B, Bekker LG, Quesniaux VJ, Fick L, Ryffel B. Correction of defective host response to *Mycobacterium bovis* BCG infection in TNF-deficient mice by bone marrow transplantation. *Lab Invest* 2000; 80:901–14.
8. Senaldi G, Yin S, Shaklee CL, Piguet PF, Mak TW, Ulich TR. *Corynebacterium parvum* and *Mycobacterium bovis* bacillus Calmette-Guérin-induced granuloma formation is inhibited in TNF receptor I (TNF-RI) knockout mice and by treatment with soluble TNF-RI. *J Immunol* 1996; 157:5022–6.
9. Roach DR, Briscoe H, Saunders B, France MP, Riminton S, Britton WJ. Secreted lymphotixin-alpha is essential for the control of an intracellular bacterial infection. *J Exp Med* 2001; 193:239–46.
10. Wallach D, Varfolomeev EE, Malinin NL, Goltsvev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. *Annu Rev Immunol* 1999; 17:331–67.
11. Jacobs M, Brown N, Allie N, Chetty K, Ryffel B. Tumor necrosis factor receptor 2 plays a minor role for mycobacterial immunity. *Pathobiology* 2000; 68:68–75.
12. Ehlers S, Holscher C, Scheu S, Tertilt C, Hohlms T, Suwinski J, Endres R, Pfeffer K. The lymphotixin beta receptor is critically involved in controlling infections with the intracellular pathogens *Mycobacterium tuberculosis* and *Listeria monocytogenes*. *J Immunol* 2003; 170:5210–8.
13. Lucas R, Tacchini-Cottier F, Guler R et al. A role for lymphotxin beta receptor in host defense against *Mycobacterium bovis* BCG infection. *Eur J Immunol* 1999; 29:4002–10.
14. Sedgwick JD, Riminton DS, Cyster JG, Korner H. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today* 2000; 21:110–3.
15. Vassalli P. The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 1992; 10:411–52.
16. Black RA, Rauch CT, Kozloski CJ et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 1997; 385:729–33.
17. Decker T, Lohmann-Matthes ML, Gifford GE. Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages. *J Immunol* 1987; 320:957–62.
18. Krieger M, Perez C, DeFay K, Albert I, Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. *Cell* 1988; 53:45–53.
19. Grell M, Douni E, Wajant H et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 1995; 83:793–802.
20. Akassoglou K, Probert L, Kostogeorgos G, Kollias G. Astrocyte-specific but non-neuron-specific transmembrane TNF triggers inflammation and degeneration in the central nervous system of transgenic mice. *J Immunol* 1997; 158:438–45.

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21 Olleros ML, Guler R, Corazza N et al. Transmembrane TNF induces an efficient cell-mediated immunity and resistance to *Mycobacterium bovis* bacillus Calmette–Guérin infection in the absence of secreted TNF and lymphotoxic-alpha. *J Immunol* 2002; 168:3394–401.

22 Olleros ML, Guler R, Vesin D et al. Contribution of transmembrane tumor necrosis factor to host defense against *Mycobacterium bovis* bacillus Calmette–Guérin and *Mycobacterium tuberculosis* infections. *Am J Pathol* 2005; 166:1109–120.

23 Alexopoulou L, Pasparakis M, Kollias G. A murine transmembrane tumor necrosis factor transgene induces arthritis by cooperative p55/p75 TNF receptor signaling. *Eur J Immunol* 1997; 27:2588–92.

24 Ruuls SR, Hoek RM, Ngo VN et al. Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. *Immunology* 2001; 15:533–43.

25 Alexopoulou L, Kranidioti K, Xanthoulea S et al. Transmembrane TNF protects mutant mice against intracellular bacterial infections, chronic inflammation and autoimmunity. *Eur J Immunol* 2006; 36:2768–80.

26 Saunders BM, Tran S, Ruuls S, Sedgwick JD, Briscoe H, Britton WJ. Transmembrane TNF is sufficient to initiate cell migration and granuloma formation and provide acute, but not long-term, control of *Mycobacterium tuberculosis* infection. *J Immunol* 2005; 174:4852–9.

