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# TNF in Host Resistance to Tuberculosis Infection

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**Abstract**

TNF is essential to control *Mycobacterium tuberculosis* infection and cannot be replaced by other proinflammatory cytokines. Overproduction of TNF may cause immunopathology, while defective TNF production results in uncontrolled infection. The critical role of TNF in the control of tuberculosis has been illustrated recently by primary and reactivation of latent infection in some patients under pharmacological anti-TNF therapy for rheumatoid arthritis or Crohn's disease. In this review, we discuss results of recent studies aimed at better understanding of molecular, cellular and kinetic aspects of TNF-mediated regulation of host-mycobacteria interactions. In particular, recent data using either mutant mice expressing solely membrane TNF or specific inhibitor sparing membrane TNF demonstrated that membrane TNF is sufficient to control acute *M. tuberculosis* infection. This is opening the way to selective TNF neutralization that might retain the desired anti-inflammatory effect but reduce the infectious risk.


Tuberculosis (TB) infection is a major public health problem caused by *Mycobacterium tuberculosis* (M.tb). The present estimate is that one third of the world population harbors M.tb in a latent form (http://www.who.int), which may be reactivated when the host immune response is suppressed such as in HIV infection [1]. Only 10% of the population which has been in contact with the pathogen develop overt clinical symptoms, while roughly 90% of the infected persons contain the infection. A recent quantification of bacterial growth and death rates showed that M.tb replicates throughout

the course of chronic TB infection in mice and is restrained by the host immune system [2]. Unraveling the host immune response during primary and chronic/latent infection is therefore a major challenge. Prominent mechanisms of the host leading to protective immunity controlling TB and reactivation of infection are associated with T cells, macrophages, interferon-γ (IFN-γ), TNF, interleukin-12 (IL-12), nitric oxide (NO), reactive oxygen and reactive nitrogen intermediates (RNIs), as reviewed in references [3–6]. While IL-23 and IL-17 contribute to host resistance [7], they do not seem essential to control acute TB infection [8].

Upon phagocytosis by macrophages, M.tb activates various pattern recognition receptors and stimulates the production TNF, IL-12, RNI as well as the expression of costimulatory molecules. This normally leads to activation of T and NK cells, and IFN-γ production augmenting the microbiocidal activity of the phagocytes [3, 4]. An essential role for IL-1 pathway in the control of acute M.tb infection has also been documented [9, 10], indicating that several proinflammatory cytokines produced during TB are nonredundant.

A simplified view of how M.tb activates antigen-presenting cells and induces T cell activation is depicted in figure 1. A concerted action of chemokines and cytokines leads to a focal accumulation of macrophages containing a few intracellular bacilli, which escaped the initial killing, surrounded by activated T cells forming the typical granulomas of M.tb infection [11]. T cell depletion and inhibition or neutralization of several mediators at different stages of TB infection leads to rapid disease progression, which may be accompanied by granuloma disruption, bacterial growth and dissemination, leading to death. Due to its multiple in vivo activities, excessive TNF may also cause a distinctive set of pathological effects in TB infection, including hyperinflammation, caseous necrosis and cachexia, all of which are correlated with elevated TNF levels [12, 13]. The occurrence of TB reactivation under TNF neutralizing therapy has shed new light on the role of TNF in the control of latent TB infection. This review focuses on the protective role of TNF in the immune response to M.tb infection.

## TNF Family

TNF is the founder member of cytokine TNF-like superfamily [for review see 14–16]. TNF is expressed by many different cell types including macrophages, dendritic cells, CD4+ and CD8+ T cells, B cells, but also by other cells such as adipocytes, keratinocytes, mammary and colon epithelium, osteoblasts, or mast cells. TNF is first synthesized as a homotrimeric 26-kDa membrane-bound protein (tmTNF). After proteolytic cleavage by TNF-α-converting enzyme, 17-kDa soluble TNF is released. Levels of circulating TNF in healthy individuals are nearly undetectable; however, they increase substantially in pathological situations [17, 18]. Lymphotoxin-α is a member of TNF superfamily and structurally the closest TNF relative. It exists as a soluble homotrimer (LTα3) or forms a membrane-bound heterotrimeric complex with the anchor LTβ [for review see 19, 20].

