Dominant-negative tumor necrosis factor protects from Mycobacterium bovis Bacillus Calmette-Guérin (BCG) and endotoxin-induced liver injury without compromising host immunity to BCG and Mycobacterium tuberculosis

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Abstract

Tumor necrosis factor (TNF) is associated with the development of inflammatory pathologies. Antibodies and soluble TNF (solTNF) receptors that neutralize excessive TNF are effective therapies for inflammatory and autoimmune diseases. However, clinical use of TNF inhibitors is associated with an increased risk of infections.

Reference


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Dominant-Negative Tumor Necrosis Factor Protects from Mycobacterium bovis Bacillus Calmette-Guérin (BCG) and Endotoxin-Induced Liver Injury without Compromising Host Immunity to BCG and Mycobacterium tuberculosis

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Background. Tumor necrosis factor (TNF) is associated with the development of inflammatory pathologies. Antibodies and soluble TNF (solTNF) receptors that neutralize excessive TNF are effective therapies for inflammatory and autoimmune diseases. However, clinical use of TNF inhibitors is associated with an increased risk of infections.

Methods. A novel dominant-negative (DN) strategy of selective TNF neutralization, consisting of blocking solTNF while sparing transmembrane TNF (tmTNF), was tested in mouse models of mycobacterial infection and acute liver inflammation. XENP1595, a DN-TNF biologic, was compared with etanercept, a TNF receptor 2 (TNFR2)–IgG1 Fc fusion protein that inhibits murine solTNF and tmTNF.

Results. XENP1595 protected mice from acute liver inflammation induced by endotoxin challenge in Mycobacterium bovis bacillus Calmette-Guérin (BCG)–infected mice, but, in contrast to etanercept, it did not compromise host immunity to acute M. bovis BCG and Mycobacterium tuberculosis infections in terms of bacterial burden, granuloma formation, and innate immune responses.

Conclusions. A selective inhibitor of solTNF efficiently protected mice from acute liver inflammation yet maintained immunity to mycobacterial infections. In contrast, nonselective inhibition of solTNF and tmTNF suppressed immunity to M. bovis BCG and M. tuberculosis. Therefore, selective inhibition of solTNF by DN-TNF biologics may represent a new therapeutic strategy for the treatment of inflammatory diseases without compromising host immunity.
using anti–murine TNF antibodies and soluble TNF (solTNF) receptors in mouse models of acute tuberculosis [19]. Although mouse models of acute tuberculosis may not fully reflect the complexities of the clinical use of anti-TNF biologics, these reports provide a potential explanation for the increased risk of mycobacterial infections associated with anti-TNF treatment.

TNF is synthesized as a 26-kDa transmembrane TNF (tmTNF) precursor that is cleaved by membrane-bound TACE (TNF-α–converting enzyme) to generate a 17-kDa solTNF molecule [20, 21]. Using genetic models (transgenic tmTNF in TNF/lymphotoxin-α (LTα)−/− and tmTNF knock-in mice), we have shown elsewhere that tmTNF induces an efficient cell-mediated immune response to Mycobacterium bovis bacillus Calmette-Guérin (BCG) infection and confers protection against acute Mycobacterium tuberculosis infection but does not fully protect against long-term M. tuberculosis infection, as also reported by others [22–25]. To date, in vivo studies of the roles played by solTNF and tmTNF in infection have been limited to such genetically modified mice. However, tmTNF is essential not only in immune responses to infection but also in the development of a normal lymphoid structure [26]. Given the abnormal development of the immune system in TNF−/− and tmTNF knock-in mice, pharmacological studies of TNF inhibitors in normal mice may better model the therapeutic use of anti-TNF biologics in humans.

The growing literature establishing the importance of tmTNF in immunity has generated support for a hypothesis that selectively inhibiting solTNF while sparing tmTNF may reduce inflammation yet maintain the host response to pathogens. A novel class of TNF inhibitors, dominant-negative (DN) TNF biologics, antagonizes solTNF but not tmTNF and is active in attenuating experimental arthritis [27]. These biologics rapidly exchange subunits with native solTNF to form inactive mixed heterotrimers, eliminating native solTNF homotrimers without inhibiting tmTNF [27, 28]. The selectivity of this class of biologics for solTNF contrasts with other anti-TNFs, including etanercept, infliximab, and adalimumab, which inhibit both tmTNF and solTNF [28–33]. Recent studies have shown that DN-TNF biologics such as XENP1595 are effective in reducing inflammation in mouse models of arthritis and Parkinson disease but, in contrast to nonselective inhibitors, do not suppress resistance to Listeria monocytogenes infection [28, 34]. The present study analyzes the effects of XENP1595 on host defense against M. bovis BCG and M. tuberculosis infections and on protection against endotoxin-induced liver inflammation in M. bovis BCG–infected mice.

