

# Neutralization of Tumor Necrosis Factor (TNF) by Antibody but not TNF Receptor Fusion Molecule Exacerbates Chronic Murine Tuberculosis

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**Tumor necrosis factor (TNF) plays an essential role in the immunologic maintenance of *Mycobacterium tuberculosis* infection. Although an increased rate of tuberculosis has been reported in humans treated with anti-TNF biological agents, disparate rates of disease have been observed between those treated with infliximab, an anti-TNF antibody, and etanercept, a TNF-neutralizing TNF receptor (TNFR) fusion molecule. We compared the effects of anti-TNF antibody and soluble TNFR fusion molecule in the murine model of tuberculosis. Systemic TNF neutralization was equivalent between these molecules, and both resulted in rapid morbidity at the initiation of infection. During chronic infection, administration of the receptor fusion molecule allowed the control of infection, whereas antibody treatment caused mice to die within a month. We provide evidence of decreased penetration into the granulomas by the receptor fusion molecule, compared with antibody. These findings begin to clarify the mechanistic difference between anti-TNF agents and their role in the exacerbation of tuberculosis.**

*Mycobacterium tuberculosis* is an intracellular, acid-fast bacillus with which one-third of the world population is infected. The majority of people infected with *M. tuberculosis* develop clinically latent infection in which mycobacteria are not transmitted. A 10% lifetime risk of reactivation exists during latent infection [1]. Data from the mouse model of tuberculosis [2–5] and human studies [6] have suggested that tumor necrosis

factor (TNF)–mediated formation and maintenance of the granuloma is fundamental to controlling *M. tuberculosis* infection.

Inhibition of TNF during chronic inflammatory diseases, such as rheumatoid arthritis and Crohn disease, by use of anti-TNF antibodies, such as adalimumab and infliximab or the soluble TNF receptor (sTNFR) fusion molecule etanercept, has resulted in the reduction in inflammation and improvement in quality of life [7]. However, TNF inhibition compromises the immune system, which increases the risk of infections in the respiratory tract, skin, bone, and joints [8]. By 2002, >300 cases of *M. tuberculosis* disease had been linked to infliximab treatment [9]. In 2004, voluntary data collected worldwide showed that the risk of reactivation of tuberculosis was statistically lower in patients who received etanercept (28/100,000 patients) than in those who received infliximab (54/100,000 patients) [10]. Although not yet elucidated, it has been proposed that the mechanisms of action between these anti-TNF molecules differ.

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The mouse model of tuberculosis has been predictive of the importance of TNF in human tuberculosis. Mice infected with *M. tuberculosis* and deficient in TNFR1, deficient in TNF ligand, or treated with the TNF-neutralizing antibody MP6-XT22 displayed granuloma disruption and rapidly died of infection [2–5]. In vitro binding competition assays showed that the association between TNF and infliximab is essentially irreversible, whereas ~90% of etanercept-TNF complexes dissociate within 10 min [11]. The increased dissociation rate associated with the TNFR fusion molecule may make neutralization incomplete under certain circumstances, thereby reducing, but not eliminating, the effects of TNF.

To model and compare the effects of anti-TNF antibody and sTNFR2-Fc during the primary and chronic phases of *M. tuberculosis* infection, we used the murine system and reagents modeled after human TNF-neutralizing drugs. Our results demonstrate that, during primary infection, both drugs exacerbated tuberculosis with similar kinetics. However, when treatment was initiated during an established infection, only mice treated with anti-TNF antibody developed overwhelming disease. By contrast, the majority of chronically infected mice treated with murine TNFR2 (mTNFR2)-Fc survived, with no evidence of exacerbated disease. We explored the effects of these test molecules during each stage of infection to identify the mechanisms by which they differ. These results have important public health implications for the use of TNF-neutralizing drugs in global regions where tuberculosis is endemic, given that rates of tuberculosis reactivation and primary disease may increase substantially after TNF neutralization without appropriate education, testing, and therapies.

## MATERIALS AND METHODS

**Mice.** Female C57BL/6 and C3-deficient (C3<sup>-/-</sup>) mice (Jackson Laboratory) were housed in a biosafety level 3 specific pathogen-free facility and were monitored for murine pathogens. All animal protocols used were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

**Mycobacteria and infection of mice.** Low-dose aerosol infection of mice (20–100 cfu) with *M. tuberculosis* strain Erdman (Trudeau Institute) has been described elsewhere [12]. The bacterial burden was determined as described elsewhere [13].

