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# Prominent role for T cell-derived Tumour Necrosis Factor for sustained control of *Mycobacterium tuberculosis* infection

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**Tumour Necrosis Factor (TNF) is critical for host control of *M. tuberculosis*, but the relative contribution of TNF from innate and adaptive immune responses during tuberculosis infection is unclear. Myeloid versus T-cell-derived TNF function in tuberculosis was investigated using cell type-specific TNF deletion. Mice deficient for TNF expression in macrophages/neutrophils displayed early, transient susceptibility to *M. tuberculosis* but recruited activated, TNF-producing CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and controlled chronic infection. Strikingly, deficient TNF expression in T-cells resulted in early control but susceptibility and eventual mortality during chronic infection with increased pulmonary pathology. TNF inactivation in both myeloid and T-cells rendered mice critically susceptible to infection with a phenotype resembling complete TNF deficient mice, indicating that myeloid and T-cells are the primary TNF sources collaborating for host control of tuberculosis. Thus, while TNF from myeloid cells mediates early immune function, T-cell derived TNF is essential to sustain protection during chronic tuberculosis infection.**

Initial events during pulmonary *M. tuberculosis* infection involve binding and uptake of *M. tuberculosis* by alveolar macrophages. Subsequent immune responses are therefore dependent on initial activation of infected macrophages through, amongst others, induction of appropriate activating molecules, production of cytokines and chemokines and synthesis of bactericidal molecules. *In vitro* studies have shown that macrophage stimulation through *M. tuberculosis* or mycobacterial antigens results in secretion of TNF which is a requirement for induction of cell activation<sup>1–4</sup>. However, coactivation by IFN $\gamma$  is required for full bactericidal status and inhibition of mycobacterial growth<sup>2,5</sup>. *In vivo* challenge studies confirmed TNF as an integral component of the host immune defence mechanism against mycobacterial infection that promotes the initiation and maintenance of granulomas during infection<sup>6–9</sup>. Amongst other family members, such as lymphotoxin (LT)  $\alpha$ , LT $\beta$ , FasL, CD40L, RANKL, CD27L and OX40L<sup>10</sup>, TNF mediated protection is non-redundant despite the potential of soluble LT $\alpha$  to also bind and signal through both TNF receptors<sup>11,12</sup>. TNFRp55 is thought to be primarily responsible for mediating most TNF mediated effects<sup>13,14</sup>. TNF signalling can induce cell death, promote cell survival and induce proinflammatory responses under defined cellular and micro-environmental conditions<sup>14</sup>. Both 26 kD membrane-anchored and 17 kD soluble TNF forms<sup>15,16</sup> contribute to the control of *M. tuberculosis* infection<sup>17–19</sup>. TNF is produced by multiple cell types including macrophages, neutrophils, dendritic cells, lymphocytes and NK cells as well as by cells of non-hematopoietic origin<sup>20</sup>. However, the precise role of TNF derived from these cells in infection remains unclear and requires investigation. The clinical importance of TNF to control mycobacterial replication was demonstrated during anti-TNF treatment of patients with rheumatoid arthritis, Crohn's disease or severe psoriasis, where, in some cases, patients developed spontaneous tuberculosis reactivation<sup>21,22</sup>. T cells and macrophages are crucial components of protective granulomas and are potentially relevant sources of TNF *in vivo*, responding to antigens or to microbial components, respectively<sup>23,24</sup>. We reported earlier that TNF derived from hematopoietic cells is essential to control mycobacterial BCG infection<sup>9</sup>. Further, cell transfer experiments suggest that TNF produced by T cells may be essential but not sufficient for antimycobacterial host defense<sup>25</sup>. It is thus



important to define the contribution and kinetics of the TNF responses issued from adaptive, T lymphocytes versus innate phagocytic, macrophage/neutrophils, in the control of acute and chronic *M. tuberculosis* infection.

In this study we examined the role of TNF produced by macrophages and neutrophils, or by T-cells, using cell-specific gene-inactivated mice<sup>26</sup> in *M. tuberculosis* infection models. We demonstrate the significance of myeloid, macrophage/neutrophil derived TNF for protective immune responses during early *M. tuberculosis* infection. This function becomes redundant during persistent infection, while T-cell derived TNF is essential for long-term control of chronic infection in a non-redundant fashion. Therefore, two major cellular sources of TNF in protection against mycobacteria display both redundant/overlapping and non-redundant/specific functions.

## Results

**TNF produced by myeloid cells is required for control of pulmonary *M. tuberculosis* replication during acute, but not persistent, infection.** To address whether TNF derived from macrophages/neutrophils is required for protective immune functions *in vivo*, we first investigated disease outcome in M-TNF<sup>-/-</sup> mice during infection with virulent *M. tuberculosis* in comparison with either immune-competent WT mice or complete TNF deficient mice. The inability to produce any TNF rendered mice highly susceptible to *M. tuberculosis* aerosol inhalation challenge resulting in rapid weight loss (Fig. 1A) and death within 42 days (Fig. 1B), confirming previous reports<sup>6,18</sup>. Strikingly, both WT- and M-TNF<sup>-/-</sup> mice were resistant to infection without notable loss of bodyweight (Fig. 1A) and survived the 7 month duration of the experiment (Fig. 1B). Thus quite unexpectedly, survival of M-TNF<sup>-/-</sup> mice clearly indicated that macrophages and neutrophils were not the unique, essential sources of TNF to elicit a sustained protective response to *M. tuberculosis*.

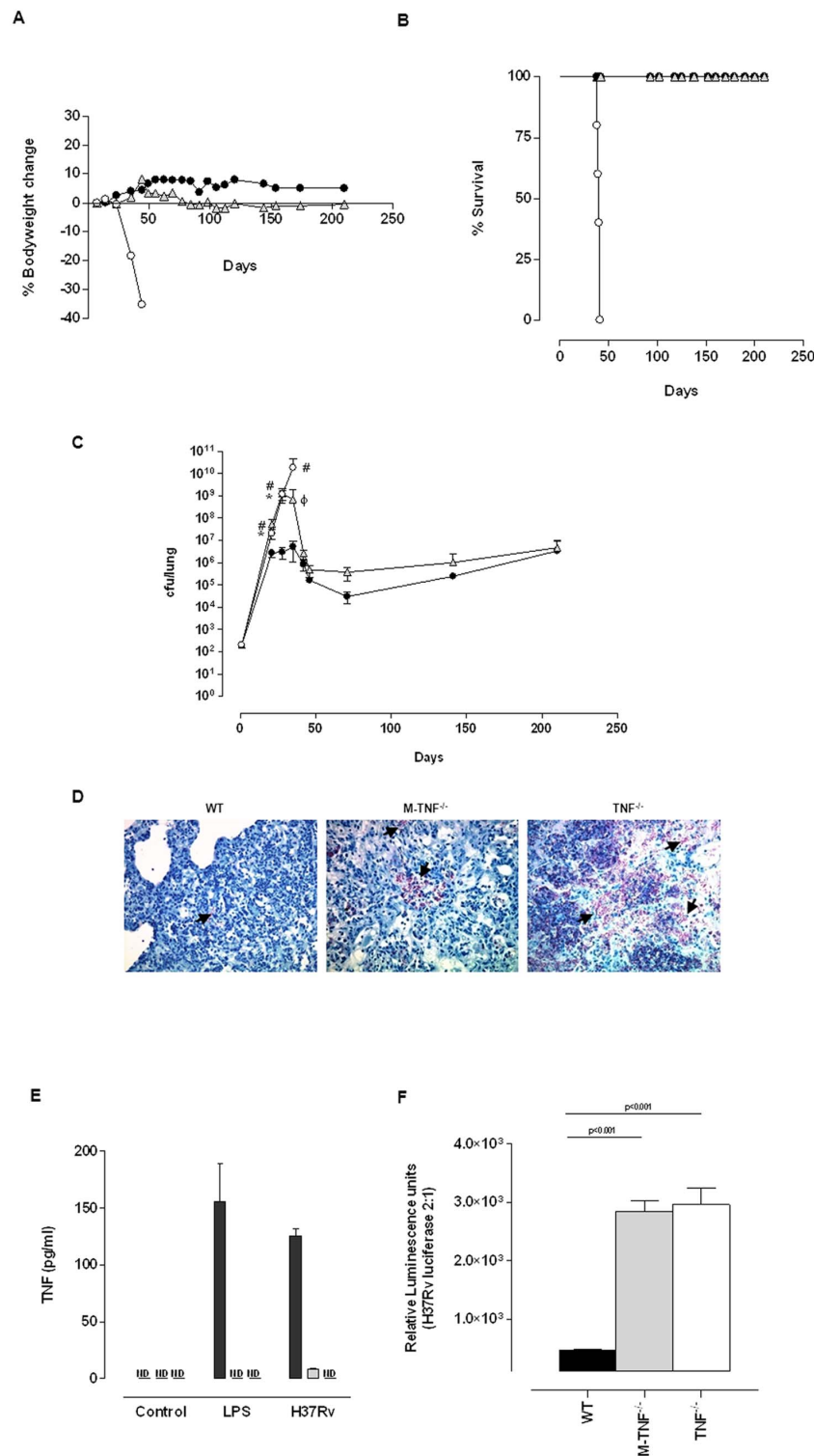
Several studies have correlated uncontrolled mycobacterial replication with high susceptibility in TNF<sup>-/-</sup> mice<sup>6,9,27</sup>. Our working hypothesis was that *M. tuberculosis* recognition may lead to suboptimal macrophage activation in the absence of TNF synthesis in M-TNF<sup>-/-</sup> mice, resulting in possible susceptibility due to a reduced capability to restrict bacterial replication during early infection. The resistant phenotype of M-TNF<sup>-/-</sup> mice was unexpected since TNF is required for optimum activation of infected macrophages to restrict *M. tuberculosis* growth<sup>2,28–30</sup>. We thus investigated the effect of TNF produced by myeloid cells on bacterial replication by comparing pulmonary bacilli growth in WT-, M-TNF<sup>-/-</sup>- and TNF<sup>-/-</sup> mice over a period of 7 months after *M. tuberculosis* challenge. Interestingly, M-TNF<sup>-/-</sup> mice failed to control pulmonary bacilli replication within the first 3–4 weeks of infection and developed high bacilli titers similar to TNF<sup>-/-</sup> mice which were 1–2 log<sub>10</sub> higher than WT mice (Fig. 1C). Ziehl-Neelsen staining of infected lung tissue confirmed distinctive differences in bacilli loads in the respective genotypes (Fig. 1D). However, the susceptibility of M-TNF<sup>-/-</sup> mice was transient and the high pulmonary bacilli burden was eventually reduced to levels similar to that of WT mice during persistent infection, whereas in TNF<sup>-/-</sup> mice bacilli replication remained unrestricted. Thus, the absence of TNF production by macrophages/neutrophils was clearly associated with defective control of bacilli growth during early infection, but it could be compensated for thereafter.