We used etanercept as a comparator biologic that inhibits both solTNF and tmTNF [4, 28–31] and, unlike the anti-TNF antibodies infliximab and adalimumab, neutralizes TNF in mice [28, 35–37]. The data show that XENP1595 efficiently protected against endotoxin-mediated hepatotoxicity in M. bovis BCG–infected mice while preserving immunity against M. bovis BCG and M. tuberculosis infections, presumably via maintenance of physiological tmTNF signaling.

**METHODS**

**Animals.** C57BL/6 mice (Charles River Laboratories) and TNF−/− mice [24] were maintained under conventional conditions in the animal facilities of the Medical Faculty, University of Geneva, and the Transgenose Institute, Orleans, France. Experiments were done in accordance with institutional guidelines and were approved by the Centre Médical Universitaire and the French Regional Ethical Committee on Animal Experimentation.

**Reagents.** The Escherichia coli–produced DN protein XENP1595 is a modified version of human solTNF (UniProtKB/ Swiss-Prot entry P01375) that contains mutations Y87H and A145R to eliminate binding to and signaling through TNF receptors TNFR1 and TNFR2 [27, 28]. The protein contains mutations C69V, C101A, and R31C to allow for site-specific pegylation at C31 to extend in vivo half-life and reduce potential immunogenicity. Etanercept, a human TNFR2–IgG1 Fc fusion protein that sequesters both solTNF and tmTNF [4, 28–31], was obtained from a pharmacy (RxUSA). The vehicle solution was PBS (pH 8) with 10% glycerol in M. bovis BCG infection and saline in M. tuberculosis infection.

**Reagent administration during M. bovis BCG and M. tuberculosis infections.** Reagents (vehicle, etanercept, and XENP1595; 30 mg/kg/dose) were administered intraperitoneally 1 day before infections and continued twice a week during the infection. The doses and dosing regimen selected are in accord with results reported elsewhere for XENP1595 and etanercept [28] and a murine homologue of etanercept [19]. Mice were infected intravenously with 1 × 107 living M. bovis BCG Pasteur strain 1173 P2 and killed 2 or 4 weeks after infection. Pulmonary infection with M. tuberculosis H37Rv (Pasteur Institute) was induced by delivering 100 bacteria split into the nasal cavities (20 μL each) of mice under xylazine-ketamine anesthesia, as described elsewhere [38], in sterile isolators in a biohazard animal unit. M. tuberculosis–infected mice were killed 3 or 9 weeks after infection.

**Determination of colony-forming units from infected organs.** The number of viable bacteria recovered from frozen organs was evaluated as described elsewhere [22, 38].

**Lipopolysaccharide (LPS) challenge.** Two weeks after M. bovis BCG infection, mice were challenged intraperitoneally with 0.1 μg of LPS from E. coli (serotype 0111:B4; Sigma) per gram of body weight and killed 8 h later.

**Evaluation of serum and spleen cytokine levels and serum enzymes.** Blood samples from retroorbital sinuses were obtained 2 and 4 weeks after M. bovis BCG infection and 8 h after LPS challenge. The amounts of solTNF, interferon (IFN)–γ, interleukin (IL)–6, and soluble TNFR1 were determined by ELISA. Spleens were homogenized in 0.04% Tween 80/saline

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buffer (125 mg of tissue per mL), as described elsewhere [39]. IL-12p70 and IL-4 were evaluated by ELISA using serial dilutions (sensitivity, 5–2000 pg/mL; Diaclone or R&D Systems). The murine TNF ELISA used does not cross-react with XENP1595, an engineered version of human solTNF. Hepatocyte damage was assessed by measuring serum enzyme activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as reported elsewhere [39].