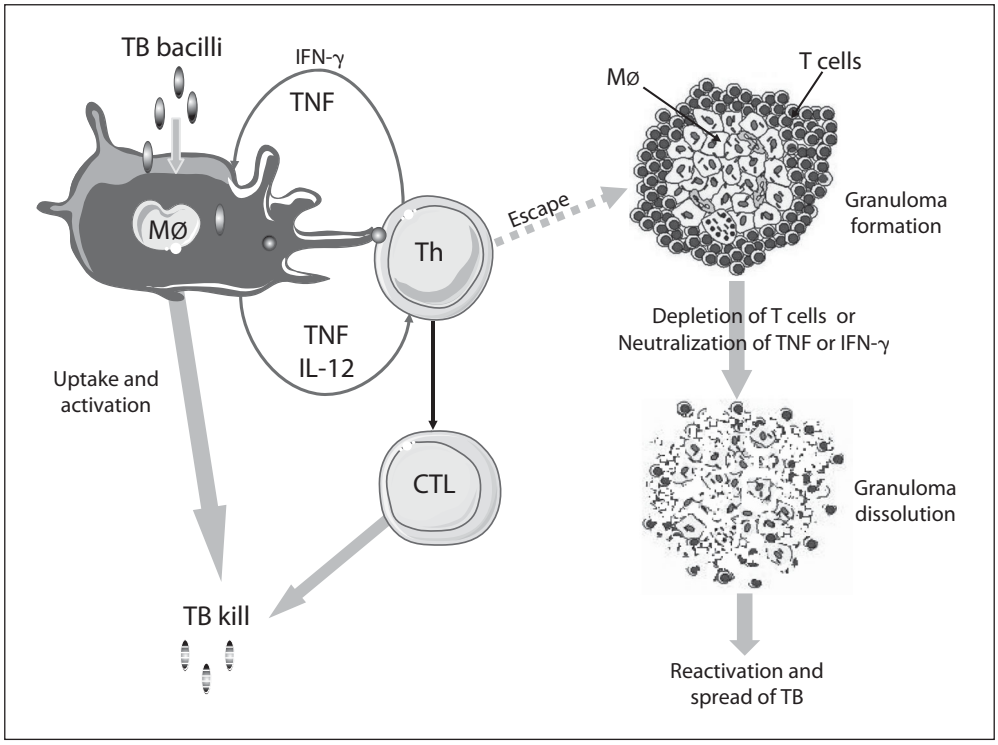


**Fig. 1.** Macrophage and T cell activation, killing of TB bacilli and granuloma formation. Macrophages are activated by TB bacilli and produce cytokines and T cell activation. Activated macrophages are mycobactericidal, but a few bacilli escape. The cell activation induces lymphocyte recruitment orchestrated by chemokines leading to the formation of granulomas which contain the bacilli. Antibody neutralization of TNF or IFN-γ or T cell depletion results in dissolution of the granuloma structure, rescue of surviving bacilli with dissemination of infection. Mø = Macrophage; Th = T helper; CTL = cytotoxic T lymphocyte.

TNF, LTα and LTβ genes are tightly clustered within 12 kb inside the major histocompatibility complex locus on murine chromosome 17 and human chromosome 6 [21, 22]. Membrane-bound as well as soluble TNF interact with two receptors, TNFR1 (p55 in mouse, p60 in humans, CD120a) and TNFR2 (p75/p80, CD120b). TNFR1, the high-affinity receptor for soluble TNF, is constitutively expressed in nearly all tissues and cell types. TNFR1 contains a protein module called 'death domain' which is essential for induction of apoptosis, as well as for other nonapoptotic functions [14]. The expression of TNFR2 is more restricted to lymphoid tissues [23]. Soluble LTα3 also binds and activates both TNFR1 and TNFR2, whereas membrane-bound LTαβ exerts its unique functions through the engagement of LTβR [for review see 15].

Receptor ligation initiates signals through a complex cascade to activate the nuclear factor NF-κB, JNK-AP1 and p38 signaling axis resulting in activation of TNF-dependent program of gene expression [for review see 24]. Both TNFR1 and

TNFR2 are constitutively shed in substantial amounts in vivo, and soluble TNF receptor shedding is likely to play an important role in regulating TNF activity under physiologic conditions [25]. Macrophage infection by M.tb was shown to induce release of soluble TNFR2 that formed inactive TNF-TNFR2 complexes and reduced TNF bioactivity [26]. In vivo infection with *Mycobacterium bovis* BCG was shown to upregulate soluble TNFR1 and TNFR2 release in the circulation following the release of TNF [27].

Another interesting mode of action that may account for some of TNF functions is the induction of reverse signaling through tmTNF [28]. Bidirectional or reverse signaling has been suggested for a number of transmembrane members of the TNF superfamily including CD40L [29], CD95L (FasL; [30]) and mTNF itself. Reverse signaling through transmembrane TNF has been shown to involve protein kinase C pathway and to induce E-selectin (CD62E) expression on activated human CD4+ T cells [31, 32], as well as for TNF release by monocytes [33]. The molecular mechanisms involved and intracellular pathways activated by reverse signaling remain largely uncharacterized.

Thus, tmTNF, soluble TNF and soluble LTα3 appear to mediate both overlapping and distinct physiological responses in vivo. Their relative roles in inflammatory models and in host defense have not been fully unraveled, in large part due to the limitations in physiologically relevant in vivo models. Membrane-bound TNF mediates cellular responses such as apoptosis [34], proliferation, B cell activation, and some inflammatory responses. To date, the main evidence for an in vivo role for tmTNF has come from genetically modified mice expressing uncleavable membrane-bound TNF [35–38]. While the role of TNF in controlling TB has been extensively studied using a panel of available mouse models [39–42], the role of LTα3 had to be implicated indirectly from the comparative phenotypes of mice deficient in LTα vs. LTβ or TNFR1/TNFR2 vs. TNF and therefore remained much less defined.

## TNF Genetic Mouse Models

A detailed understanding of the relations between different members of the TNF family is essential to appreciate the power and the limitations of the available genetic mouse models. Various models to study the role of TNF in vivo have been developed, and the most powerful tools are transgenic and gene-deficient mice. Several transgenic and gene knock-in (KI) mice expressing either human or mouse TNF systemically or in a tissue-specific manner are available, and they are relevant to the conditions of systemic TNF overproduction or local inflammation such as arthritis, colitis or chronic CNS inflammation [43]. In TB research so far the following transgenic mice and gene knockout (KO) mice have been characterized: soluble TNFR1 transgenic, soluble TNFR2 transgenic and mice deficient for TNF/LTα, TNF, LTα, LTβ, TNF/LTβ, TNF/LTα/LTβ, LIGHT, TNF-R1 and R2 and LTβR mice [44–50]. To

analyze the specific role of membrane TNF, two recent models which express a functional, normally regulated but uncleavable membrane-bound TNF were reported [37, 38], and a transgenic mouse model expressing membrane TNF in TNF/LTα KO mice [36]. Conversely, the role of the soluble TNFR1 in controlling M.tb infection can now be envisaged using mice expressing a nonsheddable p55 TNFR1 (TNFR1$^{\Delta NS}$) KI [51].