Considering earlier reports on TNF dependent restriction of *M. tuberculosis* replication we wanted to confirm that early susceptibility of M-TNF<sup>-/-</sup> was indeed a function of deficient TNF synthesis and bacterial control by macrophages from these mice<sup>31–33</sup>. TNF release by macrophages occurs upon infection or exposure to various stimuli<sup>29,34,35</sup>. WT macrophages released TNF after infection with *M. tuberculosis* H37Rv (Fig. 1E), and the growth of the bacilli was restrained, as seen with the luminescent, luciferase expressing

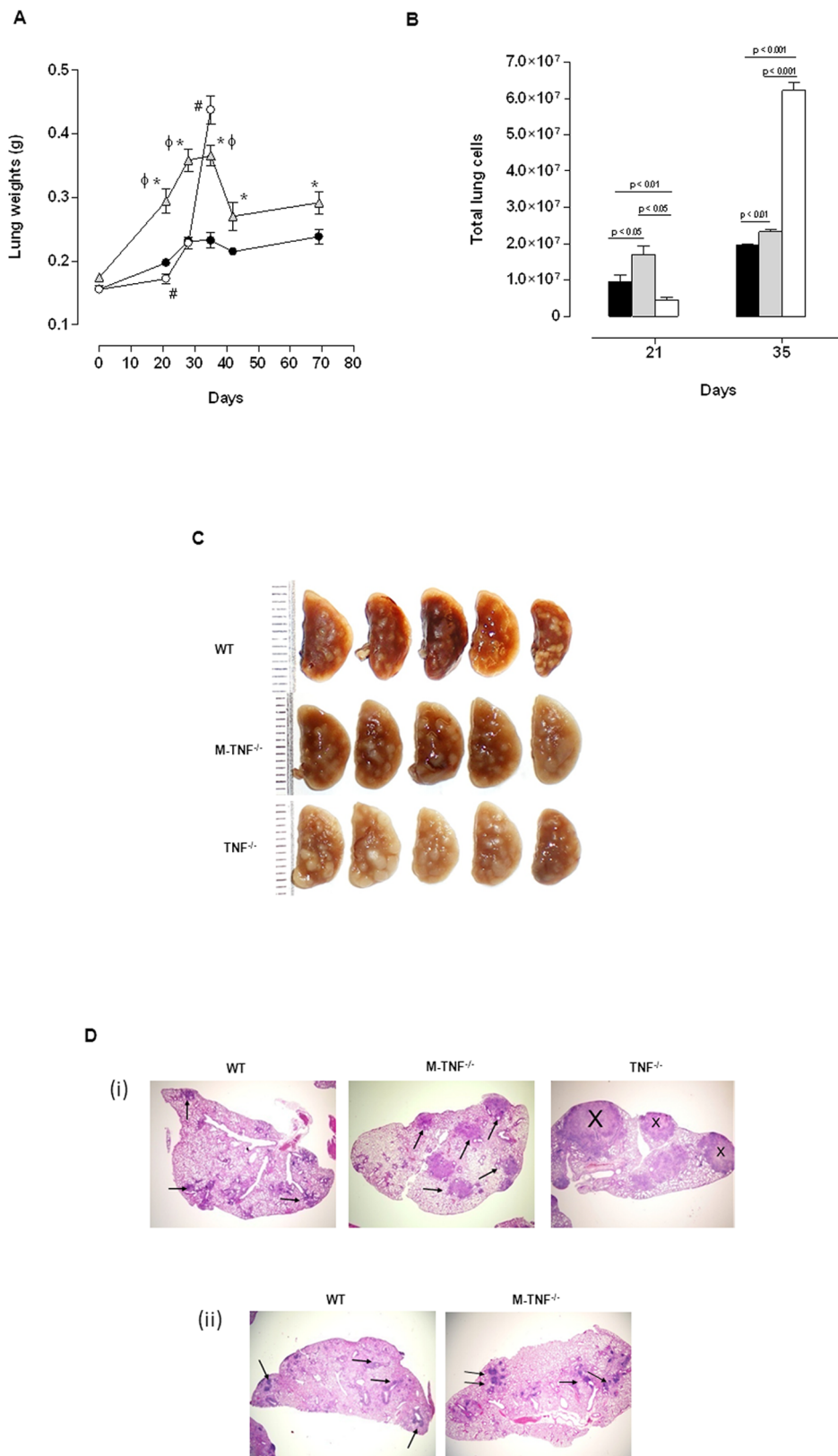
reporter *M. tuberculosis* H37Rv strain (Fig. 1F). In contrast, in macrophages from TNF<sup>-/-</sup> mice, the absence of TNF release, was accompanied by failure to restrain the bacilli growth. We demonstrate here that infected macrophages derived from M-TNF<sup>-/-</sup> mice that fail to produce TNF as shown earlier<sup>36</sup> are defective in controlling bacteria growth, similar to macrophages from TNF<sup>-/-</sup> mice. Therefore, our data clearly supports the importance of TNF, and in particular macrophage-derived TNF, in controlling mycobacterial replication.

**TNF produced by myeloid cells regulates lung inflammation but is not required for the initiation and maintenance of granuloma structural integrity.** It is well established that containment and control of bacilli growth is dependent on the establishment of functional, bactericidal granulomas. In view of uncontrolled *M. tuberculosis* replication during early infection in M-TNF<sup>-/-</sup> mice, we expected that the establishment of granulomas would be defective in the absence of TNF synthesis by macrophages and neutrophils. TNF is required not only for the initiation, but also for maintenance of granulomas and we and others have previously reported the lack of granuloma formation in the absence of TNF after mycobacterial challenge<sup>6–9</sup>. However, granulomas comprise different cell types, many capable of synthesising TNF that could potentially contribute to their structural integrity. To gain further insight into the transient nature of the immune response in M-TNF<sup>-/-</sup> mice, we investigated whether macrophages and neutrophils were an essential cellular source of TNF during the establishment of granulomas. As a correlate for infection-induced inflammation we measured lung weights (Fig. 2A) and found a transient increase of lung weights in M-TNF<sup>-/-</sup> mice which peaked at day 28 post infection, with partial resolution after day 35 post infection. In contrast, sustained, uncontrolled inflammation was observed in TNF<sup>-/-</sup> mice, at significantly higher levels than in either M-TNF<sup>-/-</sup> or WT mice on day 35 post infection, when TNF<sup>-/-</sup> mice succumbed to infection. Controlled inflammation in M-TNF<sup>-/-</sup> mice was substantiated by quantitative assessment of pulmonary infiltrating leukocytes (Fig. 2B) which was significantly higher compared to WT mice, while TNF<sup>-/-</sup> mice experienced a delay in cell recruitment at day 21 followed by excessive cell infiltration in the lung 35 days post-infection.

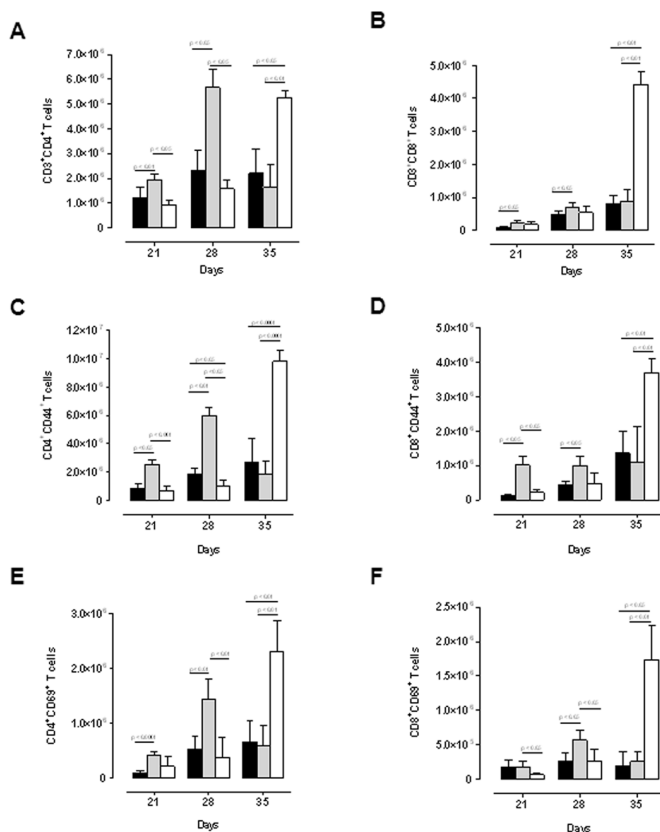
We further assessed the role of macrophage and neutrophil derived TNF for structural granuloma integrity during *M. tuberculosis* challenge. Macroscopic lung pathology showed no distinction amongst the strains after 21 days of infection (Suppl. Fig. 1), but differences were clearly evident at 28 days post-infection (Fig. 2C). Small, defined lesions were present on the pleura of WT mice while TNF<sup>-/-</sup> mice had large and in part confluent nodules. Interestingly, M-TNF<sup>-/-</sup> mice presented with an intermediate lesion phenotype not as severe as observed in TNF<sup>-/-</sup> mice but not as small and compact as in WT mice (Fig. 2C). Histological assessment showed that, although enlarged, pulmonary granulomas in M-TNF<sup>-/-</sup> mice were well structured with no necrotic areas, in contrast to TNF<sup>-/-</sup> mice which displayed effaced structures with necrotic centers on day 28 post infection (Fig. 2D). Importantly, the kinetics of granuloma formation was normal in M-TNF<sup>-/-</sup> mice with no evidence of a delay in the onset of granuloma initiation (data not shown) as previously reported for TNF<sup>-/-</sup> mice<sup>6</sup>. Examination of tissues at higher magnification clearly showed occlusion of bronchi and blood vessels in TNF<sup>-/-</sup> mice but not in M-TNF<sup>-/-</sup> or WT mice. Moreover, the differences noted in pulmonary pathology between WT and M-TNF<sup>-/-</sup> mice during early stages of infection were transient and clearly absent during chronic infection (Fig. 2Dii). At six months post-infection lung sections were pathologically indistinguishable, with defined structural granulomas present and no necrosis was evident in either M-TNF<sup>-/-</sup> or WT mice (Fig. 2Dii). Therefore, TNF produced by myeloid cells regulates inflammation and cellular