**Histological analyses.** Liver and lung samples were fixed in 4% buffered formaldehyde and embedded in paraffin for subsequent hematoxylin-eosin staining.

**Statistical analyses.** The unpaired Student’s t test was used for analyses. Differences were considered statistically significant at P < .05.

**RESULTS**

No suppression of host immunity due to blocking of solTNF with DN-TNF during *M. bovis* BCG and *M. tuberculosis* infections. To determine whether selective blocking of solTNF may alter host protection against *M. bovis* BCG, mice were infected with 1 × 10⁷ living *M. bovis* BCG and treated twice weekly (30 mg/kg) with XENP1595, a selective inhibitor of solTNF, or etanercept, which blocks both solTNF and tmTNF [4, 28, 29]. At 4 weeks after *M. bovis* BCG infection, etanercept-treated mice showed increased bacterial loads in lungs, spleen, and liver, but infected organs of XENP1595-treated mice had...
bacterial loads similar to those in control mice (figure 1A). Similarly, M. tuberculosis–infected mice treated with etanercept showed higher bacterial loads on day 22 (mainly in lungs), whereas control of bacillary growth in XENP1595-treated mice was similar to that in vehicle-treated mice (figure 1B). At this time point, etanercept-treated mice rapidly lost body weight, as did TNF/−/− mice, whereas weight loss in XENP1595-treated mice was similar to that in vehicle-treated mice (figure 1C). In addition, all 4 etanercept-treated mice died of infection between days 22 and 28, whereas only 1 of 4 XENP1595-treated mice died, on day 29; the other 3 survived the 9 weeks of the experiment. These results suggest that selective blockade of solTNF by a DN-TNF biologic during infection has minimal effect on host protection, whereas inhibition of both solTNF and tmTNF by etanercept suppresses immunity to M. bovis BCG and M. tuberculosis infections.

**Effect of blocking solTNF on M. bovis BCG–induced Th1 and Th2 cytokines.** To determine whether treatment with TNF inhibitors affects M. bovis BCG–induced Th1/Th2 cytokines, the levels of IFN-γ, IL-12p70, and IL-4 were evaluated in the serum and in the spleen. Figure 2A shows that serum IFN-γ levels in XENP1595-treated mice were lower than those induced in control mice 2 weeks after infection; in contrast, IFN-γ was not activated in etanercept-treated mice. Because levels of IL-12p70 were undetectable in serum, IL-12p70 was quantified in the spleen. At 4 weeks after infection, IL-12p70 levels were similar in vehicle- and XENP1595-treated mice but were significantly decreased in etanercept-treated mice (figure 2B). To determine the effect of TNF inhibitors on the Th2 immune response, IL-4 was quantified in the spleen 4 weeks after infection; IL-4 levels in etanercept- and XENP1595-treated mice were similar to those in control mice (figure 2C). These results suggest that selective inhibition of solTNF by XENP1595 weakens Th1 immune responses somewhat but much less than the pronounced inhibition observed when both solTNF and tmTNF are neutralized by etanercept.