**Chemicals and reagents.** All chemicals were purchased from Sigma-Aldrich, unless stated otherwise. Middlebrook 7H10 agar and 7H9 liquid medium for the growth of *M. tuberculosis* were purchased from Difco. Flow cytometry antibodies were purchased from BD Pharmingen, except for anti-C3 and its isotype control (Cedarlane Laboratories). MP6-XT22 (rat IgG1) [13, 14] was purified from cell culture of a hybridoma obtained from DNAX by the National Cell Culture Center. TNFR2-Fc [15, 16] and control IgG1 antibody were a gift from Amgen. Murine anti-TNF molecules or IgG1 were diluted in PBS and

injected intraperitoneally into mice 1 day before acute infection with *M. tuberculosis* or, for chronic studies, 4 months after *M. tuberculosis* infection and biweekly for the duration of the study.

**WEHI assay for TNF bioactivity.** The bioactivity of TNF was measured by sensitive WEHI 164 subclone 13 (American Type Culture Collection) as described elsewhere [17]. To detect systemic TNF neutralization, blood was collected by retroorbital bleed using nonheparinized capillary tubes (Drummond Scientific) and centrifuged at 13 g in a serum separator tube (Becton Dickinson). Serum was diluted in WEHI assay medium (1:6) to a final concentration of 1000 pg/mL TNF and incubated overnight with WEHI cells.

**Flow cytometry.** Lung infiltration was examined at predetermined time points as described elsewhere [3, 18].

**Histopathologic analysis.** Samples of lung were fixed in 10% normal buffered formalin and embedded in paraffin. Then, 5–6- $\mu$ m sections were stained with hematoxylin-eosin. Slides were examined in a blinded fashion. TUNEL staining was performed using the Apoptag kit (Chemicon) in accordance with the manufacturer's protocol. To track labeled molecules, lung blood and airways were cleared by perfusion and bronchoalveolar lavage. Lungs were fixed in 2% paraformaldehyde and infused with 30% sucrose. Sections were stained for 30 min with rhodamine phalloidin (Invitrogen) and DraQ5 (Biostatus). Images were captured using Olympus Flowview 500 software (Olympus).

**Preparation of fluorescein (SFX)-labeled proteins.** A 10-fold molar excess of 5(6)-SFX (6 fluorescein-5-[and -6]-carboximido hexanoic acid) succinimidyl ester in dimethyl sulfoxide was added to mTNFR2-Fc [15], MP6-XT22, or a control murine antibody (IgG1) and incubated for 1 h at a final protein concentration of 3 mg/mL. Unincorporated dye was removed by size-exclusion column chromatography (NAP 5 [GE Healthcare]) and was eluted with saline. Protein concentrations and degrees of labeling were calculated using absorbance at 280 and 495 nm, respectively.

**Statistical analysis.** Bacterial burden and cell infiltration were analyzed by a 1-way analysis of variance, whereas the log-rank test was used to compare survival.  $P < .05$  was considered to indicate significance. Experiments were repeated to demonstrate reproducibility.

## RESULTS

**Similar systemic TNF neutralization with anti-TNF antibody and mTNFR2-Fc.** MP6-XT22, a rat IgG1 monoclonal antibody, neutralizes murine TNF and is the standard anti-TNF antibody used in murine studies [13, 14]. The human reagent infliximab is a chimeric monoclonal antibody (mouse-human IgG1) that is also extremely effective at neutralizing TNF [11]. We used MP6-XT22 as a model of infliximab. mTNFR2-Fc was modeled directly on human etanercept. Etanercept is a human

soluble p75-TNF receptor–human IgG1, whereas murine etanercept is a murine soluble p75-TNF receptor–murine IgG1 [15].

To compare TNF neutralization by MP6-XT22 and mTNFR2-Fc, increasing doses of each molecule were added to WEHI clone 13 cells (a TNF bioassay) [19] in the presence of 1000 pg/mL recombinant murine TNF. TNF bioactivity was neutralized equivalently in vitro by use of both mTNFR2-Fc and MP6-XT22 (figure 1A). TNF was not reliably detectable in serum from infected mice (data not shown), so, to test the potential for systemic TNF neutralization, mice chronically infected with *M. tuberculosis* were administered 1 intraperitoneal injection of 0.1 mg of MP6-XT22, mTNFR2-Fc, or IgG1. Serum was obtained 4 h after injection and every 24 h for 3 days. Added TNF (1000 pg/mL) was completely neutralized by serum (diluted 1:6) from infected mice treated with either MP6-XT22 or mTNFR2-Fc but not from mice treated with IgG1 (figure 1B).