The generation of a novel panel of mice with cell-specific TNF deficiencies [52] and LTβ KO mice [53] allowed the investigation of in vivo functions of TNF or surface LTL produced by distinct cell types of the immune system such as macrophages/neutrophils or lymphocytes, adding yet another powerful tool to dissect TNF cytokine family functions in a more specific way.

## Nonredundant Role of TNF to Control Mycobacterial Infection

Macrophages, DC and epithelial cells are among the first cells encountering M.tb bacilli in the airway. Phagocytosis induces the transcriptional machinery resulting in the secretion of several proinflammatory cytokines, chemokines, expression of costimulatory molecules and effector molecules including NO, which has mycobactericidal activity (fig. 1). Mycobacterial proteins are degraded and presented by class II proteins to the T cell receptor inducing clonal activation of CD4 T cells. IFN-γ derived from T cells and NK or NKT cells is a potent activator of APCs, enhancing the killing of M.tb and presentation of mycobacterial peptide to T cells. The concerted action of cytokines and chemokines leads to accumulation of activated macrophages containing a few surviving bacilli surrounded by activated T cells, which constitutes the typical mycobacterial granuloma (fig. 1). Other cell types may participate in this process and include neutrophils, eosinophils, NK, NKT and mast cells and possibly γδ-T cells [3, 4, 6, 7].

Infection with the vaccine strain *M. bovis* BCG is well controlled in normal C57Bl/6 mice. However, the control of *M. bovis* BCG infection is TNF dependent as mice treated with anti-TNF antibodies showed impaired granuloma formation and increased bacillus content [54]. Transgenic mice expressing soluble TNFR1-Fc fusion protein neutralizing TNF and LTα succumbed to BCG infection [44, 55]. Using the first available TNF-LTα double-deficient mice [56], we showed that TNF and/or LTα signaling is required to activate cell of the immune system [50]. TNF-LTα double-deficient mice display high susceptibility and succumb to BCG infection between 8 and 10 weeks. The granuloma response was severely impaired with reduced T cell recruitment and macrophages expressed reduced inducible NO synthase (NOS2), a key mediator of antibacterial defense [50]. We and others further compared the susceptibility of single TNF- and LTα-deficient mice, and showed that both single gene-deficient mice succumbed to BCG infection, suggesting that both TNF and LTα are necessary and non-redundant to control BCG infection [57]. Reintroduction of LTα

as a transgene into TNF-LTα double-deficient mice prolonged survival but failed to restore resistance to BCG [57].

Although *M. bovis* BCG is an attenuated strain, the absence of TNF or TNF signaling induced a phenotype essentially similar to an infection with virulent M.tb. Indeed, mice deficient in TNF [39–42], or TNF-R1 [47], or mice treated with soluble TNFR1 or TNFR2 to neutralize TNF [44, 45, 58] have poorly formed granulomas with extensive regions of necrosis and neutrophilic infiltration of the alveoli, and an inability to control mycobacterial replication upon infection with virulent M.tb strains. Bean et al. [39] found comparable MHC class II and inducible NOS expression, serum nitrite levels, and normal activation of T cells and macrophages, while the organization of granulomas was clearly defective and not compensated by LTα. TNF was not required for granuloma formation, but rather for maintaining granuloma integrity indirectly by restricting mycobacterial growth within macrophages and preventing their necrosis in *Mycobacteruium marinum*-infected zebrafish [59]. Similarly, in a murine model of *M. bovis* BCG infection, established hepatic granuloma showed a profound decrease in size and in their population of noninfected macrophages within 2–4 days of anti-TNF treatment [60].

As observed in BCG infection studies, both TNF and LTα seemed necessary to control infection with virulent H37Rv strain of M.tb [41, 49]. However, the very close mutual proximity of genes coding for TNF, LTα and LTβ on mouse chromosome 17 raises the issue of collateral gene damage in mouse models employing targeted modifications of TNF/LT genomic locus. For example, independently generated mouse strains with TNF deficiency behave identically in a number of infection and stress models but demonstrate discrepant phenotypes with regard to the development of Peyer's patches, apparently due to differences in the configuration of the targeted locus [61]. Based on published reports, both removal of a regulatory element controlling transcription of the LT genes and their compensatory upregulation by the actively transcribed neo-resistance cassette can be envisioned. Since LT expression essential for the development of Peyer's patches has to be cell type specific and may be subject to autoregulatory feedback loops, resolution of these discrepancies proved to be a technically challenging task.

Another example of collateral gene damage, probably more relevant to TB research, is dysregulation of TNF expression in the 'conventional' LTα KO mice. Recently generated 'neo-cassette-free' $LT\alpha^{\Delta/\Delta}$ mice were fully capable of producing TNF at normal levels, whereas 'conventional' LTα KO animals displayed significant decrease in TNF synthesis in several critical types of leukocytes both in vitro and in vivo [62]. In the 'conventional' LTα KO mice, TNF deficiency could be corrected by transgenic TNF expression [63]. In agreement with the results of TNF promoter studies, the deficiency appears to be restricted to macrophages and neutrophils [62]. Defective TNF production has been noted, to various extent, by several published reports utilizing 'conventional' LTα KO mice [57, 64]. Once again, cell type-specific collateral damage to transcriptional initiation may be difficult to unambiguously discriminate from physiological mutual regulation of two closely related cytokines sharing some of their

receptors. Nevertheless, any conclusions indicating an independent protective role of soluble LTα in intracellular infections based on experiments with conventional LTα KO mice should be taken with certain caution. Our unpublished data indicate that LTα might have a less essential role than anticipated for the control of acute M.tb infection, and the phenotype observed in 'conventional' LTα KO might indeed result at least in part from additional defects such as reduced TNF expression.