**Effect of anti-TNF biologics on M. bovis BCG–induced TNF levels.** M. bovis BCG induces TNF release, which can be evaluated in serum after intravenous infection [18]. Immunoreactive TNF was therefore quantified at different time points after infection and treatment with TNF inhibitors. Etanercept treatment decreased immunoreactive TNF levels at 2 weeks after infection, whereas XENP1595 treatment dramatically increased it compared with findings in vehicle-treated control mice (figure 3A). This increase was probably due to subunit exchange of the DN-TNF trimer with mouse TNF trimer, which results in a stabilized heterotrimer lacking TNF biological activity [27, 28]. To determine whether M. bovis BCG–induced TNF could stimulate TNF receptor signaling, bioactivity was assessed using WEHI 164 cells, which are highly sensitive to TNF [40]. Serum from BCG-infected mice treated with LPS was toxic to cells because of high levels of solTNF; in contrast, serum from XENP1595-, etanercept-, and vehicle-treated mice did not exhibit bioactivity after infection (figure 3B), suggesting that solTNF formed complexes with etanercept or mouse solTNF receptor or formed heterotrimers with XENP1595, thereby preventing solTNF signaling. Soluble TNFR1 serum levels progressively increased throughout the infection, with all groups showing similar levels (figure 3C); this increase in TNFR1 levels may have contributed to the inhibition of TNF bioactivity seen in BCG-infected mice.
Effect of anti-TNF biologics on granuloma formation after *M. bovis* BCG or *M. tuberculosis* infection. Hepatic granulomas of *M. bovis* BCG–infected XENP1595-treated mice did not differ in morphology or number compared with those of control mice 2 weeks after infection, whereas those of etanercept-treated mice were smaller and fewer, contained fewer cells (figure 4A–4D), and resembled those found in mice expressing high amounts of sTNFR1-IgG, as previously reported [18]. After *M. tuberculosis* infection, acute pulmonary infection, as illustrated by an increase in lung weight, was found in etanercept-treated mice and in TNF$^{-/-}$ mice 3 weeks after infection, whereas lung weight in XENP1595-treated mice was similar to that in control mice at 3 and 9 weeks after infection (data not shown). Macroscopically, the lungs of etanercept-treated mice displayed large confluent nodules similar to those seen in TNF$^{-/-}$ mice, whereas XENP1595-treated mice had smaller, better-defined nodules similar to those in control mice (figure 5A–5D). Microscopically, the lungs of etanercept-treated mice presented large necrotic lesions and significant inflammation, comparable to findings in TNF$^{-/-}$ mice. In contrast, XENP1595-treated mice showed slightly more prominent lung pathology than did control mice, with few foci of necrosis and overall in-

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**Figure 3.** Effect of tumor necrosis factor (TNF) inhibitors on *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)–induced immunoreactive and bioactive TNF and soluble TNF receptor 1 (sTNFR1) levels. A, Immunoreactive TNF quantified by ELISA 2 and 4 weeks after *M. bovis* BCG infection ($n = 12–14$ per group). B, Bioactive TNF evaluated by cytotoxicity against WEHI 164 cells, using serum obtained from *M. bovis* BCG–infected mice 2 weeks after infection. Serial serum dilutions were tested on sensitive cells. In mice challenged with BCG alone, no serum (from vehicle-, etanercept-, or XENP1595-treated mice) showed toxicity relative to recombinant TNF (rTNF) controls. In contrast, serum from BCG/lipopolysaccharide (LPS)–challenged mice demonstrated high toxicity because of soluble TNF, as confirmed by its neutralization after addition of sTNFR1-IgG. C, sTNFR1 quantified during *M. bovis* BCG infection ($n = 7–10$ per group). Data are means ± SEs and are representative of 2 independent experiments. *$P < .01$ and **$P < .001$, for the comparison with vehicle-treated mice. OD, optical density.
Inflammatory changes comparable to those in control mice (figure 5E–5H). Lung scores for inflammation, neutrophil infiltration, and necrosis as well as the extent of free air space showed that etanercept-treated mice were as sensitive as TNF/−/− mice, whereas XENP1595-treated mice were indistinguishable from control mice, with the exception of a modest but significant increase in lung necrosis (figure 5I and 5J).

Protection conferred by anti-TNF biologics from acute liver injury induced by LPS challenge in M. bovis BCG–infected mice. After M. bovis BCG infection, formation of liver granulomas and recruitment of immune cells can mediate hepatic injury secondary to granulomatous containment of bacteria, as monitored by serum levels of AST and ALT, 2 enzymes that correlate with liver damage [39]. Etanercept-treated mice showed significantly lower levels of serum AST and ALT than did vehicle- or XENP1595-treated mice at 2 weeks after infection, which may have been due to the significant reduction in the number of hepatic granulomas compared with that in XENP1595-treated and control mice (figure 6A). Administration of endotoxin to M. bovis BCG–infected mice causes a massive release of hepatotoxic factors that is mediated by TNF and inducible nitric oxide synthase [39]. To determine whether blockade of solTNF with either etanercept or XENP1595 during M. bovis BCG infection protects from this endotoxin-induced hepatic injury, liver enzymes were quantified 8 h after LPS challenge. As expected, etanercept-treated mice were protected from secondary liver injury, owing to the reduced number of liver granulomas. Blocking of solTNF with XENP1595 showed a moderate but significant liver protection, with transaminase levels decreased compared with those in control mice (figure 6B). In addition, bioactive solTNF, IL-6, and IFN-γ serum levels were significantly reduced in M. bovis BCG–infected mice treated with etanercept or XENP1595 (figure 6C). These data confirm that both a TNFR2–IgG1 Fc fusion protein and a DN-TNF biologic protect from liver injury induced by endotoxin in M. bovis BCG–infected mice, whereas solTNF plays a larger role in the acute inflammation mediated by LPS.
DISCUSSION