**Exacerbation of acute *M. tuberculosis* infection with both anti-TNF antibody and mTNFR2-Fc.** To test whether anti-TNF antibody and soluble receptor fusion molecule treatment differentially affect control of the primary phase of *M. tuberculosis* infection, MP6-XT22 (0.5 mg), mTNFR2-Fc (0.2 mg), or IgG1 (0.5 mg) was administered intraperitoneally 1 day before infection and continued twice weekly. Both reagents exacerbated disease, and mice became moribund, with a mean  $\pm$  SD survival time of  $22 \pm 1$  days after infection (figure 2A). Control mice did not die of infection, and they survived significantly longer than both anti-TNF groups ( $P < .0001$ ). Even at a reduced dose (0.1 mg twice weekly), MP6-XT22- and mTNFR2-Fc-treated mice died of infection, with mean  $\pm$  SD survival times of  $19 \pm 5$  and  $22 \pm 0$  days after infection, respectively (figure 2A).

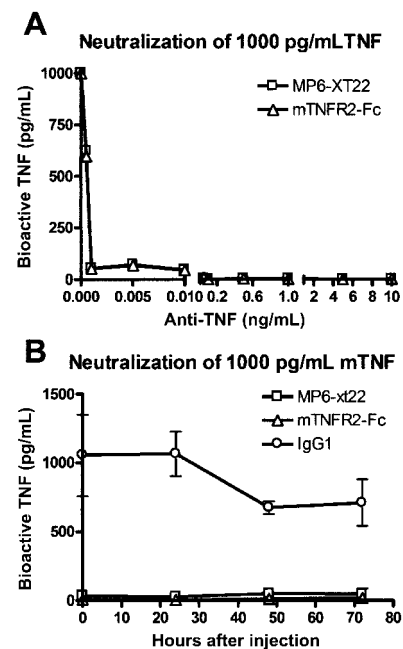
Although slightly higher in mice treated with anti-TNF reagents, the bacterial burden among all groups of mice did not differ significantly for up to  $\sim 3$  weeks after infection, when anti-TNF-treated mice died of infection (figure 2B), as reported in previous studies [3–5]. By 4 weeks, granulomas were present in control mice (figure 3A). By contrast, mice treated with either MP6-XT22 or mTNFR2-Fc had disorganized cellular infiltration and increased inflammation in the lungs (figure 3A). The degree of lung inflammation among anti-TNF-treated mice likely resulted in their poor survival. These data are consistent with previous findings indicating that TNF is required for granuloma formation and survival [3, 4].

To examine whether low-dose anti-TNF treatment during acute *M. tuberculosis* infection affected survival, mice were treated with either drug at 0.01 mg twice weekly, and these mice survived for  $>6$  months after infection (data not shown). Serum from these mice neutralized TNF in the WEHI assay, with results (data not shown) similar to those in figure 1B, which indicated that systemic levels of reagent were sufficient for TNF neutralization.

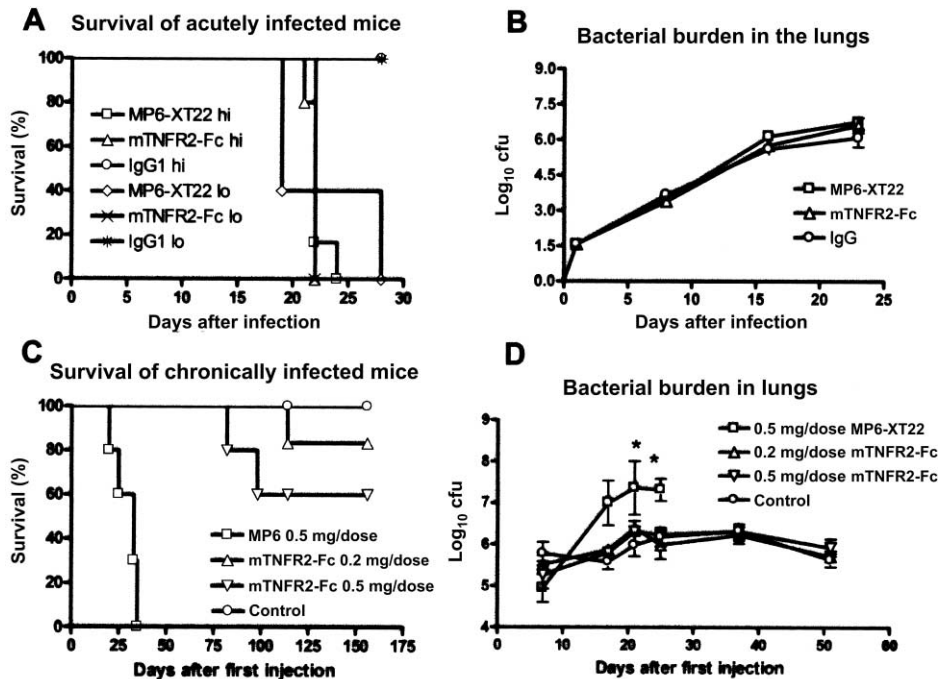
**Exacerbation of chronic tuberculosis after treatment with anti-TNF antibody but not with TNFR2-Fc.** We hypothesized that both MP6-XT22 and mTNFR2-Fc would impair control over established infection. Mice were infected with *M. tuberculosis*; after 4 months, during the chronic stage of infection, intraperitoneal injections of MP6-XT22 (0.5 mg), mTNFR2-Fc (0.2 and 0.5 mg), IgG1 (0.5 mg), or PBS were administered twice weekly. MP6-XT22-treated mice died of infection a mean  $\pm$  SD of  $33 \pm 6$  days after treatment (figure 2C). Mice given mTNFR2-Fc or control injections survived significantly longer than MP6-XT22-treated mice ( $P < .0001$ ).

