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Review

Tumor necrosis factor is critical to control tuberculosis infection

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Abstract

Tumor necrosis factor (TNF) is critical and non-redundant to control *Mycobacterium tuberculosis* infection and cannot be replaced by other proinflammatory cytokines. Overproduction of TNF may cause immunopathology, while TNF neutralization reactivates latent and chronic, controlled infection, which is relevant for the use of neutralizing TNF therapies in patients with rheumatoid arthritis.

Keywords: Tumor necrosis factor; Tuberculosis; Reactivation of infection; Gene deficient mice

1. Introduction

Tuberculosis (TB) infection is a major health problem caused by several strains of *Mycobacterium tuberculosis* (Mtb). The HIV/AIDS epidemic, increasing poverty and malnutrition allowed a massive re-emergence of active tuberculosis infection in endemic areas, especially in Sub-Saharan Africa and Asia. The present estimate is that one third of the world population harbors Mtb in a latent form, which may be reactivated when the host immune response is suppressed such as in HIV infection. Unraveling the host immune response during the primary and chronic/latent infection, is therefore a major challenge. Key immune factors controlling tuberculosis and reactivation of infection are T cells and macrophages/antigen presenting cells. Upon phagocytosis by macrophages Mtb induces tumor necrosis factor (TNF), interleukin-12 (IL-12), and reactive nitrogen intermediates (RNI) production and expression of costimulatory molecules. This in turn leads to activation of T and NK cells and interferon-γ (IFN-γ) production, augmenting the microbicidal activity of the phagocytes [1,2]. A simplified view of how Mtb interacts and activates antigen presenting cells and induces T-cell activation is depicted in Fig. 1. Activated macrophages, T cells including cytotoxic T cells together with their mediators are able to kill Mtb bacilli. 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 T cells forming the typical granulomas of Mtb infection. Depletion of T cells or inactivation of TNF or other immune factors at different stages of infection results in granuloma disruption with bacterial growth and dissemination which may lead to death. Apart from its protective effects in the immune response to Mtb infection, excess of TNF may

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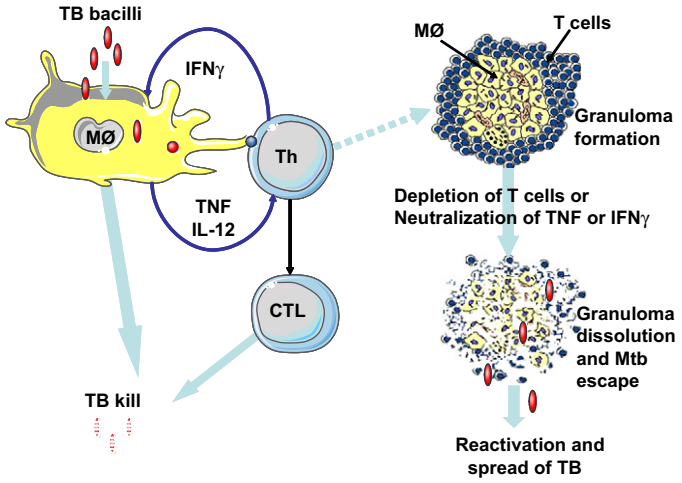

Fig. 1. Macrophage and T-cell activation, killing of TB bacilli and granuloma formation. Macrophages are activated by TB bacilli and produce cytokines and T-cell activation. Activated macrophages are mycobactericidal, but a few bacilli escape. The cell activation induces additional/further/consequent lymphocyte recruitment orchestrated by chemokines leading to the formation of granulomas which contain the bacilli. Antibody neutralization of TNF or IFNγ or T-cell depletion result in dissolution of the granuloma structure, rescue of surviving bacilli with dissemination of infection.