The extension of anti-TNF biologics for use in the treatment of inflammatory diseases beyond rheumatoid arthritis and Crohn disease increases the patient population exposed to the risk of infections and other complications associated with these therapies. Thus, to maximize the number of patients who are able to benefit from anti-TNF therapies, new therapeutic strategies are required to attenuate the deleterious effects that total TNF blockade has on the host immune system while maintaining the positive anti-inflammatory effects. In support of this goal, recent data obtained in mouse genetic models and in experimental models of inflammatory and infectious diseases suggest that selective inhibitors of solTNF that spare tmTNF may be anti-inflammatory while maintaining innate immunity to infection [22–25, 41]. Existing anti-TNF drugs have multiple targets: they can inhibit both tmTNF- and solTNF-mediated forward signaling by sequestration, stimulate tmTNF-mediated reverse signal-

Figure 5. Pulmonary lesions and pathological analyses of mice infected with *Mycobacterium tuberculosis*. Macroscopic (A–D) and microscopic (E–H) examinations of pulmonary lesions were performed 22 days after intranasal infection with 100 cfu of *M. tuberculosis* in mice receiving saline (A and E), etanercept (B and F), or XENP1595 (C and G) as well as in tumor necrosis factor (TNF)−/− mice (D and H) (original magnification, ×400). Necrotic lesions (arrows) are present in etanercept-treated mice and are more predominant in TNF−/− mice, which may develop caseous necrosis. I, Lung pathology, including inflammation, polymorphonuclear leukocyte (PMN) infiltration, and necrosis, scored 22 days after infection (0, no alteration; 1–5, increasing severity of pathological lesions). etan, etanercept. J, Free air space expressed as the area of 5 lung cross-sections (percentage). Data are means ± SEs (n = 4 per group). *P < .05, for the comparison with vehicle-treated mice.
ing by cross-linking, and trigger immune cell effector functions against tmTNF-expressing cells via their IgG1 Fc domains [4]. Etanercept can also inhibit LTα and LTαβ, which increases susceptibility to mycobacterial infections in animal models [4, 16, 42, 43]. In contrast, DN inhibitors of TNF are a new class of selective solTNF blockers that are based on variants of native human solTNF [27, 28, 34]. These agents eliminate solTNF homotrimers but do not bind to or interact with tmTNF or LTα (figure 7); consequently, they cannot block forward or reverse signaling by these cytokines. Moreover, in contrast to the 3 clinically available biologics, DN-TNFs possess no antibody Fc domain and are thus incapable of stimulating Fc-mediated immune cell effector functions.

The present study explores the in vivo consequences of selectively blocking solTNF with XENP1595, a pegylated DN-TNF biologic, versus blocking both solTNF and tmTNF with etanercept, a TNFR2-IgG1 Fc fusion protein. The effects of these anti-TNF agents were compared during M. bovis BCG and M. tuberculosis infections and in acute liver injury induced by endotoxin in M. bovis BCG–infected mice. Selective inhibition of solTNF by XENP1595 did not affect bacterial load after M. bovis BCG or M. tuberculosis infection, whereas etanercept increased the number of viable mycobacteria in infected organs. Infection by a virulent strain of M. tuberculosis resulted in a 1 log increase in bacterial load in etanercept-treated and TNF−/− mice, compared with that in the other groups. Recently, it has been shown that treatment with a murine homologue of etanercept and with an anti-TNF antibody resulted in 100% mortality during acute M. tuberculosis infection and that both inhibitory agents also sensitized mice to chronic M. tuberculosis infection, with the antibody being more immunosuppressive [19]. This finding was ascribed not to differences in TNF inhibitory mechanisms but to reduced penetration or retention of murine etanercept in granulomas relative to the anti-TNF antibody. In agreement with this report,
our data show a pronounced effect of etanercept on bacillary growth in lungs during acute *M. tuberculosis* infection, which was not detected in XENP1595-treated mice. Etanercept-treated mice infected by *M. tuberculosis* developed dramatic lung inflammation with necrotic pneumonia and disseminated infection comparable to that observed in TNF−/− mice. This finding suggests that inhibiting both solTNF and tmTNF has potent effects, whereas inhibiting solTNF has very limited effects during acute *M. tuberculosis* infection.