27 Fremond C, Allie N, Dambuza I, Grivennikov SI, Yeremeev V, Quesniaux VF, Jacobs M, Ryffel B. Membrane TNF confers protection to acute mycobacterial infection. *Respir Res* 2005; 6:136.

28 Torres D, Janot L, Quesniaux VF, Grivennikov SI, Maïllet I, Sedgwick JD, Ryffel B, Erard F. Membrane tumor necrosis factor confers partial protection to *Listeria* infection. *Am J Pathol* 2005; 167:1677–87.

29 Marino MW, Dunn A, Grail D et al. Characterization of tumor necrosis factor-deficient mice. *Proc Natl Acad Sci USA* 1997; 94:8093–8.

30 Rothe J, Lesslauer W,Lotscher H et al. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by *Listeria monocytogenes*. *Nature* 1993; 364:798–802.

31 Erickson SL, de Sauvage FJ, Kikly K et al. Decreased sensivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. *Nature* 1994; 372:560–3.

32 Car BD, Eng VM, Schnyder B et al. Interferon gamma receptor-deficient mice are resistant to endotoxic shock. *J Exp Med* 1994; 179:1437–44.

33 Müller M, Eugster HP, Le Hir M, Shakhov A, Di Padova F, Maurer G, Quesniaux VF, Ryffel B. Correction or transfer of immunodeficiency due to TNF-LT alpha deletion by bone marrow transplantation. *Mol Med* 1996; 2:247–55.

34 Czernak BJ, Sarma V, Bless NM, Schmal H, Friedl HP, Ward PA. *In vitro* and *in vivo* dependency of chemokine generation on C5a and TNF-alpha. *J Immunol* 1999; 162:2321–5.

35 Botha T, Ryffel B. Reactivation of latent tuberculosis infection in TNF-deficient mice. *J Immunol* 2003; 171:3110–8.

36 Algood HM, Lin PL, Yankura D, Jones A, Chan J, Flynn JL. TNF influences chemokine expression of macrophages in *vitro* and that of CD11b+ cells in *vivo* during *Mycobacterium tuberculosis* infection. *J Immunol* 2004; 172:6846–57.

37 Bekker LG, Moreira AL, Bergtold A, Freeman S, Ryffel B, Kaplan G. Immunopathologic effects of tumor necrosis factor alpha in murine mycobacterial infection are dose dependent. *Infect Immun* 2000; 68:6954–61.

38 Lucas R, Juillard P, Decoster E et al. Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. *Eur J Immunol* 1997; 27:1719–25.

39 Kusters S, Tiegs G, Alexopoulou L et al. In *vivo* evidence for a functional role of both tumor necrosis factor (TNF) receptors and transmembrane TNF in experimental hepatitis. *Eur J Immunol* 1997; 27:2870–5.

40 Harashima S, Horiuchi T, Hatta N et al. Outside-to-inside signal through the membrane TNF-alpha induces E-selectin (CD62E) expression on activated human CD4+ T cells. *J Immunol* 2001; 166:130–6.

41 Chan J, Xing Y, Magliozzo RS, Bloom BR. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *J Exp Med* 1992; 175:1111–22.

42 MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci USA* 1997; 94:5243–8.

43 García I, Guler R, Vesin D, Olleros ML, Vassalli P, Chvatchko Y, Jacobs M, Ryffel B. Lethal *Mycobacterium bovis* bacillus Calmette–Guérin infection in nitric oxide synthase 2-deficient mice: cell-mediated immunity requires nitric oxide synthase 2. *Lab Invest* 2000; 80:1385–97.

44 Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J Immunol* 1999; 162:5407–16.

45 Veenstra H, Dowdle EB. Multi-well cell monolayers for immunocytochemistry. An alternative to cytocentrifuge preparations. *J Immunol Methods* 1992; 146:257–8.