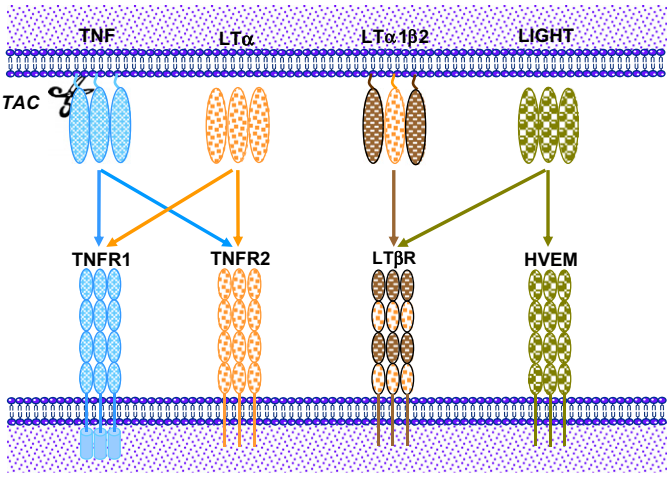

Fig. 2. Simplified overview of members of TNF ligands and receptors. Soluble trimers of TNF, LTα, and LIGHT are secreted by different cell types including T cells, while LTα-LTβ heterotrimer are membrane bound. TNF is first expressed as membrane precursor protein, which has biological activity and is then cleaved by the protease TACE to yield the soluble TNF trimer. The ligands bind to different membrane trimeric receptors as shown in the graph, which include TNF-R1 and TNF-R2, LTβR and HVEM (herpes virus entrance mediator) and are broadly expressed, but trigger differential signaling mechanisms such as activation of classical and alternative NFκB and the JNK pathways (not shown).

also cause pathology, including hyperinflammation with necrosis and cachexia, which are correlated with elevated TNF levels [3,4]. Here we review the role of TNF in the development of immunity using our own and published data in genetically modified mice.

2. The TNF family and the genetic mouse models

TNF is the founder member of cytokine TNF-like superfamily [5]. TNF gene is tightly linked with lymphotoxin (LT) α and β genes to the major histocompatibility locus on murine chromosome 17 (human 6), while the receptors (R), TNF-R1 and LTβR are clustered on mouse chromosome 6 and human chromosome 13 [6,7]. Soluble homotrimeric TNF and lymphotoxin (LT)α bind either TNF-R1 (p55) or TNF-R2 (p75), while the membrane bound LTαβ heterotrimer engages LTβ receptor. Receptor ligation initiates signals through a complex cascade to activate the nuclear factor NFκB resulting in gene activation (Fig. 2). There is a complex interaction and cross-talk between TNF-R1 and TNF-R2 signaling and presumably also with LTβR signaling [8]. The TNF family comprises several additional members such as LIGHT binding to LTβR, CD40, FAS-L and many more [9,10].

Different models to study the role of TNF cytokines *in vivo* have been explored, and the most powerful tools are transgenic and gene-deficient mice. In tuberculosis research so far the following mouse gene knockouts (KO) have been evaluated: TNF, lymphotoxin α (LTα), LTβ, TNF/LTβ, TNF/LTα/LTβ, TNF/LTα, LIGHT, as well the TNF-R1 and R2, and LTβR KO mice [11–15].

Several transgenic and gene knock-in mice expressing either human or mouse TNF systemically or in a tissue-specific manner are available [16], which are relevant to the conditions of systemic TNF overproduction or local inflammation such as arthritis, colitis or chronic CNS inflammation [16]. Finally, tissue specific TNF KO mice [17] and LTβ KO mice [18] were generated to uncover *in vivo* functions of TNF produced by different cell types of immune system such as macrophages/neutrophils or lymphocytes. Recent investigations revealed that systemic TNF in response to lipopolysaccharide was produced mostly by macrophages/neutrophils.[17]

3. Non-redundant role of TNF to control mycobacterial infection

The control of *Mycobacterium bovis* BCG induced infection depends on TNF as mice treated with anti-TNF antibodies succumbed to infection [19]. We showed that both TNF and LTα signaling is required to activate cells of the immune system [15]. TNF-LTα double deficient mice display high susceptibility and succumb to BCG infection between 8 and 10 weeks. The granuloma response was severely impaired and macrophages expressed reduced inducible nitric oxide synthase (NOS_2)—the key mediator of antibacterial defense—suggesting that both TNF and nitric oxide have bactericidal function [15]. We 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 TNF and LTα are necessary and non-redundant to control BCG infection (M Jacobs et al., unpublished data).

Mice deficient for the TNF-R1 [12], or the TNF gene [20,21] have poorly formed granulomas with extensive regions of necrosis and neutrophilic infiltration of the alveoli, and inability to control mycobacterial replication upon infection with virulent Mtb strain. Bean et al found that TNF KO animals showed

comparable MHC class II and inducible nitric oxide synthase 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. As for BCG infection, both TNF and LTα are necessary to control infection with virulent H37Rv strain of Mtb [14,21] and non-redundant as TNF and LTα double deficient mice have a comparable sensitivity to Mtb infection as single KO mice (Jacobs, in preparation). It is important to note that for the control of Mtb infection TNF provided by hematopoietic cells is critical, as revealed by bone-marrow chimeric mice [22].