**Figure 1 | TNF from myeloid cells is required to control acute *M. tuberculosis* replication in vivo.** (A–D) WT (closed circle), M-TNF<sup>-/-</sup> (grey triangle) and TNF<sup>-/-</sup> (open circle) mice were infected by aerosol inhalation with 200–500 cfu/lung of *M. tuberculosis* H37Rv and body weight changes (A) and mortality (B) (n = 10 mice/group) were monitored. Changes in bodyweight values are expressed as the mean of 4–10 mice/group. Data represents one of three independent experiments. The number of viable bacteria present in lungs was assessed at the indicated time points (C). The results are expressed as the mean  $\pm$  SD of 5 mice/group per time point and represent one of three independent experiments. \* =  $p < 0.05$  (WT versus M-TNF<sup>-/-</sup> mice), # =  $p < 0.05$  (WT versus TNF<sup>-/-</sup> mice),  $\phi$  =  $p < 0.05$  (M-TNF<sup>-/-</sup> versus TNF<sup>-/-</sup> mice). Ziehl-Neelsen staining of lungs at 35 days post infection (D). The results represent one of four similar experiments. (Magnification = 600 $\times$ ). Arrows indicate the presence of AFB. (E) WT (black), M-TNF<sup>-/-</sup> (grey) and TNF<sup>-/-</sup> (clear) bone marrow derived macrophages were stimulated either with LPS (100 ng/ml) or *M. tuberculosis* H37Rv (at a MOI of 2:1) for 48 hrs and TNF measured by ELISA. The results are expressed as the mean  $\pm$  SD of quadruplicate values and are representative of one of three independent experiments. ND: Not detected. (F) Bone marrow derived macrophages were infected with *M. tuberculosis* expressing luciferase and bacterial replication measured as relative luminescence after 100 hrs. The results are expressed as the mean  $\pm$  SD of quadruplicate values and represent one of three independent experiments.



**Figure 2 | TNF from myeloid cells regulates cellular recruitment for granuloma formation but is dispensable for initiation and maintenance of granuloma structure.** (A; B) WT (black), M-TNF<sup>-/-</sup> (grey), and TNF<sup>-/-</sup> (clear) mice were infected with *M. tuberculosis* H37Rv as described under “Materials and Methods” and lung weights (A) and cell numbers (B) assessed at the indicated time points. Data represent the mean  $\pm$  SD of 5 mice per time point and one of three independent experiments. \* =  $p < 0.05$  (WT versus M-TNF<sup>-/-</sup> mice), # =  $p < 0.05$  (WT versus TNF<sup>-/-</sup> mice.),  $\phi$  =  $p < 0.05$  (M-TNF<sup>-/-</sup> versus TNF<sup>-/-</sup> mice). (C–D). Macroscopic lung pathology (C) in WT, M-TNF<sup>-/-</sup> and TNF<sup>-/-</sup> mice at 28 days post infection (one of three independent experiments;  $n = 5$  mice/strain). Low magnification (20 $\times$ ) of pulmonary granulomas at (i) 28 days and (ii) 166 days postinfection after haematoxylin and eosin staining (D). Arrows indicate granulomatous structures and X indicates necrosis. The results represent one of 5 similar experiments.



**Figure 3 | Transiently enhanced pulmonary infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> activated T cells is independent of TNF produced by myeloid cells during *M. tuberculosis* infection.** WT (black), M-TNF<sup>-/-</sup> (grey) and TNF<sup>-/-</sup> (clear) mice were infected via aerosol inhalation with 200–500 cfu/lung of *Mycobacterium tuberculosis*. Lung cell suspensions from infected mice were analyzed by flow cytometry to determine (A) CD3<sup>+</sup>CD4<sup>+</sup> and (B) CD3<sup>+</sup>CD8<sup>+</sup> T cell pulmonary populations at day 21, 28 and 35 post-infection and activation status analysed for markers CD44 (C and D) and CD69 (E and F) on CD4<sup>+</sup> (C, E) and CD8<sup>+</sup> (D, F) T cells by flow cytometry. The results are expressed as the mean  $\pm$  SD of 5 mice per group and are from one experiment, representative of two independent experiments.

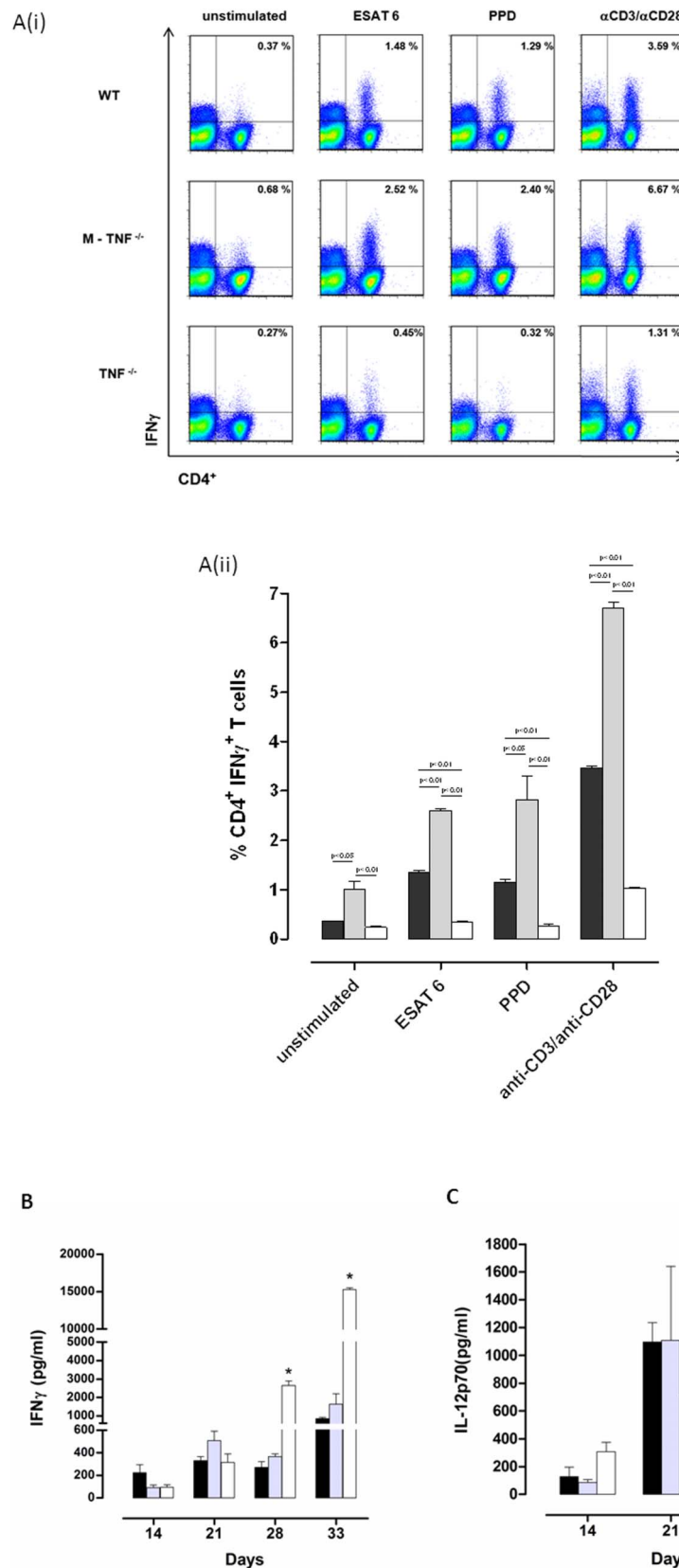
recruitment to the lung but is not required for initiating and maintaining the structural integrity of granulomas.

**TNF produced by myeloid cells regulates pulmonary lymphocytic recruitment during early *M. tuberculosis* infection.** Protective immunity in response to *M. tuberculosis* is cell-mediated<sup>37,38</sup> and the recruitment of T cells, especially CD4<sup>+</sup> and CD8<sup>+</sup> T cells, is critical to control the growth of bacilli<sup>39,40</sup>. TNF is an important mediator of cellular traffic through the regulation of adhesion molecule expression, dendritic cell maturation and chemokine induction<sup>27,41</sup>. To understand the mechanism responsible for the transient nature of susceptibility in M-TNF<sup>-/-</sup> mice, we asked whether lymphocytic recruitment and activation were affected by the lack of macrophage/neutrophil derived TNF. Here, we first measured migration of lymphocytes to local sites of infection and found that M-TNF<sup>-/-</sup> mice had a small but significantly higher recruitment of both CD4<sup>+</sup> (Fig. 3A) and CD8<sup>+</sup> (Fig. 3B) T cells to the lung at 21 and 28 days post infection as compared to WT mice. Enhanced lymphocytic recruitment in M-TNF<sup>-/-</sup> mice was transitory and levels similar to those in WT mice were recorded at 35 days post infection. Conversely, TNF<sup>-/-</sup> mice seemed to control cell migration during the early stages of infection, but 2–4-fold increased CD4<sup>+</sup> and CD8<sup>+</sup> T cell levels were measured on day 35, correlating with the overwhelming bacilli burden at this latter stage

of infection. The transient increase in pulmonary CD4<sup>+</sup> and CD8<sup>+</sup> T cells seen during early infection in M-TNF<sup>-/-</sup> mice was effectively controlled thereafter, suggesting that the control of excessive lymphocytic response was independent of macrophage/neutrophil derived TNF but likely dependent upon another source of TNF like T-cell derived TNF. We further assessed the activation status of the lung infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets by flow cytometry for expression of the leukocyte homing and activation marker CD44 (Fig. 3C and 3D) and the early activation marker CD69 (Fig. 3E and 3F). CD4<sup>+</sup> and CD8<sup>+</sup> T cells recruited to the lung after *M. tuberculosis* infection expressed CD44 and CD69 in all the respective strains. Thus, the absence of TNF production by myeloid cells favoured early recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> activated T cells to the lung in response to *M. tuberculosis* infection.

