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Tumor Necrosis Factor Neutralization Results in Disseminated Disease in Acute and Latent *Mycobacterium tuberculosis* Infection With Normal Granuloma Structure in a Cynomolgus Macaque Model

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Objective. **An increased risk of tuberculosis has been documented in humans treated with tumor necrosis factor α (TNFα)–neutralizing agents. In murine models, impaired signaling by TNF causes exacerbation of both acute and chronic infection associated with aberrant granuloma formation and maintenance. This study was undertaken to investigate immune modulation in the setting of TNF neutralization in primary and latent tuberculosis in a non-human primate model.**

Methods. **Cynomolgus macaques 4 years of age or older were infected with *Mycobacterium tuberculosis* and subjected to clinical, microbiologic, immunologic, and radiographic examinations. Monkeys were classified as having active or latent disease 6–8 months after infection, based on clinical criteria. Monkeys used in acute infection studies were randomized to receive either adalimumab (prior to and during infection) or no treatment. Monkeys with latent infection that were randomized to receive TNF-neutralizing agent were given either an inhibitor of soluble TNF, recombinant methionyl human soluble TNF receptor I (p55-TNFRI), or adalimumab. Control monkeys with latent infection were given no treatment or saline. Data from previously studied monkeys with active or latent disease were also used for comparison.**

Results. **Administration of TNF-neutralizing agents prior to *M tuberculosis* infection resulted in fulminant and disseminated disease by 8 weeks after infection. Neutralization of TNF in latently infected cynomolgus macaques caused reactivation in a majority of animals as determined by gross pathologic examination and bacterial burden. A spectrum of dissemination was noted, including extrapulmonary disease. Surprisingly, monkeys that developed primary and reactivation tuberculosis after TNF neutralization had similar granuloma structure and composition to that of control monkeys with active disease. TNF neutralization was associated with increased levels of interleukin-12, decreased levels of CCL4, increased chemokine receptor expression, and reduced mycobacteria-induced interferon-γ production in blood but not in the affected mediastinal lymph nodes. Finally, the first signs of reactivation often occurred in thoracic lymph nodes.**

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Conclusion. **These findings have important clinical implications for determining the mechanism of TNF neutralization–related tuberculosis.**

In humans, an increased incidence of tuberculosis (presumably reactivation of latent infection) is observed in patients receiving tumor necrosis factor (TNF)–neutralizing agents for inflammatory conditions (1,2). The granuloma, the histopathologic hallmark of *Mycobacterium tuberculosis* infection, represents both an immunologic and a physical barrier by which to contain the infection. Poor granuloma structure has been associated with disseminated disease (3). TNFα plays a critical role in the control of acute and chronic *M tuberculosis* infection in murine models, characterized by disorganized granuloma structure contributing to poor control of infection (4,5). Other mechanisms by which TNF affects the response to *M tuberculosis* include macrophage activation (6), apoptosis (7,8), and chemokine (9,10) and adhesion molecule (11,12) expression. Patients who developed tuberculosis after treatment with TNF-neutralizing agents often had few clinical signs of tuberculosis, leading to difficulty in diagnosis and ultimately poor outcome. There was a striking predominance of extrapulmonary and disseminated tuberculosis unlike the more typical (pulmonary) pattern of reactivation (13). As TNF-neutralizing agents are introduced in countries with higher endemic rates of tuberculosis, the potential risk of tuberculosis, both primary and reactivation, may be greatly increased.

The standard murine models used for the study of tuberculosis are inbred strains, with varying patterns of resistance and disease (14). While the mouse is crucial for investigating immune responses and pathogenesis, there are 2 major limitations to this model. First, unlike humans, mice do not establish latent infection, but instead develop chronic disease and will eventually die of progressive primary tuberculosis. Second, the common inbred strains of mice produce granulomas that are best termed granulomatous infiltrations: collections of macrophages and lymphocytes that lack the architectural organization seen in humans. No mouse strains generate the spectrum of granulomas observed in humans.