TNF has long been regarded as a protective cytokine involved in antimicrobial Th1 immunity. We and others have shown that mice lacking TNF or TNF signaling quickly succumb to Mtb infection with respiratory failure due necrotic pneumonia [12,15]. TNF-R1 expression has been shown as important in control of *M. avium* infection [11]. Disrupted TNF–TNF-R1 signaling leads to massive inflammation with necrosis [11]. Tissue destruction may be the result of an uncontrolled T helper type 1 immune response characterized by expansion of activated and antigen-specific CD4 and CD8 T cells, and overproduction of IFNγ and IL-12 as shown recently [23]. Depletion of CD4 and CD8 T cells decreased IFNγ production, prevented granuloma and tissue necrosis, and prolonged the survival of TNF-deficient mice. Early reconstitution of TNF by gene transfer reduced the frequency of antigen-specific T cells and improved survival [23]. TNF controlled the exaggerated type 1 immune activation at least in part by suppressing T-cell proliferation. Heightened type 1 immune response also occurred in TNF-deficient mice treated with dead mycobacteria, live replication-deficient mycobacteria, mycobacterial cell wall components or heat-killed *Corynebacterium parvum*. These studies and our unpublished results suggest that TNF has a regulatory role on Th1 cytokine expression preventing a detrimental type 1 immune response [23]. Therefore, complete absence of TNF results in an uncontrolled Th1 cytokine response.

4. Correction of TNF deficiency

Since TNF is critical in the host resistance to mycobacterial infection, we asked whether TNF from hematopoietic or stromal cells confer protection. Therefore, we generated mixed radiation bone marrow chimeras, and tested their susceptibility to mycobacterial infection. We demonstrated that the defective host response was corrected by the transplantation of wild-type (WT) bone marrow cells into irradiated TNF-deficient mice (BM-WT > TNF KO). Reconstituted TNF-deficient mice were able to clear bacilli as wild-type control mice (Fig. 3A), developed bactericidal granulomas and survived infection [22]. Conversely, reconstitution of irradiated WT mice with TNF-deficient bone marrow heightened their susceptibility to infection (BM-TNF > WT). These results demonstrate that TNF derived from hemopoietic cells rather than from mesenchymal origin are essential for a normal host response to BCG infection [22]. Reconstitution of TNF in the host by adenoviral gene transfer improved survival of TNF-deficient

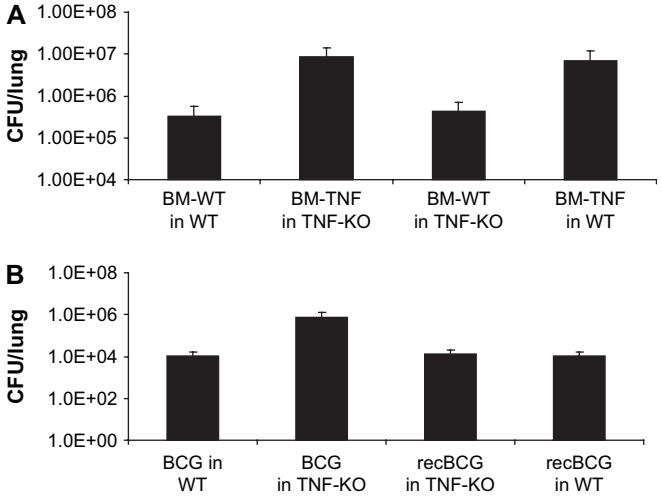

Fig. 3. Correction of heightened susceptibility of TNF-deficient mice to infection. Two different approaches were chosen: Infection in mixed radiation bone marrow cells (BM) chimeric mice (A) or use of BCG (recBCG) expressing recombinant TNF (B). (A) Correction of TNF KO mice susceptibility to *Mtb* infection by WT BM reconstitution or transfer of susceptibility to BCG infection by TNF KO BM reconstitution (BM-TNF) in WT mice as described [22]. (B) Correction of susceptibility of TNF-deficient mice by BCG expressing TNF, modified after Bekker et al. [3].

mice as reported recently [23]. On the other hand, 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 [19], indicating that TNF should be present locally.