***M. tuberculosis* specific T cell-priming is independent of TNF produced by myeloid cells.** Resistance to *M. tuberculosis* is highly dependent on the host's capability to induce an effective Th1 immune response<sup>40,42,43</sup>. Our studies indicated a down-regulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment by TNF synthesized by myeloid cells with no clear effect on lymphocyte activation. To further assess how distinct cellular sources of TNF may affect Th1 immune function we next investigated IFN $\gamma$  release by primed CD4<sup>+</sup> and CD8<sup>+</sup> T cells in response to mycobacterial antigens in M-TNF<sup>-/-</sup> mice. Single cell suspensions from lungs harvested at 21 day post *M. tuberculosis* infection were restimulated *ex vivo* with purified protein derivative (PPD), early secreted antigen of 6 kDa (ESAT-6), or anti-CD3/anti-CD28 antibodies. Restimulation of lung infiltrating CD4<sup>+</sup> T cells from TNF<sup>-/-</sup> mice showed compromised IFN $\gamma$  expression in response to ESAT-6, PPD or anti CD3/CD28 antibodies, to only one third of that seen in WT cells, while cells from M-TNF<sup>-/-</sup> mice had a 2-fold increased IFN $\gamma$  response (Fig. 4A(i and ii)). We found no differences in IFN $\gamma$  synthesis of restimulated CD8 T cells amongst the mutant and control strains (data not shown). Moreover, the level of IFN $\gamma$  in whole lung homogenates was increased in M-TNF<sup>-/-</sup> mice at 21 days post-infection and contracted thereafter to concentrations equivalent to WT mice, while pulmonary IFN $\gamma$  in TNF<sup>-/-</sup> mice were equivalent to WT mice during the first 3 weeks and highly increased at 4 weeks (Fig. 4B) Effective control of *M. tuberculosis* infection is only possible when IL-12 synergizes with IFN $\gamma$  to induce a Th1 immune response<sup>28,44</sup>. Pulmonary IL-12p70 levels increased strongly between 2 and 3 weeks post-infection in both WT and M-TNF<sup>-/-</sup> mice but were halved in TNF<sup>-/-</sup> mice (Fig. 4C). Therefore, the absence of TNF production by myeloid cells did not compromise *M. tuberculosis* specific priming and restimulation of lung infiltrating CD4<sup>+</sup> T cells.

**TNF release by lung infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells during *M. tuberculosis* infection is not compromised by the absence of TNF from myeloid origin.** The overall protection against *M. tuberculosis* infection seen in M-TNF<sup>-/-</sup> mice indicated that TNF derived from an alternative cellular source, other than macrophages/neutrophils contributed to protective immune functions. We thus next addressed the contribution of T cells, a major cellular component of bactericidal granuloma, as a source of TNF during *M. tuberculosis* infection that could contribute to control bacilli replication. Adoptive transfer experiments previously illustrated that TNF secretion by lymphocytes is protective against *M. tuberculosis* challenge<sup>25</sup>. Moreover, it is known that T cells produce TNF upon antigen specific activation and the addition of CD4<sup>+</sup> T cells to *M. tuberculosis* infected monocytes enhances TNF production by both T cells and monocytes<sup>45</sup>. We first investigated whether T cells do respond to *M. tuberculosis* challenge by expressing TNF *in vivo* and how this may be influenced by the absence of TNF from myeloid origin. TNF expression by CD4<sup>+</sup> or CD8<sup>+</sup> T cells isolated from *M. tuberculosis* infected WT mice 21 days post-infection was analyzed



**Figure 4 | Enhanced *M. tuberculosis* specific priming of pulmonary CD4<sup>+</sup> T cells in infected M-TNF<sup>-/-</sup> mice.** (A) Lung cell suspensions from *M. tuberculosis* H37Rv infected WT, M-TNF<sup>-/-</sup>, and TNF<sup>-/-</sup> mice harvested at 21 days post infection were restimulated with ESAT 6, PPD or antibodies to CD3 and CD28 for 72 hrs and intracellular IFN $\gamma$  produced by CD4<sup>+</sup> T cells assessed by flow cytometry. Fig. 4A(i) are representative dot blots of single mice while the results in Fig. 4A(ii) are expressed as the mean  $\pm$  SD of 4 mice per group and represent one of two similar experiments. (B, C) Lung concentrations of IFN $\gamma$  (B) and IL-12p70 (C) were determined by ELISA in lung homogenates of *M. tuberculosis* H37Rv infected WT (black), M-TNF<sup>-/-</sup> (grey) and TNF<sup>-/-</sup> (open) mice at different time points. The data are expressed as the mean and  $\pm$  SD of 4 mice/group for each time point and represent one of two similar experiments.



by flow cytometry. TNF production was absent in CD4<sup>+</sup> T cells isolated from TNF<sup>-/-</sup> mice, as expected, while it was present or slightly increased in CD4<sup>+</sup> or CD8<sup>+</sup> T cells isolated from M-TNF<sup>-/-</sup> mice (Supplementary Fig. 2). Therefore, T cell derived TNF may compensate for the lack of TNF production by myeloid cells *in vivo* during *M. tuberculosis* infection.

**T cell derived TNF is required for sustained control of pulmonary *M. tuberculosis* during chronic infection.** Guided by our hypothesis that CD4<sup>+</sup>- and CD8<sup>+</sup> T cell derived TNF may compensate for the absence of TNF produced by myeloid cells during early infection, we next addressed the specific role of T-cell derived TNF in protective immunity to *M. tuberculosis* and disease outcome. T cell specific TNF<sup>-/-</sup> (T-TNF<sup>-/-</sup>) mice<sup>26</sup> were infected with *M. tuberculosis* and the absence of TNF release by CD4<sup>+</sup> or CD8<sup>+</sup> T cells verified on day 21 post-infection (Suppl. Fig. 2). Strikingly, while WT mice survived *M. tuberculosis* infection with no apparent loss of body mass and TNF<sup>-/-</sup> mice succumbed within 40 days with rapid bodyweight loss (Fig. 5A, B), T-TNF<sup>-/-</sup> mice survived significantly longer than TNF<sup>-/-</sup> mice, but eventually succumbed between 130–150 days post-infection accompanied by severe loss of body mass (Fig. 5A, B). Thus, ablation of TNF production by both CD4<sup>+</sup>- and CD8<sup>+</sup> T cells led to an inability of the host to sustain immune protection during chronic *M. tuberculosis* infection.

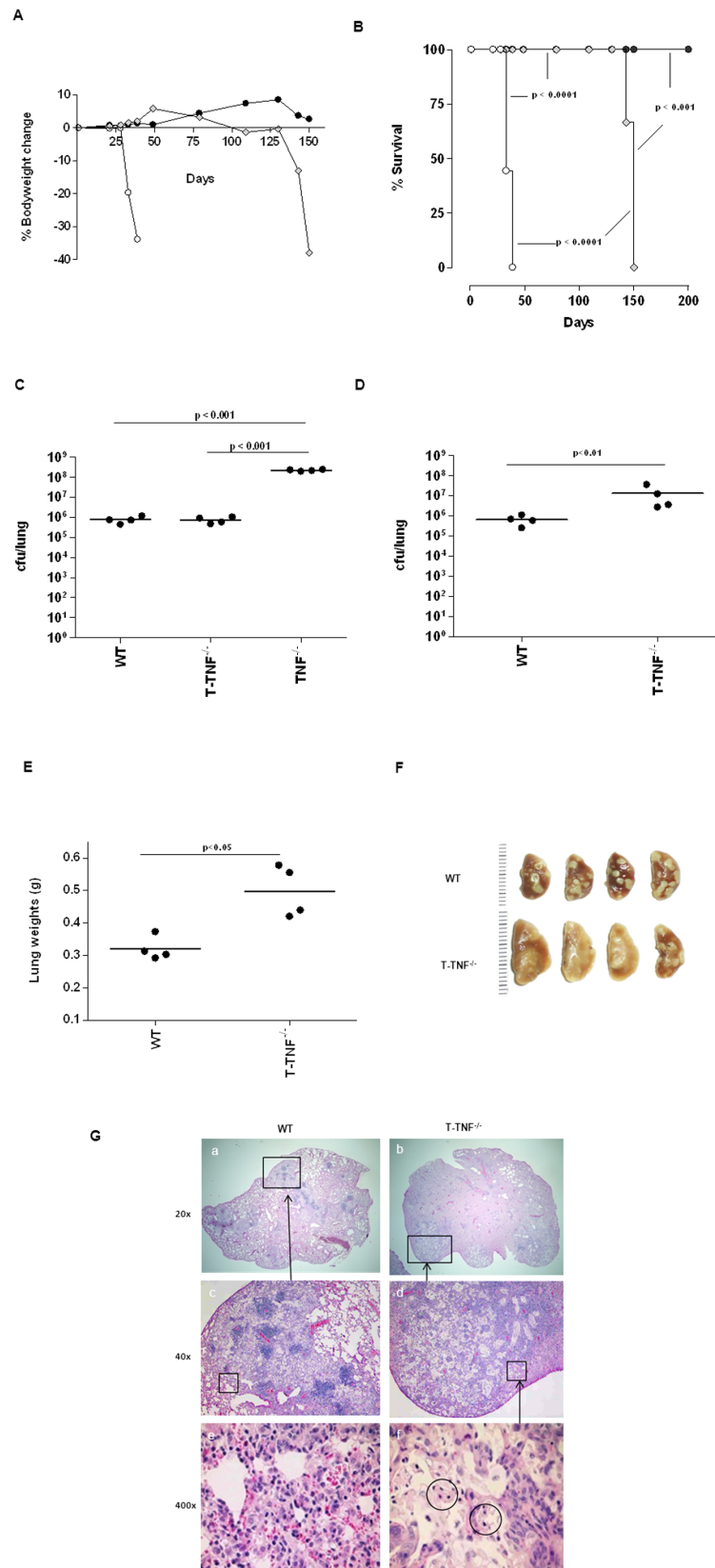
We next investigated whether T cell derived TNF was implicated in the control of *M. tuberculosis* replication during the generation of protective immune responses. Pulmonary bacilli burden measured in T-TNF<sup>-/-</sup> mice on day 33 post-infection was similar to that seen in WT mice, ca 5 log<sub>10</sub> cfu per lung, in contrast to the 10<sup>2</sup>–10<sup>3</sup> fold higher bacterial loads of TNF<sup>-/-</sup> mice (Fig. 5C). This indicated an efficient early control of bacilli replication, independent of T cell derived TNF during acute infection. Indeed, there was no significant alteration of overall pulmonary TNF mRNA and protein expression in cell-specific M-TNF<sup>-/-</sup> or T-TNF<sup>-/-</sup> mice, as compared to WT mice, 4 weeks post *M. tuberculosis* infection (Suppl. Figure 3). Considering mortality of T-TNF<sup>-/-</sup> mice at 150 days post-infection we evaluated bacilli burden during chronic infection (Fig. 5D). Pulmonary bacterial burden was 10-fold higher in T-TNF<sup>-/-</sup> mice compared to WT control animals suggesting an importance of T cell derived TNF for inhibiting pulmonary bacterial replication during persistent/chronic infection (Fig. 5D). Thus, although TNF is produced by many cell types, T cell derived TNF has a non-redundant function to sustain protection during chronic mycobacterial infection that cannot be compensated for by other sources of TNF, such as myeloid cells.