Three weeks of MP6-XT22 treatment in chronically infected mice resulted in a significantly higher bacterial burden than that in mTNFR2-Fc-treated or control mice ( $P < .05$ ), as reported elsewhere [3, 5] (figure 2D). Consistent with previous findings [2], lung sections revealed a loss of granuloma organization after 4 weeks of MP6-XT22 treatment, whereas control and mTNFR2-Fc-treated mice maintained granuloma structure (figure 3B) after  $>4$  months of mTNFR2-Fc treatment (figure 3C).

To investigate the difference between MP6-XT22 and mTNFR2-Fc treatment during chronic *M. tuberculosis* infection, leukocytes isolated from the lungs of treated mice at serial time points were analyzed by flow cytometry. No differences in the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, macrophages, or neutrophils were observed (data not shown).



**Figure 1.** Efficient neutralization of tumor necrosis factor (TNF) bioactivity by both MP6-XT22 and murine TNF receptor 2 (mTNFR2)-Fc. *A*, Increasing MP6-XT22 or mTNFR2-Fc added to WEHI clone 13 cultures with 1000 pg/mL TNF. *B*, Mice injected at time 0 with 0.1 mg MP6-XT22, mTNFR2-Fc, or IgG1. Serum was collected and incubated with WEHI clone 13 and 1000 pg/mL TNF.