### TNF Controls Hyper-Inflammatory Response

Although TNF has been mainly considered as a major proinflammatory cytokine, from accumulating studies it appears that TNF mediates both pro- and anti-inflammatory activities which are necessary first for a rapid recruitment of cells to infected sites and then to attenuate this process in order to limit lesions and tissue injury. Evidence was obtained with TNF deficient mice for anti-inflammatory activities of TNF in autoimmune-mediated demyelination [65]. Upon injection with heat-killed *Corynebacterium parvum*, $TNF^{-/-}$ mice showed very little response in the early phase, but later, they developed a strong lethal inflammatory response, whereas wild-type mice exhibited a prompt inflammatory response that resolved [66]. Similarly, infections with M.tb and *M. bovis* BCG in mice unable to use TNF resulted in a delay or very little response during early infection, whereas at late infection, exacerbated inflammatory reaction and disorganized granulomas were observed; [67–69] Mohan, 2001 No. 115; Guler, 2005 No. 1709; Florido, 2007 No. 1748; Zganiacz, 2004 No. 887; Flynn, 1995 No. 119; Jacobs, 2000 No. 17. This exaggerated inflammatory response following TNF deprivation was also observed when the bacterial load in infected organs was low [45]. The pathologies and tissue destruction observed in infected mice were attributed to an excess of IL-12 and IFN-γ production, suggesting that TNF acts as a negative regulator of Th1 immune responses [42]. Infection of $TNFR1^{-/-}$ mice with *Mycobacterium avium* also resulted in granuloma disintegration that was lethal and dependent on IL-12 expression in association with an excess of T cells. In this report, treatment with anti-IL-12 antibodies led to resolution of the exacerbated response in $TNFR1^{-/-}$ mice similar to that observed in wild-type mice [46]. A recent study has shown that granuloma disintegration observed in *M. avium*-infected $TNF^{-/-}$ mice was associated with upregulation of TRAIL, another member of the large TNF family of ligands [70]. These data support the anti-inflammatory in vivo role of TNF in mycobacterial infections which seems to be predominant in granuloma resolution and is associated with a TNF-regulated control of IL-12 and IFN-γ expression. Alternatively, in vitro macrophage infection with M.tb induced TNF but the TNF bioactivity was reduced due to the release of soluble TNFR2 and the formation of inactive TNF-TNFR2 complexes in an IL-10-dependent way [26]. These studies and our unpublished results suggest that TNF has a regulatory role in Th1 cytokine expression preventing a detrimental type 1 immune response. Therefore, complete absence of TNF results in an uncontrolled Th1 cytokine response.

### Correcting Experimental TNF Deficiency

Multiple injection of soluble recombinant TNF systemically in vivo did not result in any improvements in sick or infected TNF KO animals or anti-TNF-treated animals [54], indicating that TNF should be present locally. We thus reconstituted TNF deficiency by infecting the TNF-deficient host with recombinant BCG expressing TNF [12]. Indeed, in TNF-deficient mice infected with low doses of BCG expressing TNF, bacillary growth was controlled, granulomas were small and well differentiated, and the mice survived, unlike TNF-deficient mice infected with the wild-type BCG [12]. Therefore, local and not systemic production of TNF at the site of infection enabled a normal response controlling infection. However, infection with high inocula of BCG-expressing TNF induced severe inflammation in the lungs and spleen and earlier death despite a more rapid bacterial clearance. The relative amount of TNF at the site of infection seems to determine whether the cytokine is protective or destructive [12]. It has since been shown that reconstitution of TNF in the host by adenoviral gene transfer improved survival of TNF-deficient mice [42].

### Cell-Specific Response and TNF Control of Tuberculosis

The T cell response is critical to control mycobacterial infection. Indeed, antibody-mediated depletion of CD4 T cells in immunocompetent B6 mice leads to uncontrolled infection similar to what is observed in T cell-deficient mice [71]. Depletion of CD4 cells may also lead to reactivation of silent, chronic TB infection, despite almost normal levels of IFN-γ [72]. One of the explanations would be that the TNF produced by CD4 cells is critical for host resistance. Antigen-specific CD8 T cell responses to culture filtrate protein-10 (CFP10) were documented both in human volunteers and in M.tb-infected mice, where CFP10-specific T cells were detected as early as week 3 after infection and reached 30% of CD8 T cells in the lung with long persistence [73]. T cell subsets induced by M.tb infection include Th1 and Th17 cells, but the role of Th17 is still unclear as only the absence of Th1 cells but not of Th17 alters the protective response [74]. In vaccinated animals, however, absence of memory Th17 cells results in loss of accelerated memory Th1 response and protection [8]. Thus, Th1 and Th17 responses seem to cross-regulate each other during mycobacterial infection [8, 74].

Other cells involved in the control of infection, include γδ-T cells and NK cells. γδ-T cells, are recruited into the lung [75] and produce large amounts of IL-17 and may contribute to the host protection [7, 76]. NK cells are associated with early resistance against intracellular pathogens and potent producers of IFN-γ. Aerosol M.tb infection increased NK cell recruitment and activation, and IFN-γ secretion. However, in vivo depletion of NK cells using a lytic antibody had no influence on M.tb clearance. Therefore, NK cells appear to have a minimal role in the host resistance to M.tb [77].

By contrast, NKT cells may play a role in M.tb infection control. Activation of NKT cells by α-galactosylceramide in vivo augmented host resistance to M.tb in mice, which may also be mediated in part by the production of IFN-γ [78]. Moreover, activation of CD1 restricted human T cells increased killing, probably via granulolysin [79]. Indeed, some mycobacterial antigens can be presented to NKT cells in a context of CD1 nonclassical MHC, including mycobacterial PIM [80, 81], thereby mediating interaction of NKT cell with infected cells.