Lung weights were significantly higher in T-TNF<sup>-/-</sup> mice compared to WT mice at day 150 post-infection (Fig. 5E), indicative of enhanced inflammation. During chronic infection WT mice displayed discrete lesions under the pleura; while lesions appeared coalescent and severe in T-TNF<sup>-/-</sup> mice covering most of the lung surface (Fig. 5F). Indeed, WT mice showed discrete granulomatous lesions with aggregates of lymphocytes in lung tissue (Fig. 5G), while T-TNF<sup>-/-</sup> mice demonstrated severe pulmonary histopathology with large necrotic areas, coalescent with no clear demarcated areas of lymphocytic aggregates (Fig. 5G). At higher magnification the free alveolar and airway spaces seen in WT mice were largely occluded in T-TNF<sup>-/-</sup> mice. Cellular degeneration was readily noticeable within pulmonary tissue in the T-TNF<sup>-/-</sup> mice indicating extensive necrosis. Therefore, T cell derived TNF appears critical for the structural maintenance of the granuloma during chronic *M. tuberculosis* infection and overall resistance to the pathogen.

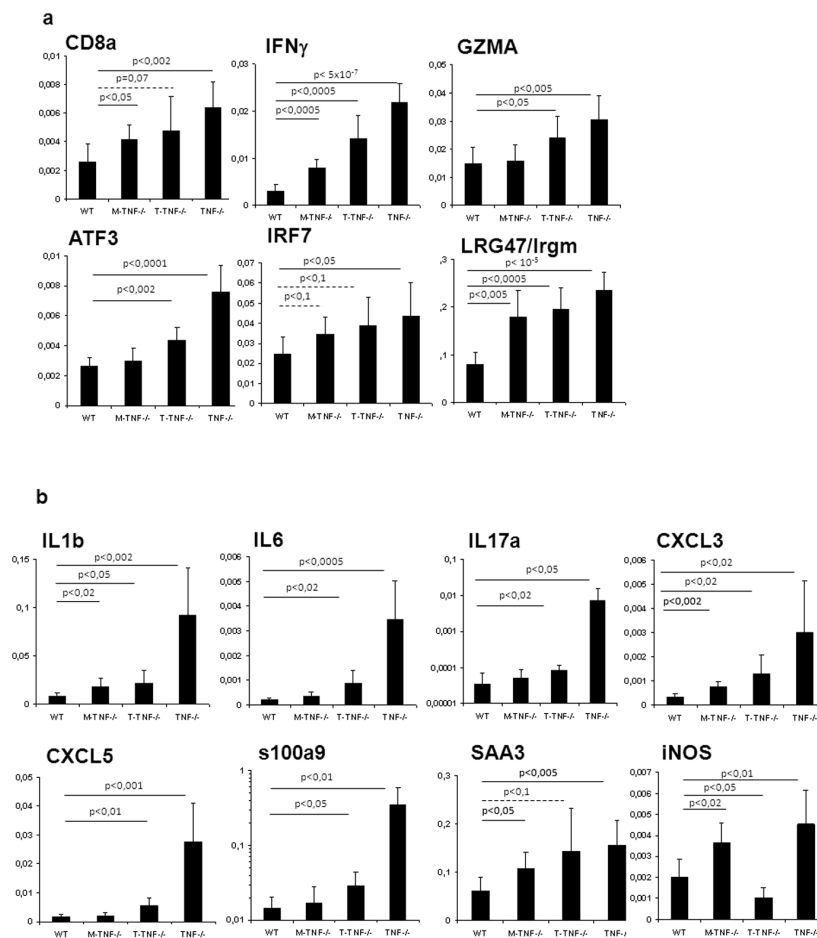
**Differential regulation of inflammatory genes by myeloid versus T-cell derived TNF during acute *M. tuberculosis* infection.** The molecular mechanisms providing a non-redundant function of TNF produced by T-cells and/or macrophages remain unclear. Gene expression signatures in *M. tuberculosis* infected mice may

provide an insight in this respect. We assessed TNF-dependent gene expression during *M. tuberculosis* infection and identified approximately 200 genes that were up- or down-regulated in TNF<sup>-/-</sup> mice as compared to WT mice at 2 and 3 weeks post-infection. Amongst these, we selected genes expressed preferentially in either macrophages or T-cells, and assessed the expression by quantitative PCR in *M. tuberculosis* infected M-TNF<sup>-/-</sup>, T-TNF<sup>-/-</sup> or TNF<sup>-/-</sup> mice (Fig. 6). Cytotoxic T cells signature consisting of CD8a, IFN $\gamma$  and granzyme A as well as IFN $\gamma$ -induced GTPase Irgm1/LRG47 transcription factors IRF7 or ATF3 was abnormally high in complete TNF<sup>-/-</sup> mice 4 weeks post *M. tuberculosis* infection, and less so in M-TNF<sup>-/-</sup> mice, while T-TNF<sup>-/-</sup> mice showed an intermediate phenotype (Fig. 6A), suggesting a limited influence of T-cell derived TNF on the regulation of these genes. Interestingly, pro-inflammatory cytokines and chemokines such as IL-1b, IL-6, IL-17a, CXCL3 or CXCL5 or s100a9 which were highly overexpressed in complete TNF<sup>-/-</sup> mice 4 weeks post-infection, were only modestly up-regulated in T-TNF<sup>-/-</sup> mice, while M-TNF<sup>-/-</sup> behaved essentially like WT mice (Fig. 6B), indicating a strong compensation of either source of TNF in this inflammatory regulation. Acute phase protein Serum Amyloid A3 (SAA3) was overexpressed in TNF<sup>-/-</sup> mice and rather strongly expressed in either T-TNF<sup>-/-</sup> or M-TNF<sup>-/-</sup> mice 4 weeks post-infection. The expression of iNOS, a key anti-mycobacterial enzyme, was strongly increased in M-TNF<sup>-/-</sup> as well as in complete TNF<sup>-/-</sup> mice, while T-TNF<sup>-/-</sup> mice had a low expression compared to infected WT mice 4 weeks post-infection. Therefore we showed that TNF derived from either myeloid or T cells have differential effects on genes known to contribute to the control of acute *M. tuberculosis* infection.

**Deficiency in both myeloid- and T-cell derived TNF confers high susceptibility to *M. tuberculosis* infection and reconstitutes the phenotype of TNF<sup>-/-</sup> mice.** Our data in *M. tuberculosis* infected M-TNF<sup>-/-</sup> mice indicated a transient dependence on TNF derived from myeloid cells to induce protection which could eventually be compensated for by other cellular sources of TNF. T cells could provide a compensatory cellular source of TNF and data from T-TNF<sup>-/-</sup> mice demonstrated the dependence on lymphocyte derived TNF for sustained protection against chronic *M. tuberculosis* infection. To investigate whether T cells were indeed the “compensatory” cellular source of TNF essential for early control of *M. tuberculosis* infection in the absence of macrophages and neutrophils TNF, we challenged mice deficient for both myeloid- and T-cell derived TNF (MT-TNF<sup>-/-</sup> mice). Strikingly, MT-TNF<sup>-/-</sup> mice were highly susceptible to *M. tuberculosis* infection with rapid bodyweight loss and death occurred within 40 days post-infection, similar to complete TNF<sup>-/-</sup> mice, while mice deficient for T-cell derived TNF succumbed by day 150 and M-TNF<sup>-/-</sup> mice survived the duration of the experiment (Fig. 7A,B). Lung weights, an indication of pulmonary inflammation, were markedly increased in MT-TNF<sup>-/-</sup> and TNF<sup>-/-</sup> mice, and less so in T-TNF<sup>-/-</sup> mice, compared to M-TNF<sup>-/-</sup> and WT mice at day 39 post-infection (Fig. 7C). MT-TNF<sup>-/-</sup> mice were unable to control bacterial replication, similar to TNF<sup>-/-</sup> mice, with a 100–1000 fold higher pulmonary bacterial burden compared to WT control mice (Fig. 7D). We confirmed that M-TNF<sup>-/-</sup> and T-TNF<sup>-/-</sup> mice had pulmonary bacterial burdens similar to that of WT mice at 39 days post-infection (Fig. 7D). Differences in pulmonary bacilli burden were also visible microscopically since lung sections of MT-TNF<sup>-/-</sup> and TNF<sup>-/-</sup> mice displayed widely dispersed aggregates of acid fast bacteria that appeared to be predominantly extracellular, while WT, M-TNF<sup>-/-</sup> or T-TNF<sup>-/-</sup> mice presented primarily with single acid fast bacilli sparsely distributed in alveolar macrophages (Fig. 7E). Enlarged, necrotic nodules characteristic of TNF<sup>-/-</sup> mice were also present in the lungs of MT-TNF<sup>-/-</sup> on day 39 post infection (Fig. 7E). Histopathologically, well defined granulomas were