Although etanercept treatment increased the number of viable *M. bovis* BCG by only 2-fold, an inhibitory effect on IFN-γ and IL-12p70 was observed, suggesting a modification of Th1 immune responses. Systemic IFN-γ was also decreased in DN-TNF–treated mice 2 weeks after infection, but the extent of this effect was equivalent to that found in mice expressing only tmTNF (TNF/LTα−/−), which are able to survive similar experimental *M. bovis* BCG infection, as previously reported [22]. These data suggest that, compared with etanercept, XENP1595 better preserves the Th1 immune response induced by acute *M. bovis* BCG infection. In addition, our data show that *M. bovis* BCG liver granuloma formation was not altered by treatment with XENP1595. In contrast, etanercept-treated mice presented with poor granuloma formation and deficient cell recruitment, correlating with the higher number of living mycobacteria in infected organs than in other experimental groups and suggesting that tmTNF activity is important in the development of bactericidal granulomas.

Possibly because of a reduction in the number of inflammatory and immune cells in the liver, *M. bovis* BCG–infected mice treated with etanercept showed decreased hepatic injury compared with control and DN-TNF-treated mice, as assessed by serum transaminase levels. These data suggest that XENP1595, by preserving tmTNF activity, allows granuloma cell recruitment and thereby maintains anti-mycobacterial host defense. However, it was critical to clarify whether this solTNF-selective biologic remained able to protect *M. bovis* BCG–infected mice.
from acute liver injury caused by endotoxin-induced TNF. XENP1595-treated mice, like etanercept-treated mice, were significantly protected from M. bovis BCG/LPS–induced liver damage, as assessed by a reduction in serum transaminase levels as well as in bioactive TNF, IL-6, and IFN-γ levels. This result for the DN-TNF biologic is in agreement with our previous findings showing that M. bovis BCG/LPS–induced liver damage is dependent on sOTNF, because mice lacking sOTNF but expressing tmTNF also showed protection [22].

TNF is involved in the pathogenesis of human inflammatory and autoimmune diseases but is also essential in host defense mechanisms. Its nonselective neutralization is known to compromise host immunity and is associated with new tuberculosis infections and reactivation of latent infections in humans and mice [2, 5–10]. Notably, computational modeling of tuberculosis infection rates for etanercept and infliximab suggests that these agents sensitize humans to both newly acquired and latent tuberculosis [44, 45]. New infection represented almost half of all tuberculosis cases in patients treated with etanercept, whereas reactivation was predominant for infliximab [45]. Our study investigated only acute tuberculosis infection in mice, but another recent mouse study using a virus-based vaccine selectively targeting sOTNF [46] lends support to our hypothesis; its findings showed that by inhibiting only sOTNF one can achieve protection from arthritis without inducing either newly acquired or reactivated tuberculosis. Given that nonselective anti-TNF therapies, such as decoy receptors and monoclonal antibodies, have proved efficacious for severe inflammatory disease, the development of selective inhibitors of sOTNF may represent a logical next-generation strategy. Data showing that DN-TNF biologics attenuate arthritis without suppressing immunity to L. monocytogenes in mouse models further support our hypothesis [28]. These 2 independent reports from studies using a vaccine and a biologic, along with extensive data from mouse genetic models, suggest that selective inhibition of sOTNF does not suppress immune responses to granulomatous infections. It will be valuable to repeat these studies using mouse models of tuberculosis reactivation, to compare all 3 classes of anti-TNF biologics and ultimately to determine whether these findings will have clinical relevance.

In conclusion, the present study shows that a DN-TNF biologic that selectively inhibits sOTNF did not suppress host immunity to M. bovis BCG and M. tuberculosis infections but did protect mice from M. bovis BCG/LPS–induced liver injury. This suggests that the risks associated with the first generation of nonselective TNF inhibitors might be reduced by use of a DN-TNF biologic that spares the protective effects of tmTNF. A reduction in the infection risks associated with current anti-TNF drugs may also allow the safer use of novel anti-TNF therapies in other inflammatory diseases.

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