Mast cells are abundant in the lung and interact directly with a wide variety of infectious agents, including M.tb, triggering the release of histamine and β-hexosaminidase, TNF and IL-6, the latter being critically involved in antimycobacterial resistance. M.tb appears to interact with CD48 on mast cells inducing histamine release, which is inhibited by anti-CD48 antibodies. Therefore, M.tb and its antigens recognize and activate mast cells [82]. Recent studies using mast cell degranulation revealed reduced M.tb induced inflammation and reduce host resistance [83]. Further investigations in mast cell-deficient mice are necessary to define the role of mast cells in host response to M.tb infection.

Using mixed radiation bone marrow chimera, we demonstrated that TNF derived from hematopoietic cells rather than stromal cells of mesenchymal origin are essential for a normal host response to BCG infection [84].

Further, using T cell vs. macrophage/neutrophil-specific TNF-deficient mice we are currently analyzing the relative contribution of TNF originating from the different cell types in the control of M.tb infection.

## Molecular Mechanisms of Mycobacterial Killing/Resistance

Activation of macrophages and dendritic cells by M.tb induces several proinflammatory cytokines including TNF, LTα and IL-12, and expression of costimulatory molecules that enhance antigen presentation and activation of T cells. Activated T cells produce TNF, IFN-γ and LTα inducing further activation of macrophage and likely other cells including stromal cells. Activated macrophages express NOS2, producing NO and RNIs, which are critical for killing and inhibiting growth of virulent M.tb and BCG [27, 85, 86].

Mycobacteria may inhibit phagosome maturation and fusion with lysosomes, thereby escaping killing [87–90]. Activated macrophages recruit T cells to form granulomas, which contain bacterial growth. The granuloma is a dynamic structure, which requires a permanent signal from activated T cells and macrophages [91]. Any perturbation of this signaling such as neutralization of TNF causes dissolution of granulomas [54] and allows reactivation and spread of infection (fig. 1). Activated T cells not only provide help, but acquire cytotoxic functions, which eradicate bacilli, although the relative contribution of CD4 versus CD8 cells to control TB infection is not fully established.

In order to better understand the effect of TNF on intracellular replication of mycobacteria, we investigated the growth of the vaccine strain BCG in TNF-deficient macrophages. BCG infection resulted in logarithmic growth of the intracellular bacilli, while recombinant BCG-expressing TNF led to bacillary killing associated with production of NO. Therefore, TNF contributes to the expression of NOS2 and to bacterial growth inhibition indirectly [92].

IFN-γ has been shown to be an essential component of immunity to TB. It activates infected host macrophages to directly inhibit the replication of M.tb [3]. Although IFN-γ-inducible NOS2 is considered the principal effector mechanism, other pathways exist. M.tb has developed several mechanisms to escape eradication including inhibition of phagosome maturation [93]. Mycobacteria blocking $Ca^{2+}$ signaling and phagosome maturation in human macrophages or inhibiting sphingosine kinase may allow the escape from eradication in the phagocyte [94–96]. Role of autophagy and ensuing inhibition of phagolysosome formation [97] may be considered, as well as Coronin-1 inhibition as an alternative pathway to prevent phagosome maturation [98]. Autophagy can be induced by IFN-γ and by the immunity-related guanosine triphosphatases (GTPases) or LRG-47 (Irgm-1) which is a member of the IFN-γ-induced 47-kDa GTPase family [99]. LRG-47/Irgm-1 has been linked to autophagosome and autolysosome formation and killing of mycobacteria [100, 101].

Defensins such as cathelicidin (LL37) have an important anti-mycobacterium activity in human macrophages. Liu et al. [102] have reported that activation of Toll-like receptors (TLRs) upregulates the expression of the vitamin D receptor and the vitamin D-1-hydrolase generating $1,25(OD)_2D3$, the active form of vitamin D, and leading to induction of the microbicidal peptide cathelicidin and killing of intracellular M.tb in human macrophages. Granulysin contained in CD8 T and NK T cell granules as well as the perforin/granzyme system contribute to elimination of infected macrophages and mycobacteria [79, 103]. Exogenous ATP induces the killing of intracellular M.tb and *M. bovis* BCG in macrophages and involves the purinergic P2X7 receptor which is regulated by IFN-γ [104–107].

Mycobacteria induce apoptosis of macrophages and cause the release of apoptotic vesicles that carry mycobacterial antigens to uninfected antigen-presenting cells, including dendritic cells which are indispensable for subsequent antigen cross-presentation through MHC-I and CD1b. This new pathway for presentation of antigens from a phagosome-contained pathogen illustrated the functional significance of infection-induced apoptosis in the activation of CD8 T cells specific for both protein and glycolipid antigens in TB [108].

Induction of TNF and other proinflammatory cytokines is mediated through several mycobacterial motives triggering different pattern recognition receptors, including TLR2, TLR4 or TLR9. However, while the control of acute TB infection was severely compromised in the absence of MyD88 [109, 110], TLR2, TLR4 and/or TLR9 do not seem essential for the control of acute TB infection but may interfere in the control of chronic infection [111–113]. MyD88 pathway may thus contribute rather

through IL-1R signaling to control acute TB [9]. TLR/MyD88-dependent signaling is also required for phagosome maturation [114].

In summary, TNF participates in resistance to mycobacteria in the following ways: (1) activation of macrophages, (2) induction of chemokines and cell recruitment, (3) activation of T cells, (4) killing by macrophages, T and other cells and (5) regulation of apoptosis and signals from TLR/MyD88/IL-1R pathway that contribute to the host response. Since separating the effects of these different TNF functions in vivo is presently difficult or impossible, a computational model was applied to understand specific roles of TNF in control of TB in a single granuloma. The model predicted that macrophage activation is a key effector mechanism for controlling bacterial growth within the granuloma, TNF and bacterial numbers represent strong contributing factors to granuloma structure, and TNF-dependent apoptosis may reduce inflammation at the cost of impaired mycobacterial clearance [115].