**Figure 5** | T-TNF<sup>-/-</sup> mice succumb to chronic *M. tuberculosis* infection. (A; B) WT (closed circle), T-TNF<sup>-/-</sup> (grey diamond), and TNF<sup>-/-</sup> (clear circle) mice were infected via aerosol inhalation with 200–500 cfu/lung of *M. tuberculosis* H37Rv are represented (n = 10 mice/strain). Change in bodyweight values (A) are expressed as the mean of 4–10 mice/group. Differences in mortality rates (B) were analysed by the student's logrank test. (C; D). The number of viable bacteria present in lungs were assessed at 33 days (C) and 150 days (D) post-infection. The results are expressed as the mean of 4 mice/group. (E; F and G). Lung weights (E), macroscopic lung pathology (F) and haematoxylin and eosin staining of lung sections (G) from WT-, and T-TNF<sup>-/-</sup> mice infected with *M. tuberculosis*. Images of left lobes of WT and T-TNF<sup>-/-</sup> mice at 150 days post infection are presented (n = 4 mice/group). The data is from one experiment representative of three independent experiments.



**Figure 6 | Inflammatory gene expression during acute *M. tuberculosis* infection in cell-specific *TNF*<sup>-/-</sup> mice.** (A; B) Analysis of selected inflammatory gene expression was performed by quantitative RT-PCR on day 28 after *M. tuberculosis* infection and normalized to b-actin. (n = 3–4 mice per point). Genes include CD8a, IFN $\gamma$ , granzyme A, Irgm1/LRG47, IRF7 and ATF3 (Fig. 6A), the proinflammatory cytokines and chemokines IL-1b, IL-6, IL-17a, CXCL3 or CXCL5 and s100a9, the acute phase reactant, SAA and iNOS (Fig. 6B).

observed in WT mice, in contrast to both MT-*TNF*<sup>-/-</sup> and *TNF*<sup>-/-</sup> mice which presented with severe and necrotic lesion structures (Fig. 7F).

Therefore, absence of TNF from both T-cells and myeloid cells rendered mice essentially as susceptible as fully *TNF*-deficient mice, demonstrating that these are the main cellular sources involved in host control of *M. tuberculosis* infection. The fact that TNF production by T-cells or by macrophages/neutrophils can partially compensate each other to control of acute infection, points to some redundant functions which may be provided by either source of TNF.

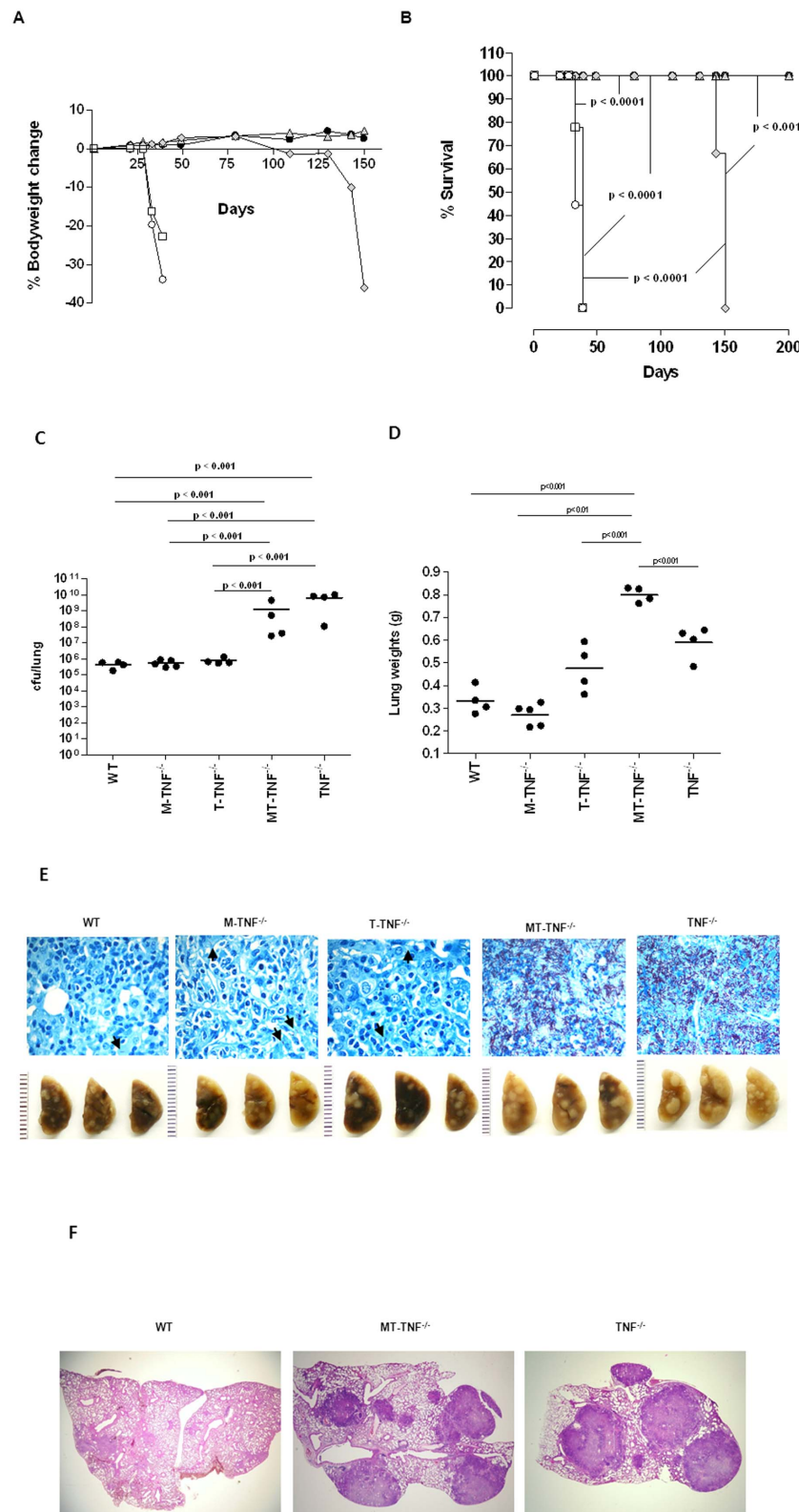
## Discussion

TNF is essential for the effective control of mycobacterial infection in several host species including humans<sup>7–9,22,27,46–48</sup>. We and others have shown that *TNF* deficient mice are highly susceptible to *M. bovis* BCG<sup>7,9</sup> and *M. tuberculosis*<sup>6,8</sup>. Although both immune and non-immune cells are capable of TNF synthesis, the identity of the critical cell types contributing to immune protection remains undefined. In this study we investigated the role of TNF from distinct cellular sources in mediating immune protection, focusing on the contribution of myeloid cells, such as macrophages and neutrophils, and CD4<sup>+</sup>/CD8<sup>+</sup> T cells in the host's immune response against *M. tuberculosis*.

Synthesis of TNF by various cell types has been demonstrated as integral to the host's immune response against pathogen challenge<sup>46</sup>. Early host immune responses against *M. tuberculosis* infection are characterized by leukocyte recruitment in which macrophages and

neutrophils constitute >70% of inflammatory cells<sup>47</sup>. Particularly relevant to this study is the widely accepted paradigm that monocytes/macrophages are significant sources of TNF during bacterial infections and are therefore deemed to be critical for host resistance to pathogens<sup>2,31,48–51</sup>. When macrophages are infected with live bacilli or presented with either *M. tuberculosis* derived ligands or heat killed bacilli *in vitro*, rapid production of TNF is initiated, suggesting that they may contribute significantly to the host immune response *in vivo*<sup>6</sup>. Several studies described neutrophil mediated control of *M. tuberculosis*<sup>52,53</sup> and evidence for a TNF dependent cooperative mechanism between neutrophils and alveolar macrophages to contain *M. tuberculosis* replication has been reported<sup>54</sup>. Furthermore, optimum macrophage activation and mycobacterial growth inhibition are acquired through synergistic effects of TNF and IFN $\gamma$  production by T cells to induce nitric oxide bactericidal mechanisms<sup>5</sup>.