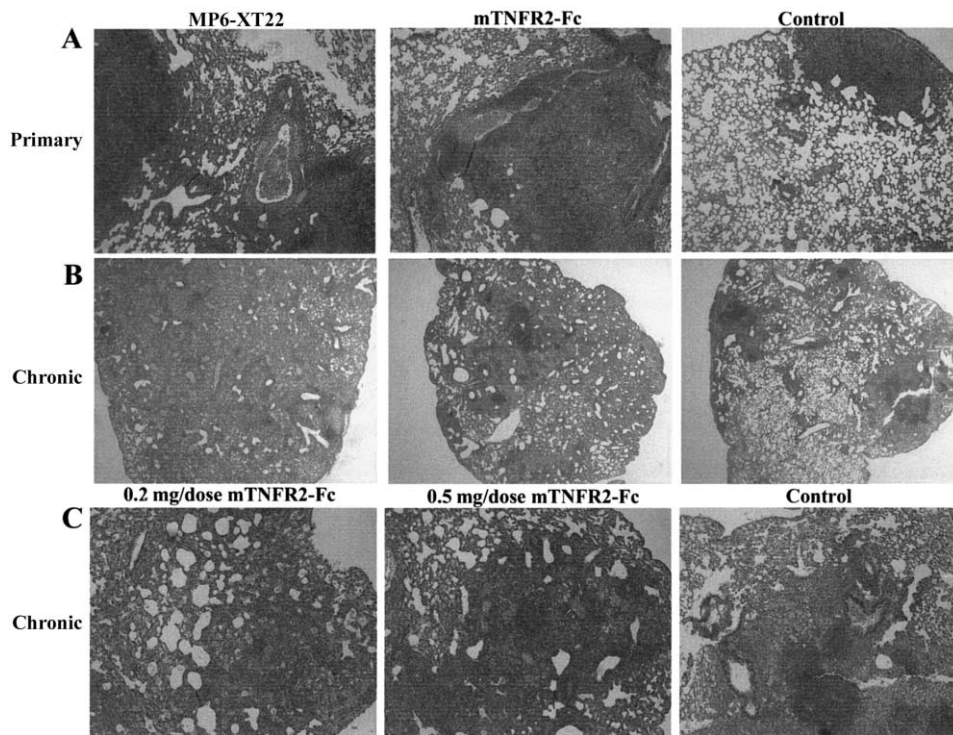


**Figure 2.** Survival and bacterial burden in mice treated with anti-tumor necrosis factor (TNF). Mice were acutely (*A* and *B*) or chronically (*C* and *D*) infected with *Mycobacterium tuberculosis* and treated with 0.5 mg of MP6-XT22, 0.2 or 0.5 mg of murine TNF receptor 2 (mTNFR2)-Fc, or control PBS injections twice weekly. *A*, 0.1 mg/dose low-dose treatment. \* $P < .05$ .

**Increase in complement protein C3 on T cells in the lungs resulting from anti-TNF antibody.** It has been hypothesized that if transmembranous TNF is bound by anti-TNF antibody, the Fc portion could cause complement (C3b) deposition, resulting in the death of TNF-expressing cells [20, 21]. Although TNF could not be detected on the surface of cells (data not shown), flow cytometry revealed increased C3 deposition on CD4<sup>+</sup> T cells in lungs of mice treated with MP6-XT22, compared with that in mTNFR2-Fc-treated or control mice, during acute infection (figure 4B). There were significantly fewer CD4<sup>+</sup> T cells (figure 4C), but not macrophages (data not shown), in the lungs of MP6-XT22-treated mice, compared with that in control or mTNFR2-Fc-treated mice, during primary infection but not chronic infection (figure 4A; data not shown). We did not observe a similar loss of CD4<sup>+</sup> T cells in C3<sup>-/-</sup> mice after MP6-XT22 treatment, which supports the notion that C3b deposition contributes to a loss of CD4<sup>+</sup> T cells. However, the C3<sup>-/-</sup> mice were equally susceptible to MP6-XT22-induced exacerbation of tuberculosis (data not shown).

Conflicting reports in the literature have suggested that anti-TNF antibody may affect the apoptosis of T cells and macrophages [20–23]. In both acutely and chronically infected mice, annexin V staining by flow cytometry (figure 4D) and TUNEL staining of lung sections (figure 4E) revealed no obvious differences in apoptotic cells between groups.

**Higher levels of MP6-XT22 than of TNFR2-Fc in established granulomas.** Although the potential for systemic neutralization of TNF was similar with both MP6-XT22 and mTNFR2-Fc, drug penetration and local neutralization within the lung tissue may differ. Fluorescently labeled mTNFR2-Fc or MP6-XT22 was injected into chronically infected mice. Labeling of these molecules did not impair TNF-neutralization ability in vitro (data not shown). After 2 days, both molecules were detectable by confocal microscopy (representative sections are shown in figure 5A). Occasional aggregations of fluorescently labeled MP6-XT22 were observed in sections from lung lobes from all mice in this group. By contrast, few sections of lung had visible mTNFR2-Fc-SFX or IgG1-SFX (figure 5A; data not shown). To quantitatively compare levels of each molecule in lungs, random sections of lobes were assessed, and the ratio of labeled molecule to the number of nuclei in each section was calculated using Metamorph software (version 7; Molecular Devices) (figure 5B). Significantly more fluorescently labeled anti-TNF antibody than fluorescently labeled mTNFR2-Fc or IgG1 was detected in the lungs of mice. We conclude from these findings that, compared with MP6-XT22, there was reduced penetration or retention of mTNFR2-Fc in granulomas during established infection. This may lead to less efficient neutralization of TNF within the granuloma and to improved



**Figure 3.** Granuloma formation and maintenance during anti-tumor necrosis factor (TNF) treatment. Representative hematoxylin-eosin-stained lung sections (magnification,  $\times 20$ ) are shown 4 weeks after infection (A) and in chronically infected mice treated for 4 weeks (B) or 4 months (C). mTNFR2-Fc, murine TNF receptor 2-Fc.