### Role of Other Members of the TNF Family

In order to dissect the respective roles of soluble LTα3 and membrane-bound LTα-LTβ in the host response to aerosol M.tb infection, and to avoid the complication presented by the absence of secondary lymphoid organs in LT-deficient mice, Roach et al. [49] prepared bone marrow chimeric mice. LTα-deficient chimeras, which lack both secreted LTα3 and membrane-bound LTαLTβ, were highly susceptible and succumbed 5 weeks after infection, while LTβ-deficient chimeras, which lacked only the membrane-bound LTβ, controlled the infection similar to wild-type chimeric mice. T cell responses to mycobacterial antigens and macrophage responses in LTα-deficient chimeras were equivalent to those of wild-type chimeras, but granuloma formation was abnormal with perivascular and peribronchial location of T cells. These studies thus suggested that secreted LTα3 is essential for the organization of functional granulomas and control of pulmonary TB [49]. These data should probably be reevaluated now, in light of the potential defective TNF expression in these 'conventional' KO, as opposed to the more recent 'neo-cassette-free' LTα KO (see above). The role of the LTαβ-LTβR pathway in the control of mycobacterial infection was further studied. Treatment of BCG-infected mice with LTβR-Ig resulted in reduction of iNOS activity and increased bacterial growth [116]. Mice deficient in either LTα or LTβ, which form the LTαβ heterotrimeric ligand of LTβR, showed reduced resistance to M.tb infection, and LTβR KO had increased bacterial load with widespread pulmonary necrosis 35 days after infection, although they expressed normal levels of TNF and IFN-γ and recruited similar numbers of T cells in the pulmonary granulomatous lesions as compared to wild-type mice [117]. Furthermore, inhibition of the LTαβ pathway with soluble LTβR-Fc fusion proteins also compromised immunity against mycobacterial infections [116]. By contrast, LIGHT-deficient mice proved to be resistant to M.tb infection [117]. In conclusion, several members of the TNF family are critically involved in the host response to M.tb infection.

## Membrane TNF Biological Activity Controls Acute *Mycobacterium tuberculosis* Infection

Although a key role of TNF in controlling intracellular bacterial infections is uncontested, it is only recently that the specific function of membrane TNF has been appreciated. Membrane TNF is cleaved by the metalloproteinase-disintegrin TNF-α-converting enzyme [118] into secreted, soluble trimeric TNF. Several functions of membrane TNF have been described, such as cytotoxicity, polyclonal activation of B cells, induction of IL-10 by monocytes, ICAM-1 expression on endothelial cells and liver toxicity [34, 37, 119, 120]. The transgenic expression of membrane TNF suggested an in vivo role of membrane TNF [35]. Olleros et al. [67, 69] investigated the resistance to mycobacterial infection in transgenic mice expressing a membrane TNF (Δ12-10; Δ-2-+1; one substitution +11) under the control of proximal TNF promoter and on a TNF-LTα-deficient background. In this model, membrane, TNF had a fully protective effect against *M. bovis* BCG but only partial protective effect against M.tb infection. The recent generation of mice with functional, normally regulated and expressed membrane-bound TNF represents a major advance and allowed interesting insights into the role of membrane TNF in lymphoid structure development and inflammation. KI mice expressing the uncleavable KΔ1-9, K11E TNF [37] and TNF-deficient mice [66] were compared in their resistance to mycobacterial infection. As previously reported for membrane TNF transgenic mice, we and others demonstrated that membrane TNF has important biological functions and substitutes soluble TNF to a large extent [121–124]. Membrane TNF KI mice survived a M.tb aerosol infection for 3 months, were able to recruit and activate macrophages and T cells, generate granuloma and partially control mycobacterial infection in the early stage, unlike complete TNF-deficient mice [122, 123]. However, during the chronic phase of infection, membrane TNF KI mice demonstrated reduced bacterial clearance and succumbed to infection [122]. In another model of targeted mutagenesis in mice, the shedding of membrane TNF was prevented by deleting its cleavage site [38]. Mice expressing noncleavable and regulated Δ1-12 TNF allele partially controlled *M. bovis* BCG infection, with recruitment of activated T cells and macrophages and granuloma formation, while mice with complete TNF deficiency succumbed [125]. It was confirmed that membrane TNF conferred partial protection against virulent *M.tb* infection, and intercrossing these mice with TNF-R1 or TNF-R2 KO mice showed that tmTNFxTNFR2 KO mice were very sensitive, essentially as much as TNF KO mice, while tmTNFxTNFR1 KO mice behaved more like tmTNF mice, suggesting that the protective effect of membrane TNF against acute *M.tb* infection is mediated through TNF-R2 signaling [125].

Therefore, data from the genetic mouse models suggest that membrane-expressed TNF is sufficient and soluble TNF may be dispensable to control the first phase of acute TB infection. However, during the chronic phase membrane TNF alone is not sufficient and soluble TNF seems to be required to control chronic TB infection. The

reason for the progressive loss of infectious control is unclear. As previously discussed, soluble TNF may be required to negatively control the Th1 type cytokines. This TNF function may become important during the chronic phase of infection by regulating excess production of IL-12 and IFN-γ by DC and T cells.