We chose yet another approach reconstituting TNF deficiency by infecting the TNF-deficient host with recombinant BCG expressing TNF [3]. Infection of TNF-deficient mice with BCG containing TNF vector (BCG-TNF vector) at a low dose led to increased bacillary load in all organs and an extensive granulomatous response in the lungs and spleen. In TNF-deficient mice infected with low doses of BCG-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 (Fig. 3B). Therefore, local and not systemic production of TNF at the site of infection enabled a normal response controlling infection [3,19]. However, infection with high inocula of BCG-TNF induced severe inflammation in the lungs and spleen, and earlier death, despite a more rapid bacterial clearance. Therefore, the relative amount of TNF at the site of infection determines whether the cytokine is protective or destructive [3].

5. Role of other members of the TNF family

To investigate the role of lymphotoxins in TB infection we infected LTα-deficient mice and found a comparably augmented susceptibility to aerosol infection with death within 4 weeks as in TNF-deficient mice, as described [14] (Jacobs et al., submitted). In order to dissect the roles of secreted LTα3 and membrane-bound LTα-LTβ in the host response to aerosol Mtb infection, and to avoid the complication of the

absence of secondary lymphoid organs in lymphotoxin-deficient mice, Roach and colleagues prepared several bone marrow chimeric mice [14]. LTα deficient chimeras, which lack both secreted LTα3 and membrane-bound LTβ, were highly susceptible and succumbed 5 weeks after infection, while LTβ-deficient chimeras, which lack only the membrane-bound LTβ, controlled the infection as did 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. Therefore, these studies suggest that secreted LTα3 is essential for the organization of functional granulomas and control of pulmonary tuberculosis [14].

Ehlers et al. investigated the role of the LTαβ–LTβR pathway upon Mtb infection [24]. Mice deficient for LTβR developed significantly increased bacterial load and exhibited widespread pulmonary necrosis as soon as day 35 after intranasal Mtb infection. Furthermore, mice deficient for either LTα or LTβ, which form the LTαβ heteromeric ligand of LTβR, showed as expected reduced resistance to Mtb infection [24]. The discrepant results for a role of LTαβ from the previous study is unresolved [14]. By contrast, LIGHT-deficient mice proved to be resistant to Mtb infection. Therefore the LTαβ–LTβR-signaling axis appears to be an essential prerequisite for containment of Mtb infection. With respect to TNF-R1 and TNF-R2 signaling, Flynn et al. demonstrated that TNF-R1 is critical to control infection [12], while TNF-R2 may not be involved.

Another member of the TNF family, CD40, is upregulated in antigen presenting cells upon mycobacterial infection. Interestingly, CD40-deficient mice succumbed to low-dose aerosol infection with Mtb, with deficient IL-12 and IFNγ T-cell responses, while CD40 L deficient mice were resistant to infection [25]. Therefore mycobacteria might activate directly CD40, which could explain the differential sensitivity to Mtb infection. Mycobacterial heat shock protein Hsp70 was shown to activate CD40 and this response is abrogated in the absence of CD40. Therefore the data suggest that mycobacterial Hsp70 serves as an alternative ligand for CD40 [25].

In conclusion, several members of the TNF family are critically involved in the host response to Mtb infection.

6. Membrane TNF has biological activity controlling acute Mtb infection

Although a key role of TNF in controlling intracellular bacterial infections is uncontested, the function of membrane TNF, which is subsequently cleaved by the metalloproteinase-disintegrin TACE (TNF converting enzyme) into the secreted trimeric TNF was unknown in mycobacterial host resistance.

Several biological functions of membrane TNF have been described, such as cytotoxicity, polyclonal activation of B cells, induction of IL-10 by monocytes, and ICAM-1 expression on endothelial cells. Olleros et al. investigated the resistance to mycobacterial infection in membrane TNF transgenic mice on a TNF-LTα deficient background, and showed that membrane TNF has a partial protective effect [26]. However, transgenic expression of high-level membrane TNF is artificial and may cause non-physiological pathology. The recent generation of a mouse with functional, normally regulated and expressed membrane-bound TNF, which was obtained by knocking-in an uncleavable Δ1-9,K11E TNF allele, allowed interesting insights in the role of membrane TNF in lymphoid structure development and inflammation [27]. The host resistance to mycobacterial infection of Δ1-9,K11E TNF knock-in mice [27] was compared with TNF-deficient mice. Using this model it was demonstrated that membrane TNF has important biological functions and substitutes soluble TNF to a large extent [28,29]. TNF knock-in mice were able to recruit and activate macrophages and T cells, generate granuloma and partially control mycobacterial infections unlike complete TNF-deficient mice. Unexpectedly, 3 months post infection mice expressing solely the membrane TNF progressively lost control of infection with increased bacterial burden, and developed inflammation and succumbed to chronic infection. The reason for the progressive loss of infectious control is unclear, but one hypothesis is that there is a continuous exhaustion of T cells during the chronic phase of infection.