Utilizing mice with highly efficient, cell lineage restricted inactivation of *TNF*<sup>26</sup>, we were able to show here that TNF produced by myeloid cells has an important non-redundant function during early control of *M. tuberculosis*, but unexpectedly becomes redundant during the latter stages of infection. Previous studies have shown that virulent *M. tuberculosis* induces TNF production by macrophages to mediate apoptosis and escape<sup>55</sup> whereas neutrophil-derived TNF was reported as important for activation of macrophages<sup>54</sup>. The absence of TNF from myeloid origin in our infection model could therefore result in reduced activation and bactericidal capacity of infected macrophages permitting increased bacterial replication during early stages of infection. Interestingly, the onset of adaptive immune



**Figure 7 | MT-TNF<sup>-/-</sup> mice are highly susceptible and succumb to acute *M. tuberculosis* infection.** (A; B) WT (closed circle), M-TNF<sup>-/-</sup> (grey triangle), T-TNF<sup>-/-</sup> (grey diamond), MT-TNF<sup>-/-</sup> mice (clear square) and TNF<sup>-/-</sup> (clear circle) mice infected as described were monitored for change in bodyweight (A; expressed as the mean of 4–10 mice/group) and survival (B; n = 10 mice/group) Data is from one experiment representative of three independent experiments. (C; D) The lung weights (C) and number of viable bacteria present in lungs (D) were assessed at 39 days post-infection. The results are expressed as the mean  $\pm$  SD of 4–5 mice/group and are from one experiment, representative of two independent experiments. (E; F) Pulmonary bacilli burdens on day 39 were confirmed by Ziehl-Neelsen staining (E; magnification 600 $\times$ ). Arrows indicate the presence of single AFB within macrophages. Images of left lobes illustrating macroscopic pulmonary pathology in WT-, M-TNF<sup>-/-</sup>, T-TNF<sup>-/-</sup>, MT-TNF<sup>-/-</sup> and TNF<sup>-/-</sup> mice. (n = 5 mice/group). Images are representative of one of three independent experiments. Haematoxylin and eosin staining show enlarged, necrotic nodules in the lungs of MT-TNF<sup>-/-</sup> and TNF<sup>-/-</sup> mice at day 39 post infection (F). Images are representative of one of three independent experiments.



responses coincided with a reduction in bacterial growth eventually resulting in burden equivalent to WT control mice. From previous studies it was concluded that the recruitment of effector antigen specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells is critical to further enhance infected macrophage killing capacity at the site of infection and the subsequent killing of the intracellular bacilli<sup>56,57</sup>. Our data show that in the absence of TNF from macrophages and neutrophils M-TNF<sup>-/-</sup> mice have enhanced pulmonary effector CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment during early infection. While the increased recruitment was associated with increased bacterial burden elevated numbers of T cells likely contributed with TNF production to compensate the lack of macrophage/neutrophil-derived TNF. Susceptibility in M-TNF<sup>-/-</sup> mice was therefore not due to the lack of T cells at local sites of infection, unlike the situation in complete TNF<sup>-/-</sup> mice where CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment was initially delayed, but became unregulated during terminal stages of infection. Rather, this increased influx of CD4<sup>+</sup> and CD8<sup>+</sup> T cells early on in M-TNF<sup>-/-</sup> mice points to an important function of TNF produced by macrophages and neutrophils in the regulation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment. Nonetheless, we postulated that although T cell recruitment was not impaired in M-TNF<sup>-/-</sup> mice, the effector function of recruited cells may have been compromised. Our data demonstrated that recruited CD4<sup>+</sup> and CD8<sup>+</sup> T cells were fully mature effector cells, expressing CD44 and CD69 activation markers. Moreover these lymphocytes were primed against *M. tuberculosis* and hence could potentially initiate macrophage bactericidal mechanisms. Previous studies have shown that Th1 immune function is mediated predominantly by *M. tuberculosis* antigen specific IFN $\gamma$  expression by CD4<sup>+</sup> and CD8<sup>+</sup> T cells that can upregulate the antimycobacterial function of macrophages at the site of infection<sup>40</sup>. These primed pulmonary CD4<sup>+</sup> and CD8<sup>+</sup> T cells of M-TNF<sup>-/-</sup> mice were also capable of synthesising TNF and IFN $\gamma$  which resulted in the induction of nitric oxide synthesis in *M. tuberculosis* infected macrophages<sup>43,58,59</sup>. We therefore postulated that key to the eventual resistance to *M. tuberculosis* infection of M-TNF<sup>-/-</sup> mice was the potential substitution of TNF produced by myeloid cells with TNF from recruited activated T cells resulting in an effective and protective adaptive immune response.

Indeed, the transient nature of disease susceptibility in M-TNF<sup>-/-</sup> mice indicated that compensatory mechanisms and other cellular sources of TNF allowed for the eventual establishment of full protective immunity. Several studies highlighted T cells as sources of TNF that impacted on disease outcome during bacterial challenge<sup>60,61</sup>. T cell derived TNF in association with GM-CSF induce macrophage aggregation that may contribute to the formation of granulomas during host immune protective responses against mycobacterial infection<sup>61</sup>. Furthermore, a modest increase in resistance of *M. tuberculosis* infected RAG<sup>-/-</sup> and TNF<sup>-/-</sup> mice after transfer of T cells capable of producing TNF was reported<sup>25</sup>. Adoptive transfer approaches have inherent limitations and did not allow for direct interrogation of the role of T cell derived TNF. Here we used a loss of function approach through the use of T cell specific deletion of TNF gene in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells which has distinct advantages over the adoptive transfer approach, especially for long-term studies of chronic infection studies. Previous reports documented distinct and non-redundant functions of TNF produced by T cells, macrophages and neutrophils for primary defence against *Listeria monocytogenes* whereas T cell derived TNF appeared subservient in function<sup>26</sup>. Our present findings support a clear and distinctive non-redundant function for TNF derived from T cells during persistent infection, which may be dispensable during initial immune responses. Interestingly, mortality in T-TNF<sup>-/-</sup> mice was associated with a relatively modest increase in bacterial burden compared to WT mice, as opposed to TNF<sup>-/-</sup> mice which succumbed with significantly higher bacilli burdens. However, moribund T-TNF<sup>-/-</sup> mice presented with an aggressive pulmonary pathology, indicating

that T cell derived TNF is required primarily for down regulation of inflammation during persistent/chronic *M. tuberculosis* infection rather than for bactericidal mechanisms. Increased susceptibility and eventual mortality of TNF<sup>-/-</sup> mice may thus be a consequence of pulmonary pathology induced by an unregulated immune response. Increased recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T cells have previously been reported by Zganiacz et al, where the authors elegantly illustrated the regulatory function of TNF mediated through both TNFRp55 and TNFRp75<sup>62</sup>. Neutralisation of TNF led to disregulated pulmonary pathology accompanied by enhanced production of specific proinflammatory cytokines responsible for the control and migration of different leukocytes including macrophages and T cells. Although the presence of TNF produced by macrophages and/or other cell types within the granulomatous structures in T-TNF<sup>-/-</sup> mice were able to contain early mycobacterial infection, the absence of TNF production by T cells during chronic infection resulted in inefficient recruitment of effector T cells, leading to inefficient TNF dependent signalling within the microenvironment of the granuloma. Here, we convincingly demonstrated dependence on CD4<sup>+</sup> and/or CD8<sup>+</sup> T cell derived TNF for sustaining structural and functional integrity of granulomas during chronic infection, in spite of the fact that it was not required for its initial establishment. Egen et al., 2008 have elegantly shown that effector T cells are rapidly recruited to existing granulomas and are retained within these structures with limited egress from lesions<sup>60</sup>. The authors further demonstrated that T cells within granulomas are highly motile, actively interacting with resident macrophages and apparently provided signals essential for the maintenance of granuloma structure. Anti-TNF treatment both inhibited recruitment of effector T cells to granulomas and restricted movement of T cells within granulomas. It is therefore interesting to speculate on the contribution of TNF produced by T cells in providing T cell-macrophage interaction for the maintenance of structural granuloma integrity. The lack of structural granuloma integrity in T-TNF<sup>-/-</sup> mice would argue strongly for an essential role of T cell derived TNF, possibly in its membrane-bound form, in driving T cell-macrophages interaction during structural granuloma formation. In fact, we and others showed earlier that not only soluble TNF but also membrane-bound TNF is important for the control of *M. tuberculosis* infection<sup>17,19</sup>. Thus, local, cell-associated TNF interactions are likely important for the multi-fold effects of TNF, and measuring soluble TNF in the lung might not give the full picture. This was also documented in an earlier study using TNF expressing BCG, where a limited, local production of TNF at the site of infection conferred protection against *M. bovis* BCG in TNF-deficient mice<sup>63</sup>. We report low levels of TNF expression in wild-type, M-TNF<sup>-/-</sup> or T-TNF<sup>-/-</sup> mice. Indeed, it is our experience that the pulmonary protein concentration of soluble TNF remains low, even after 1–3 months of *M. tuberculosis* infection, as compared to other pro-inflammatory cytokines/chemokines such as IL-1, MCP1 or RANTES which are more up-regulated and reach levels in the ng/lung range<sup>64,65</sup>).

The initial establishment of granuloma to facilitate myeloid-T cell interaction is significantly dependent on controlled cellular inflammation in response to infection. Th1 polarisation of the adaptive response to *M. tuberculosis* infection has been illustrated at the transcriptional level by persistent induction of CD8a, IFN $\gamma$  and granzyme A transcripts 15–100 days post-infection<sup>66,67</sup>. Here, we show that in complete absence of TNF there was a very high induction of molecules indicative of cytotoxic T-cell signature such as CD8a, IFN $\gamma$  and granzyme A, of ATF3, a positive regulator of IFN $\gamma$  expression<sup>68</sup>, of IFN $\gamma$ -induced GTPase *irgm1*/LRG47 and IFN $\gamma$  signalling pathway IRF7, 4 weeks post *M. tuberculosis* infection, as compared with wild-type mice. The over-induction of these genes was similar or only slightly less marked in mice deficient for T-cell derived TNF, indicating that TNF from T-cell origin is dispensable for this up-regulation. However, the over-induction of cytotoxic T cell signature



genes was more limited in M-TNF<sup>-/-</sup> mice, indicating that TNF from myeloid origin is essential for the up-regulation of these genes. A strong and persistent inflammatory response was documented as early as 2–3 weeks post *M. tuberculosis* infection, by the transcriptional up-regulation of pro-inflammatory cytokines including TNF itself, IL-1 $\beta$  or IL-6, of chemokines such as CXCL5, but also of acute response proteins such as SAA3 or S100a9, and of iNOS, a key antimycobacterial enzyme source of reactive nitrogen<sup>66,67</sup>. We show that the inflammatory signature was strongly increased in complete TNF-deficient mice 4 weeks post *M. tuberculosis* infection, which may result from the very high infectious burden in these mice at this time point. Interestingly, IL-1 $\beta$ , IL-6, IL-17a, CXCL3, CXCL5 or S100a9 were much less up-regulated in T-TNF<sup>-/-</sup> mice, and M-TNF<sup>-/-</sup> behaved essentially like WT mice. iNOS was strongly over-expressed in mice fully deficient for TNF or in M-TNF<sup>-/-</sup> mice, while T-TNF<sup>-/-</sup> mice had a low expression, as compared to infected WT mice. Thus, TNF *per se* is dispensable for the induction of these inflammatory mediators and might be compensated for by other up-regulated inflammatory cytokines. The data correlates with the phenotypic profiles of the respective strains and supports the relative importance and differential effects of TNF produced by macrophages and T cells in the control of acute *M. tuberculosis* infection.