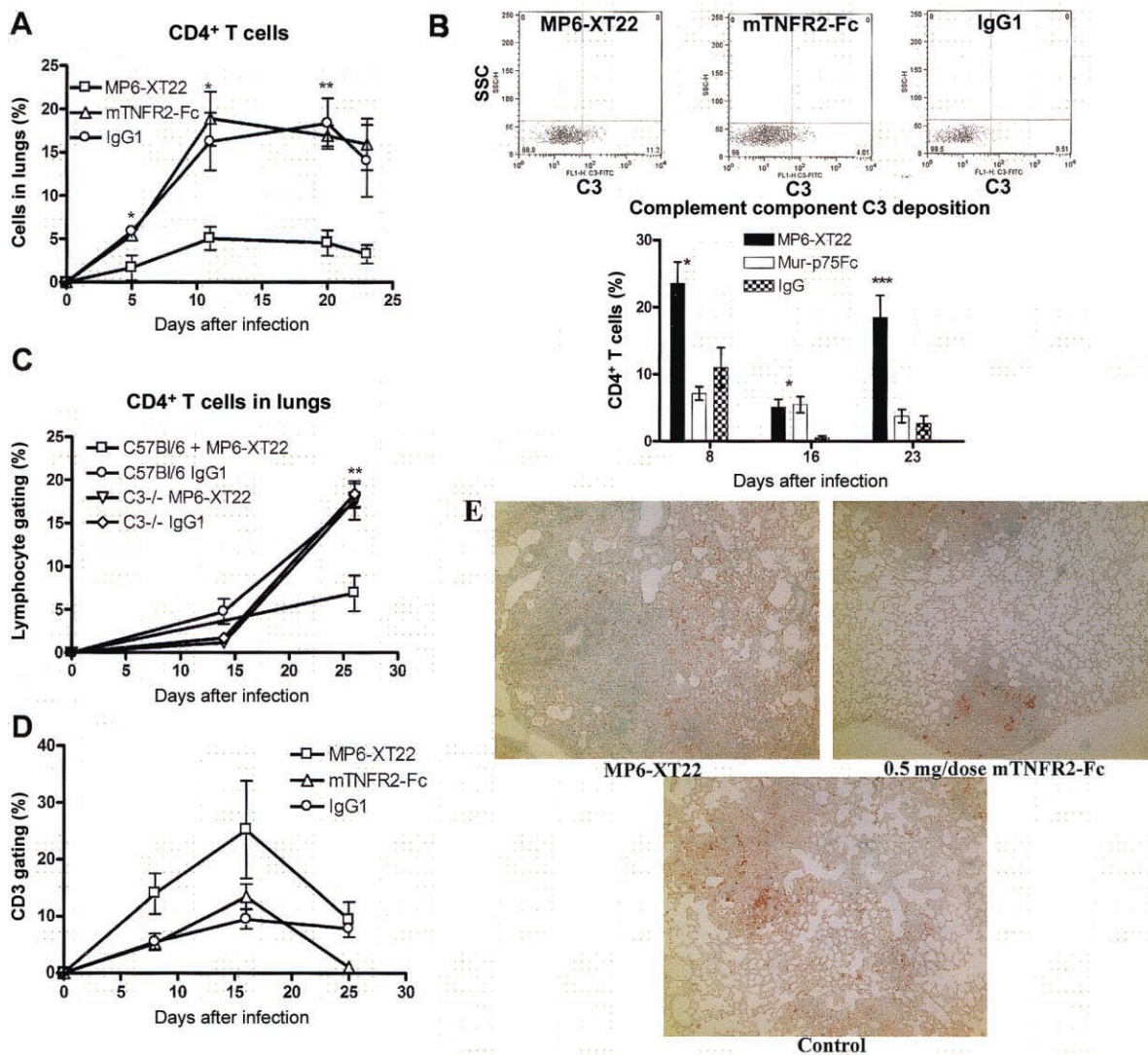
outcomes among chronically infected mice given mTNFR2-Fc, compared with those given MP6-XT22.

## DISCUSSION

The murine model of *M. tuberculosis* infection is an established and valuable tool for studying the host immune response during primary and chronic infection. During primary infection, the bacterial burden increases until the immune response is established ( $\sim 3$ – $4$  weeks). The appearance of granulomas in the lungs coincides with the control of bacterial numbers. In primary human infection, poor granuloma formation is associated with a poor clinical outcome [24]. Mice do not clear the infection or develop true latent infection; instead, a state of chronic infection is achieved with loosely organized granulomas and a relatively high bacterial burden ( $\sim 10^6$  cfu/lung) [25]. Although the cellular components of granulomas in mice and humans are similar, humans have very structured granulomas not seen in the murine model. Despite these differences, the recent reports of increased tuberculosis observed in patients treated with anti-TNF reagents were predicted by the murine model [2, 4, 5]. In the present study, the clinical disparity in terms of reactivation tuberculosis in response to anti-TNF agents may provide important insights into the use of these agents in certain clinical settings.