Herein we demonstrate that cynomolgus macaques receiving TNF-neutralizing agents had uncontrolled and disseminated disease by 8 weeks after *M tuberculosis* infection. TNF-neutralizing agents also induced a high rate of reactivation tuberculosis among macaques with latent infection (15). Extrapulmonary disease occurred in both acute and reactivation tuberculosis. In sharp contrast to murine data, normal granuloma architecture, similar to that seen in active tuberculosis, was observed in monkeys that received TNF-neutralizing agents, suggesting that mechanisms of TNF-associated susceptibility to tuberculosis may be different than in murine models (16).

MATERIALS AND METHODS

Animals. Cynomolgus macaques (*Macaca fascicularis*) 4 years of age or older (from Labs of Virginia, Yemassee, SC; Shin Nippon Biomedical Laboratory, Summerville, SC; Valley Biosystems, Sacramento, CA; and Covance, Vienna, VA) were studied within a biosafety level 3 primate facility (17). All animal protocols and procedures were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Monkeys were infected with *M tuberculosis* (Erdman strain) via bronchoscopic instillation of ~25 colony-forming units (CFUs) to the lower lung lobe (17). Infection was confirmed by tuberculin skin test conversion and/or lymphocyte proliferation assay (18). Serial clinical, microbiologic, immunologic, and radiographic examinations were performed (15). Based on defined clinical criteria (15), monkeys were classified as having latent or active disease 6–8 months after infection. To be classified as having active disease, monkeys had to have abnormal findings on chest radiographs, *M tuberculosis* growth evident on gastric aspirate or bronchoalveolar lavage (BAL), cough, weight loss, and/or elevated erythrocyte sedimentation rate (ESR) beyond 3 months after infection (15,19). In contrast, monkeys classified as having latent infection had no radiographic, microbiologic, or clinical signs of disease (15,19). Control monkeys with latent and active disease were used for comparison. (Some data on these monkeys have been published previously [19].)

Anti-TNF agents. For acute infections, monkeys were given 4 mg/kg adalimumab (Abbott, Abbott Park, IL; provided by Amgen, Thousand Oaks, CA), a humanized monoclonal antibody obtained via pharmacy, subcutaneously 2 days prior to infection and every 10 days until necropsy. This dose is ~1.8-fold higher than the loading dose for a human with Crohn's disease. Monkeys with latent infection were given either an inhibitor of soluble TNF, recombinant methionyl human soluble TNF receptor I (p55-TNFRI) (20) (provided by Amgen) (monkeys 7104 and 6604) or adalimumab (monkeys 17905, 9605, 16605, 10605, 12102, 23802, and 25503). Monkeys received adalimumab every 10 days for 4–8 weeks. Monkeys received p55-TNFRI 10 mg/kg subcutaneously weekly for 19 weeks. Control monkeys with latent infection were given saline or no treatment.

Immunologic assays. *Immunogenicity against TNF agents.* Monkey-derived antibody against the humanized neutralizing agent was assayed by enzyme-linked immunosorbent assay. Plates were coated with the anti-TNF agent, and serial plasma dilutions were used to estimate the anti-neutralizing agent present (http://www.bidmc.harvard.edu/display.asp?node_id=3770). To determine whether the macaque-derived antibody neutralized the effects of the anti-TNF agent, a functional assay was developed using WEHI var 13 cells (21),