The similarity in phenotype between WT and T-TNF<sup>-/-</sup> mice together with the disparate phenotypes between WT and M-TNF<sup>-/-</sup> mice during acute *M. tuberculosis* infection indicated that TNF produced by myeloid cells is more relevant for early host immune defence as compared to T cell derived TNF. Nonetheless, we hypothesised that recruited T cells capable of TNF synthesis could contribute to rescue *M. tuberculosis* infected M-TNF<sup>-/-</sup> mice from early defect in resistance to infection. We therefore characterized host immune function against *M. tuberculosis* in MT-TNF mice, in which case macrophages/neutrophils, and T cells were incapable of TNF synthesis. We argued that deletion of TNF synthesis capability from CD4<sup>+</sup>- and CD8<sup>+</sup> T cells in M-TNF<sup>-/-</sup> mice would render the host unable to draw on the secondary TNF cellular source to induce immune protection. The extent of susceptibility of *M. tuberculosis* infected MT-TNF<sup>-/-</sup> mice was indistinguishable from TNF<sup>-/-</sup> mice indicating that macrophage/neutrophils and T cells are the main cellular sources of TNF required to generate complete protection against *M. tuberculosis* infection. Combined TNF deficiency in myeloid cells and T cells resulted in exacerbated disease progression with uncontrolled bacterial growth in organs and severe pulmonary pathology and mortality, clearly pointing out to redundant essential functions of TNF from these distinct cellular sources.

In conclusion, this study provides significant insights on TNF cellular source requirements for host immune responses against *M. tuberculosis* challenge. Although widely acclaimed as the major cellular source of TNF, macrophages have a surprisingly limited role in restricting bacterial replication through TNF dependent synthesis during *M. tuberculosis* infection. Disease outcome resides rather with T cell derived TNF whose function appears as non-redundant. Our data imply that inhibiting TNF synthesis in T cells during latent *M. tuberculosis* infection may lead to an imbalance in the cytokine profile within the granuloma microenvironment with subsequent potential for reactivation and immunopathology. TNF neutralizing therapies used in severe inflammatory diseases should thus spare T-cell derived TNF and this may reduce the risk of tuberculosis reactivation. We clearly demonstrated the existence of labour division between myeloid cells and T cells in provision of protective TNF which implicates both redundant and non-redundant functions of this cytokine from distinct cellular sources.

## Methods

**Mice.** All animal strains used in this study were previously described<sup>26</sup>. TNF “floxed” (WT control) mice were crossed with MLys-Cre deleter mice to generate myeloid, macrophage/neutrophil TNF deficient mice (M-TNF<sup>-/-</sup>) and similarly crossed with CD4-Cre deleter mice to generate T-cell TNF deficient mice (T-TNF<sup>-/-</sup>). M-TNF<sup>-/-</sup>

and T-TNF mice were in turn intercrossed to generate TNF ablation in both myeloid cells and T cells (MT-TNF<sup>-/-</sup>). In addition, complete TNF deficient (TNF<sup>-/-</sup>) mice were used as controls. Animals were bred under specific pathogen free conditions at the University of Cape Town or Transgenic institute (CNRS UPS44, Orleans, France) and used between 6–12 weeks. Infected animals were maintained in individually ventilated cages or isolators under biosafety level 3 conditions.

**Ethics statement.** All animal experimental protocols complied with South African or French regulations. Approval was obtained from the Animal Research Ethics Committee, University of Cape Town, South Africa in accordance with the South African National Standard 10386-*The care and use of animals for scientific purposes* or were approved by the “Ethics Committee for Animal Experimentation of CNRS Campus Orleans” (CCO), registered (N°3) by the French National Committee of Ethical Reflexion for Animal Experimentation, under the N° CLE CCO 2011-029 in compliance with the French ethical and animal experiments regulations (see Charte Nationale, Code Rural R 214–122, 124).

**Mycobacteria and infection.** *Mycobacterium tuberculosis* H37Rv was grown in Difco Middlebrook 7H9 medium containing 0.5% glycerol and enriched with 10% OADC. Cultures were incubated at 37°C and grown until log phase, aliquoted and maintained as frozen stocks at –70°C. An aliquot of *M. tuberculosis* H37Rv was rapidly thawed at 37°C, passed 30 $\times$  through a 29.5G needle and diluted in sterile saline. Mice were infected at doses of 200–500 cfu/lung using a Glas-Col Inhalation Exposure System Model A4224. The pulmonary infection dose was confirmed by sacrificing 10 mice 1 day after infection and plating the homogenised lung tissue on Difco Middlebrook 7H10 agar plates in 10 fold serial dilutions. Plates were semi-sealed in plastic bags, incubated for 17–21 days at 37°C and the number of mycobacterial colonies was counted.

**Single cell preparation, cell restimulation, intracellular cytokine staining and analysis by flow cytometry.** Lungs were perfused by injecting 5 ml of cold PBS containing 20 U/ml heparin in the right ventricle of the heart. Lungs were removed, sectioned on ice and incubated in PBS containing 50 U/ml collagenase I (Worthington Biomedical Corporation, Lakewood, NJ) and 13 ug/ml DNase I (Boehringer-Mannheim, Germany) at 37°C for 90 minutes. For single cell suspensions lung tissue was passed through a 70  $\mu$ m nylon cell strainer (Beckton and Dickinson), washed 2 $\times$  with PBS and the cell concentration determined by counting in the presence of trypan blue. Cells were labelled using the following antibodies: anti-mouse CD3 (Clone 17A2; Pharmingen); anti-mouse CD8a (Clone 53–6.7; Pharmingen); anti-mouse CD4 (Clone RM4-5; Pharmingen), anti-mouse CD44 mouse (Clone IM7; Pharmingen) and anti-mouse CD69 (Clone H1.2F3; Pharmingen). 1  $\times$  10<sup>6</sup> cells were incubated for 20 minutes with anti-CD16/32 (Clone 2.4G2; Pharmingen) to block Fc receptors in 96 well round bottom plates (Sterilin). Cells were washed with PBS/0.1% BSA/0.01% NaN<sub>3</sub> and incubated with 2 ug/ml of appropriate antibodies for 20 minutes in the dark. Excess antibodies were removed by washing cells 2 $\times$  with PBS/0.1% BSA/0.01% NaN<sub>3</sub>, then pelleted cells were fixed for 18–24 hours in 10% PBS buffered formalin.

For intracellular staining, single cell suspensions of lungs were restimulated with plate-bound anti-mouse CD3 (5  $\mu$ g/ml; clone 145-2c11) and anti-mouse CD28 (5  $\mu$ g/ml; clone 37.51), ESAT-6 (10  $\mu$ g/ml; Statens Serum Institute) and PPD (10  $\mu$ g/ml; Statens Serum Institute) for 6 hours in the presence of Brefeldin A (5  $\mu$ g/ml). Cells were labelled with either anti-mouse CD8a (Clone 53–6.7; Pharmingen) or anti-mouse CD4 (Clone RM4-5; Pharmingen) and anti-mouse IFN $\gamma$  (Clone XMGI.2; Pharmingen). Analysis was done using FACScalibur (Beckton and Dickinson) incorporating Cellquest software.

For cytokine measurements samples were subjected to ELISA using antibody and protein standards obtained from RnD Systems Inc., (Minneapolis, MN). Absorbance was measured using a Versamax Microplate Reader (Molecular Devices, LLC, CA).

**Lung morphology.** Lungs were fixed in phosphate buffered formalin and embedded in paraffin wax. Tissues were sectioned at 2  $\mu$ m, stained with hematoxylin and eosin and Ziehl-Neelsen, and subsequently mounted with Canada balsam. Images of stained tissue sections were captured using an Olympus E330 digital camera attached to Olympus Model BX40F microscope. Macroscopic lung images were captured with Pentax Optio M40 camera.

**Analysis of mRNA gene expression.** Total RNA were isolated from lungs by TRIzol reagent (Sigma, St. Louis, MO) and purified by RNeasy Mini Kit (Qiagen, Valencia, CA) at the indicated times after *M. tuberculosis* infection. For quantitative RT-PCR 1  $\mu$ g of total RNA was pretreated by DNaseI and converted to cDNA by ImProm-II<sup>TM</sup> Reverse Transcriptase (Promega, Madison, WI) using random nonamer primers. Q-PCR reactions were performed using Brilliant II SYBR QPCR kits and a Stratagene Mx3005P thermocycler (Agilent, Palo Alto, CA). The following program was used: 95°C for 15 min and 40 cycles 95°C for 10 sec and 60°C for 30 sec. Primers used for quantitative PCR are provided in Supplementary Table 1.

**Statistical analysis.** Statistical analysis was performed by ANOVA or the Student's t test. For mortality studies, analysis was performed using the logrank test. For all tests, a *p* value of <0.05 was considered significant.



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## Author contributions

N.A., S.G., R.K., N.H., M.B., N.C., C.F., Y.S. and V.Y. performed experiments while N.A., S.G., Y.S., S.G., S.N., V.Q. and M.J. were responsible for experimental design and data analysis. B.R., S.N. and M.J. provided materials. M.J., B.R., V.Q., S.N., S.G. and N.A. wrote the main manuscript text and all the authors reviewed the manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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