We compared the ability of soluble TNFR2-Fc fusion protein and anti-TNF antibody treatment in C57BL/6 mice during *M. tuberculosis* infection. Murine TNF-neutralizing agents were used as surrogates for the human reagents: MP6-XT22 is a rat IgG1 monoclonal antibody against murine TNF that efficiently neutralizes TNF in vitro and in vivo [13]. The human anti-TNF antibodies are human-mouse chimeric monoclonal IgG1 (infliximab) and humanized monoclonal IgG1 (adalimumab). mTNFR2-Fc was modeled after and is functionally similar to human etanercept [15, 16]. Despite both compounds providing systemic neutralization of circulating TNF, disease outcomes were dependent on the stage of infection and the therapeutic agent. During primary infection, treatment with either reagent was equally detrimental, resulting in disorganized granuloma formation and poor survival. This suggests that, during the establishment of tuberculosis, neutralization of circulating TNF prevents the control of infection.

Disease was exacerbated in chronically infected mice treated with anti-TNF antibody but not in those treated with mTNFR2-Fc. Despite equivalent systemic neutralization of circulating TNF, significantly more MP6-XT22 was detected in granulomas, compared with mTNFR2-Fc. The disparity in detection of these 2 reagents may be due to less penetration by mTNFR2-Fc into the granuloma and/or to the formation of immune com-



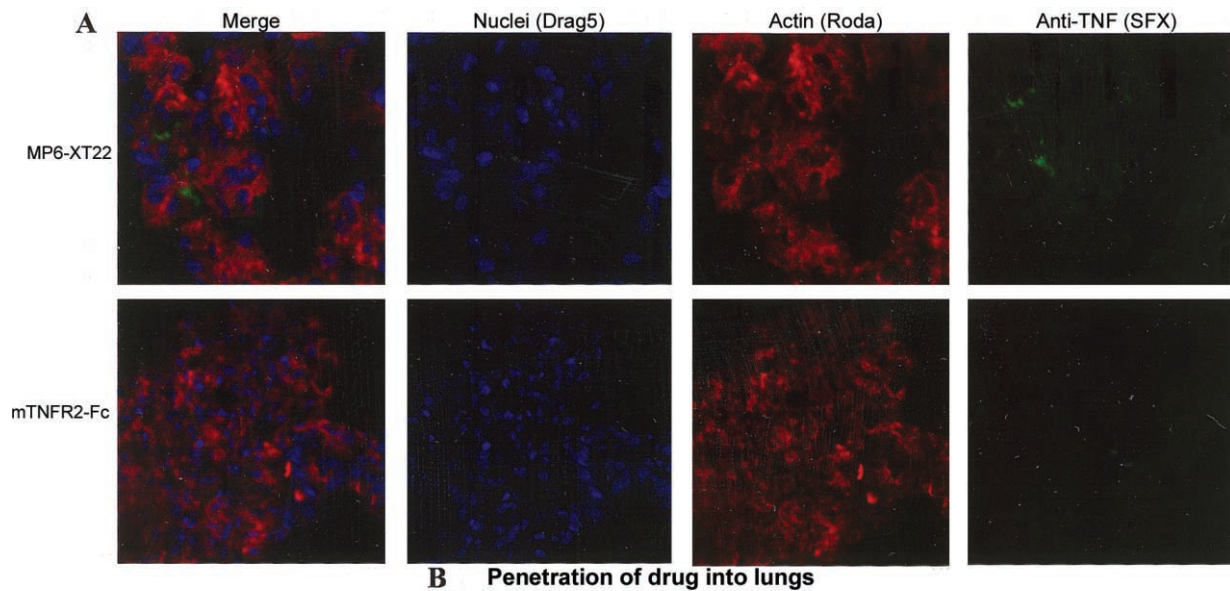
**Figure 4.** Complement C3 deposition and apoptosis. *A*, Percentage of CD4<sup>+</sup> T cells in lung infiltrate of control mice treated with MP6-XT22 or murine tumor necrosis factor receptor 2 (mTNFR2)-Fc or infected acutely with *Mycobacterium tuberculosis*. \**P* < .05, \*\**P* < .01. *B*, Complement C3 staining gated on CD4<sup>+</sup> lymphocytes in MP6-XT22-, mTNFR2-Fc-, and control-treated mice. \**P* < .05, \*\**P* < .001. *C*, CD4<sup>+</sup> T cells in C3<sup>-/-</sup> or control mice treated with MP6-XT22 or IgG1. *D*, Flow cytometry analysis of annexin V staining in lungs of mice injected with MP6-XT22, mTNFR2-Fc, or control. *E*, TUNEL staining of lung sections from mice chronically infected with *M. tuberculosis*. FITC, fluorescein isothiocyanate; SSC, side scatter.

plexes of TNF and anti-TNF antibody. Maintenance of granuloma structure and increased survival in mTNFR2-Fc-treated mice may be due to incomplete neutralization of TNF within the granuloma. Systemic expression of TNF appears not to be required to control chronic *M. tuberculosis* infection, which highlights the localized nature of tuberculosis and the critical function of granulomas once *M. tuberculosis* infection has been established.