7. Latent/persistent infection and reactivation of TB infection

Over a million people are currently receiving TNF neutralizing therapy for the treatment of severe rheumatoid arthritis. The most common complication of TNF blockade is the emergence of opportunistic infection and TB reactivation in patients without a clinical history of active TB infection. Therefore, these data suggest the normal immune system is able to control, but not to eradicate a primary infection, and TNF appears to play a role in the long-term containment of residual Mtb in tissues.

Several experimental attempts have been made to study the factors leading to reactivation of chronic or chemotherapy controlled latent infection. Two basic models have been described to date, the Cornell model and the low-dose infection model. The Cornell model consists of an intravenous administration of Mtb H37Rv and treatment with pyrazinamide and isoniazide (INH) for 12 weeks. The mice appeared to have cleared the bacilli from organs, but in a substantial proportion of mice the *Mtb* infection spontaneously reactivated with acute disease upon cessation of chemotherapy. Since the original publication of the Cornell model a few variations on this model have been reported [30,31]. The low-dose model involves infection with tubercle bacilli in the absence of chemotherapy with the ensuing infection exclusively controlled by the host [31,35]. Although the latter is considered to better reflect the human host response, bacterial numbers in the organs of these mice remain high during the chronic persistent phase of infection. To date, these models have yielded significant information on the immune effector mechanisms participating in latent or chronic persistent and reactivated tuberculosis.

We established the first aerosol infection model of drug-induced latent and reactivated murine tuberculosis using rifampicin and isoniazid [32]. In this model, latency is defined

as almost undetectable levels of bacilli in mouse organs for a prolonged period of time. Reactivation of infection can be achieved by inhibiting nitric oxide synthase activity by aminoguanidine. Using this low-dose aerosol infection model, we showed that a 4 weeks rifampicin and isoniazid administration cleared infection as assessed by viable bacterial accounts in the organs in both wild-type and TNF-deficient mice. Upon cessation of therapy a massive spontaneous reactivation of Mtb infection occurred within 4 weeks in TNF-deficient mice with necrotic pneumonia and death, while wild-type mice displayed mild subclinical reactivation [32].

Experimental reactivation using antibodies and other inhibitors has been reviewed [31]. Administration of neutralizing TNF antibody or soluble TNF receptor was able to reactivate latent infection. A novel approach is to compete for natural TNF by the use of dominant negative mutant TNF, which deserves further testing [33].

Therefore, the experimental model allows testing the potential risk of diverse TNF neutralizing therapies to induce reactivation of TB.

8. Clinical reactivation of TB infection

Clinical tuberculosis 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 Mtb reactivation. Recently, therapy with neutralizing TNF antibody, infliximab, or soluble TNFR, Etanercept, was complicated by reactivation of latent Mtb bacilli and overt clinical disease. Reactivation occurs within 12 weeks of commencement of TNF neutralizing therapy [34] and has often extrapulmonary disease manifestations (disseminated infection in lymph node, peritoneum and pleura). The frequency of tuberculosis in association with infliximab therapy was much higher than the reported frequency of other opportunistic infections associated with this drug. Therefore, active tuberculosis may develop quite soon after the initiation of treatment with infliximab or Etanercept [34].

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) represents a global health issue in Southern Africa and Asia and the major complication is reactivation of infection in endemic TB areas (see WHO). However, this is not within the scope of this review, but suffices to recall that depletion of CD4 cells and cytokines allows the reactivation of latent/persistent *Mtb* infection. Further to the tragedy for individuals affected with both plagues, the costs of TB and HIV/AIDS are catastrophic for most households. The urgent need for a substantial increase in health sector investments to expand access to preventive and curative health services and international economic support is critical to control TB and HIV/AIDS infection.

9. Conclusions and perspectives

In conclusion, TNF is critical and non-redundant to form microbicidal granulomas and to control Mtb infection. The reason for the need for several cytokines for the control of Mtb infection is not understood. We propose that TNF and other cytokines may work in sequence, although their exact sequential functions are still largely elusive.

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. At a global level the most significant cause of TB reactivation is HIV infection with depletion of CD4 T cells and likely TNF and related cytokines. Clearly, this form of TB reactivation and its relation to TNF depletion and, most importantly, the access to preventive and curative therapies, should receive our utmost attention.

Acknowledgments

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