### TNF in Reactivation of Tuberculosis Infection

Clinical TB in humans may be due to a primary infection or reactivation of latent controlled infection. Secondary immunosuppression due to HIV/AIDS is the most common cause of M.tb reactivation. In the recent years, over a million patients received TNF-neutralizing therapy for the treatment of severe rheumatoid arthritis, Crohn's disease or severe psoriasis. The most common complication of TNF blockade has been the emergence of opportunistic infection and TB. Both reactivation of latent TB and increased susceptibility to new TB in patients without a clinical history of active TB infection was observed [126]. In some patients, TNF-neutralizing antibody, infliximab, or soluble TNFR2-IgG1 Fc fusion protein, etanercept, yielded reactivation of latent TB within 12 weeks and overt clinical disease [127–129], often with extrapulmonary disease manifestations (disseminated infection in lymph node, peritoneum and pleura). The frequency of TB in association with infliximab therapy was higher than the reported frequency of other opportunistic infections associated with this drug [129]. Reactivation of latent TB and primary infection in patients treated with TNF inhibitors are still difficult to clearly define in many cases. Anti-TNF antibody may be more associated with latent TB reactivation than etanercept. The majority of etanercept-associated cases of TB appeared late (90% after 90 days of treatment), suggesting that these cases may have occurred as a result of the inability to control new M.tb infection while 43% of infliximab associated cases of TB occurred during the first 90 days of treatment, indicating that they likely represent reactivation of latent infection [126, 130]. The reactivation of latent TB under TNF-blocking therapy indicates that the normal immune system is able to control, but not able to eradicate, a primary infection, and that TNF plays a role in the long-term containment of residual M.tb in tissues.

In order to study the factors leading to reactivation of chronic or chemotherapy-controlled latent infection, several experimental models have been developed [131]. In the Cornell model, after an intravenous administration of M.tb H37Rv and treatment with pyrazinamide and isoniazide for 12 weeks, mice appear to have cleared the bacilli from organs, but a substantial proportion of animals spontaneously reactivate with acute disease upon cessation of chemotherapy. Since the original publication of the Cornell model, a few variations have been reported [132, 133]. In the low-dose model, infection is exclusively controlled by the host in the absence of chemotherapy [133]. Although considered to better reflect the human host response, bacterial numbers in the organs of these mice remain high during the chronic persistent

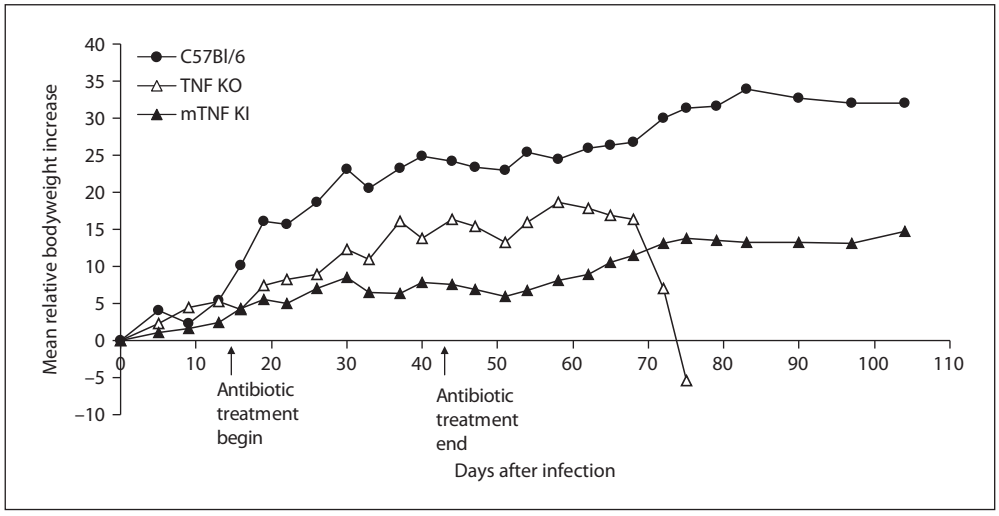


**Fig. 2.** Comparison of susceptibility of membrane TNF KI and conventional TNF KO reactivating chronic M.tb infection. Wild-type, TNF KO and mTNF KI mice were infected with M.tb (ca 100 CFU i.n.) and treated for 4 weeks with rifampicin and isoniazide (days 14–42) to control the infection. TNF KO mice started to die 6 weeks after the end of the antibiotic treatment, while all mTNF KI mice survived as wild-type mice. In parallel groups infected with M.tb but not treated with antibiotics, TNF KO had to be euthanized at 3 weeks, while 6 out of 8 mTNF KI survived with no marked bodyweight loss.

phase of infection. To date, these models have yielded significant information on the immune effector mechanisms participating in latent or chronic persistent and reactivated TB.

We established the first aerosol infection model of drug-induced latent and reactivated murine TB using rifampicin and isoniazide [132, 134]. In this model, latency was defined as almost undetectable levels of bacilli in mouse organs for a prolonged period of time. Reactivation of infection could be achieved by inhibiting NOS activity by aminoguanidine [132]. Using this model, we showed that a 4 weeks' rifampicin and isoniazide administration cleared infection as assessed by viable bacterial accounts in the organs in both wild-type and TNF-deficient mice. Upon cessation of therapy, massive spontaneous reactivation of M.tb infection occurred within several weeks in TNF-deficient mice with necrotic pneumonia and death, while wild-type mice displayed mild subclinical reactivation [134]. This model allows us to study the role of TNF neutralization in a reactivating infection in the presence of an established specific adaptive immune response.

The role of soluble versus membrane TNF was then studied in this model (fig. 2 and unpubl. data). Although TNF KO mice rapidly lost weight and had to be terminated within 6 weeks after the end of the antibiotic treatment with uncontrolled infection, mTNF KI mice survived as wild-type mice. Therefore, membrane TNF

suffices to provide some control of the M.tb infection after reduction of the bacterial burden by an antibiotic treatment, while complete absence of TNF results in rapid progression of the infection.