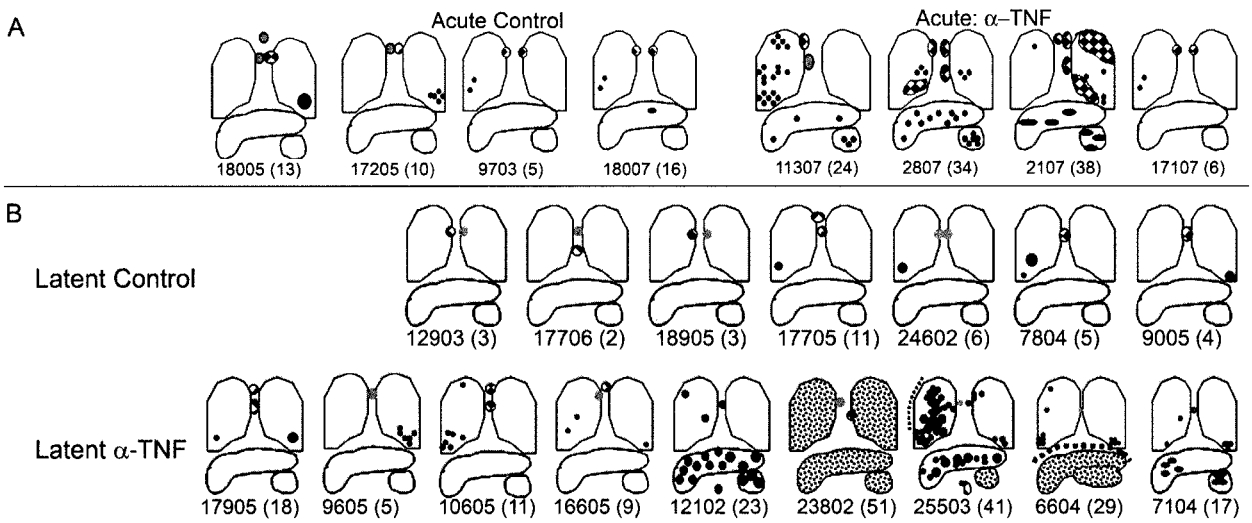

Figure 1. Tumor necrosis factor (TNF) is necessary to control initial and latent *Mycobacterium tuberculosis* infection. **A,** Gross pathologic evaluation of disease in macaques that were infected with low-dose *M tuberculosis* and were left untreated (acute control) or were treated with TNF-neutralizing antibody for 8 weeks (acute α-TNF). Control monkeys had granulomas (solid circles) localized to a single lung lobe and mediastinal lymph node enlargement (shaded circles) with or without granulomas (checkered circles). In 3 of 4 monkeys treated with anti-TNF reagent, aggressive and disseminated disease was observed, including multiple granulomas in lungs, liver, and spleen and tuberculous pneumonia (checkered areas in the lungs). **B,** Gross pathologic evaluation of disease in macaques that had latent infection and were left untreated (latent control) or were treated with TNF-neutralizing agent (latent α-TNF). Monkeys with latent infection showed limited disease: a few granulomas in the lung, and mediastinal lymph node enlargement with or without granulomas. In contrast, monkeys with latent infection that were treated with TNF-neutralizing agents ranged from normal to severely affected with disseminated disease. The liver and spleen (in monkeys 12102, 23802, 25503, 6604, and 7104) had multiple granulomas to miliary disease (stippled pattern). Disseminated miliary disease was observed in all lung lobes and the liver and spleen in monkey 23802. Multiple granulomas were observed on the diaphragm (dotted line) of monkey 6604, and extensive pleural disease (dotted line) was observed in monkey 25503. The numbers below each schema identify individual monkeys. The numbers in parentheses are necropsy scores.

under the following conditions: positive control (recombinant human TNF; 10–10,000 pg/ml); negative control (media alone); recombinant human TNF (10–10,000 pg/ml) preincubated with monkey serum (dilutions 1:100 and 1:1,000) and adalimumab (10 μg/ml); and recombinant human TNF (10–10,000 pg/ml) preincubated with adalimumab (10 μg/ml) alone. Sera from control monkeys were compared with sera from monkeys that received adalimumab. In this assay, no biologically active TNF could be detected after adalimumab and recombinant human TNF were added together, and the presence of anti-adalimumab antibody would neutralize these effects, resulting in levels of TNF detectable by WEHI assay.