These findings in the mouse model are consistent with the human literature, in which higher rates of tuberculosis have been associated with patients receiving anti-TNF antibody (infliximab), compared with TNFR2-Fc (etanercept) [6, 9, 10]. These data have important public health implications for the use of these drugs in regions where tuberculosis is highly en-

demic. On the basis of our data, we hypothesize that an increased rate of reactivation of tuberculosis could occur among patients treated with TNF neutralizing antibody rather than with TNFR2-Fc. In addition, therapies with either TNF antibody or TNFR2-Fc may pose an increased risk for dissemination and perhaps for the development of primary tuberculosis after exposure to persons with active disease.

The pharmacodynamic differences between these 2 drugs likely play an important role in the overall activity against systemic versus localized diseases. Although the increased dissociation of TNF from TNFR2-Fc, compared with antibody [11], may not be relevant in the periphery, where the drug is at a higher concentration relative to TNF, TNF dissociation may be



**Figure 5.** Penetration of treatment into the lungs with established *Mycobacterium tuberculosis* infection. *A*, Representative lung sections (magnification,  $\times 20$ ) from mice receiving fluorescein (SFX)-labeled drug and staining for actin (red) and nucleus (blue). *B*, Random sections of lung from mice injected with anti-tumor necrosis factor (TNF) or IgG1-SFX analyzed for the presence of labeled drug. Data are reported as the SFX:nuclei ratio.  $**P < .001$ . FITC, fluorescein isothiocyanate; mTNFR, murine TNF receptor.

a very important factor in the microenvironment of the granuloma. If TNF dissociates from the TNFR2-Fc in the granuloma, it may rapidly be bound again by the drug. However, TNF is just as likely to be bound to the large numbers of TNFRs present on leukocytes in close proximity in the granuloma. This is consistent with the fact that infliximab and etanercept are both effective in the treatment of rheumatoid arthritis but that only infliximab is effective in ameliorating the symptoms of Crohn disease [26]. Perhaps penetration of the 2 drugs into the inflammatory foci (similar to granulomas) of the intestines, as is seen many cases of Crohn disease, resembles that in our murine studies, where the antibody penetrates tissue better than the TNFR fusion protein.

There are other differences between these 2 anti-TNF agents that might contribute to the reactivation of tuberculosis. Membrane-bound TNF is reported to be bound more tightly by anti-TNF antibody than is mTNFR2-Fc [11]. Mice that express only membrane-bound TNF were similar to wild-type mice in the control of acute infection, but they had exacerbated disease during the chronic phase [27]. We were unable to detect

membrane-bound TNF on cells from the lungs of infected mice (data not shown), which suggests that membrane-bound TNF is transient and quickly cleaved by TNF- $\alpha$ -converting enzyme. We also did not detect antibody binding to cells from lungs of mice treated with SFX-labeled TNF-inhibiting drugs by flow cytometry (data not shown). Although it is possible that differential binding of TNF by anti-TNF antibody, compared with mTNFR2-Fc, accounts for the different outcomes in chronic infection, we did not observe increased cell death, which would be expected for reverse signaling [28] or antibody binding to cells. Although other researchers have demonstrated that TNF itself can induce apoptosis of *M. tuberculosis*-infected macrophages [29] and that neutralization of TNF may result in increased survival of infected macrophages in the granuloma, differences in macrophage apoptosis were not observed in acutely or chronically infected mice with any treatment. Immune complex formation with anti-TNF antibody treatment (which would not occur with TNFR2-Fc) may lead to increased inflammation or even increased levels of interleukin (IL)-10 production if these immune complexes interact with Fc recep-

tors on alternative macrophages [30]. This is an area that requires further investigation, given that increased IL-10 production in mice exacerbates chronic tuberculosis [31].

TNF is a major contributor to many facets of the immune response, including macrophage activation, granuloma formation and maintenance, and regulation of cytokines and chemokines [32–34]. It is likely that the neutralization of TNF affects several pathways important in the control of tuberculosis. The data presented here suggest that TNF-inhibiting molecules may have different effects during acute, compared with chronic or perhaps even latent, tuberculosis and that incomplete neutralization of TNF allows the host to maintain control of the infection. These agents provide models for understanding the mechanisms by which TNF is important in the control of tuberculosis.

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