## Pharmacological TNF Neutralization and Tuberculosis Control

The experimental models of TB reactivation described above allow us to test the potential risk of diverse TNF-neutralizing therapies to induce reactivation of TB. Administration of neutralizing TNF antibody but not of soluble TNF receptor was able to reactivate experimental latent infection [135]. TNF neutralization resulted in marked disorganization of the tuberculous granuloma and enhanced expression of specific proinflammatory molecules [68]. A computational approach suggested that TNF bioavailability following anti-TNF therapy is the primary factor for causing reactivation of latent infection and that even very low level of soluble TNF is essential for infection control [136].

Novel approaches to experimentally block soluble TNF are being tested in murine models of TB. One approach is to compete for natural TNF by the use of dominant negative mutant TNF (DN-TNF; see fig. 3) reported to block soluble TNF while sparing membrane TNF [137]. In vivo, DN-TNF attenuated arthritis without suppressing innate immunity to *Listeria monocytogenes* [138]. Similarly, DN-TNF protected mice from acute liver inflammation, without compromising host control of *M. bovis* BCG and M.tb infections [139]. This was in contrast to TNFR2-IgG1 etanercept that inhibits murine soluble and membrane TNF as well as LTα, which severely compromised the host response to M.tb infection [139]. Another novel approach is an active immunization selectively targeting soluble TNF. Vaccination with a virus-like particle linked to a TNF N-terminal peptide resulted in high titers of autoantibodies against soluble TNF. It protected mice from arthritis without inducing reactivation of latent TB [140], while immunization against the entire TNF molecule yielded enhanced reactivation of latent TB. This difference was attributed to recognition of only soluble TNF vs. recognition of both transmembrane and soluble TNF by the elicited antibodies. Thus, specifically targeting soluble TNF has the potential to be effective against inflammatory disorders while overcoming the risk of opportunistic infections known to be associated with the currently available TNF antagonists.

## Conclusions and Perspectives

TNF is an essential mediator for the integrity of microbiocidal granulomas and the control of M.tb infection. Experimental TB infection of gene-deficient mice has demonstrated the nonredundant contribution of several proinflammatory

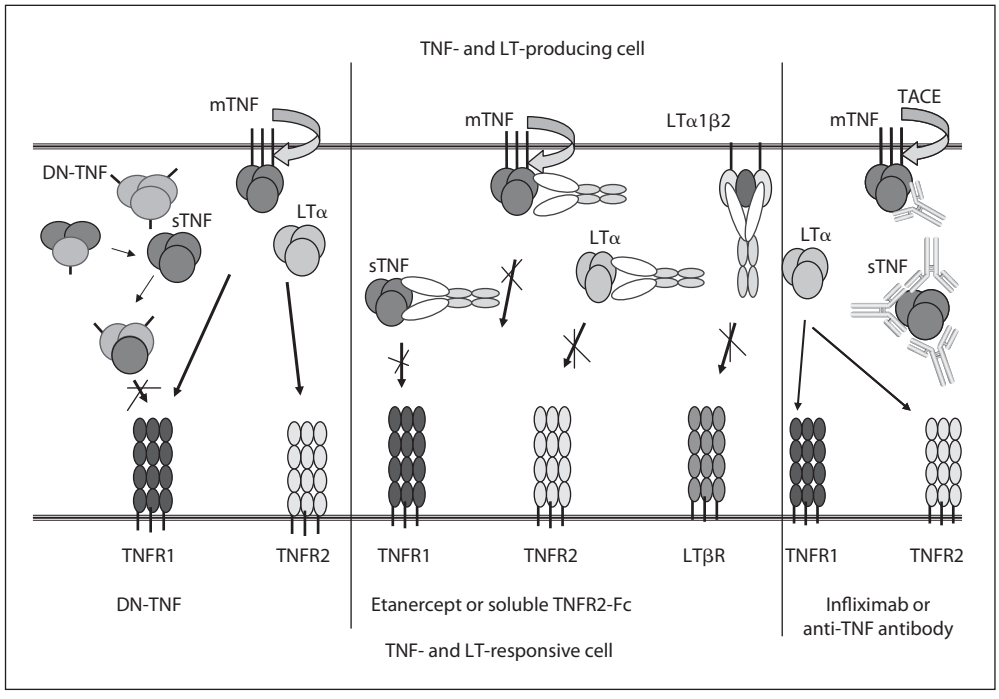


**Fig. 3.** Mechanisms of action of dominant-negative TNF (DN-TNF) biologics, soluble TNFR2-Fc (etanercept), or anti-TNF antibodies. Left: DN-TNF, a mutated form of human solTNF with disrupted receptor-binding interfaces eliminates solTNF by a subunit exchange mechanism, but is unable to interact with tmTNF and LTα. Center: solTNF, tmTNF, LTα and LTαβ can be neutralized by etanercept, inhibiting interaction with the corresponding receptors. Right: Monoclonal antibodies directed against human TNF neutralize both membrane-bound and soluble TNF, while sparing LTα. Thus, DN-TNF (XENP1595) inhibits solTNF receptor signaling without suppressing tmTNF- or LTα responses to TNFR1 and TNFR2, mediating inflammatory and immune responses.

cytokines such as TNF, IL-12, IFN-γ or IL-1 to the host response to M.tb infection [9, 131]. An important notion is the fact that latent mycobacterial infection can be reactivated by TNF neutralization. The finding that membrane TNF confers partial protection and abrogates the hyperinflammatory syndrome is significant. Sparing membrane TNF in neutralizing TNF therapy used in rheumatic arthritis or Crohn's disease may diminish the infectious complications and reactivation of latent TB infection.


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