Interferon-γ (IFNγ) enzyme-linked immunospot assay (ELISpot). Mycobacteria-induced production of IFNγ was measured by ELISpot with peripheral blood mononuclear cells (PBMCs) and BAL fluid samples at baseline and serial intervals, and at necropsy. Briefly, PBMCs or BAL fluid samples were cocultured with autologous PBMC-derived dendritic cells and mycobacterial antigen peptide pools (17), and the frequency of IFNγ-producing cells was measured (in spot-forming units per well).

Assays at necropsy. At necropsy, monkeys were maximally bled, and terminal BAL was performed. Monkeys were killed using pentobarbital and phenytoin (Beuthanasia; Schering-Plough, Kenilworth, NJ). Gross pathologic findings were described by a board-certified veterinary pathologist (EK). To quantify gross pathologic disease, we developed a necropsy score worksheet in which tuberculosis disease from each lung lobe, lymph node, and visceral organ was recorded and enumerated, and an overall score was determined (19). Points were given for number, size, and pattern of granulomas distributed in each lung lobe and mediastinal lymph node and in other organs (19). Representative sections of each tissue were placed in formalin for histologic analysis or homogenized into single-cell suspensions for immunologic studies, flow cytometric analysis, and bacterial burden, as previously described (15,17).

Histologic analysis. Microscopic histopathologic analysis of tissue sections was reviewed by a veterinary pathologist (EK) with specific focus on the following granuloma characteristics: overall architectural appearance, type of granuloma (caseous [local organization of lymphocytes and epithelioid macrophages with a central core of eosinophilic protein, or caseum], non-necrotizing solid [regional area of inflammatory cells like caseous granulomas but with a central area of macrophages instead of caseum], or suppurative [a caseous granuloma with >50% of the caseous area filled with neutrophils]), distribution pattern (focal, multifocal, coalescing, or invasive), and cellular composition (19). The results of microscopic histopathologic analysis were compared in a blinded manner between groups of monkeys with acute infection,

latent infection, and active infection and those receiving TNF-neutralizing agents.

ELISpot and phenotypic expression. At necropsy, PBMCs and cells from BAL, granulomatous and non-granulomatous lung tissue, and mediastinal lymph nodes were used in ELISpot assays (15,17). Flow cytometry using markers for T cells (CD3, CD8, and CD4), T cell activation (CD69 and CD29), chemokine receptors (CCR5 and CXCR3), and macrophages (CD14, CD11b, and CD11c) (17) was performed using CellQuest Software (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed with Flow Jo software (Tree Star, Ashland, OR).

Cytokine analysis of tissue homogenates. Luminex-Beadlyte human multi-cytokine detection (Upstate Millipore, Billerica, MA) was performed on tissue homogenates of the lungs and mediastinal lymph nodes, according to the recommendations of the manufacturer (22).

Bacterial burden. Bacterial burden was assessed in BAL specimens obtained monthly and at necropsy and in 20–40 tissue samples obtained at necropsy. BAL fluid and tissue homogenates were serially diluted and plated on 7H10 media, and CFUs were enumerated on day 21 (17). A CFU score was derived (as a summation of the log transformation of CFU/gram of each sample obtained from each tissue) as a measure of overall bacterial burden (19). Bacterial dissemination was reflected as the percentage of samples that grew *M tuberculosis* from tissue obtained at necropsy. These 2 methods of determining bacterial burden were validated to distinguish monkeys with latent disease from those with active disease (19).

Statistical analysis. Pairwise analysis between monkeys that received TNF-neutralizing agents and control monkeys was performed using Student's *t*-test (for normally distributed data) and the Mann-Whitney test (for non-normally distributed data). *P* values less than or equal to 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). When >2 groups were compared, one-way analysis of variance was used with Bonferroni post hoc analysis or other nonparametric equivalent.

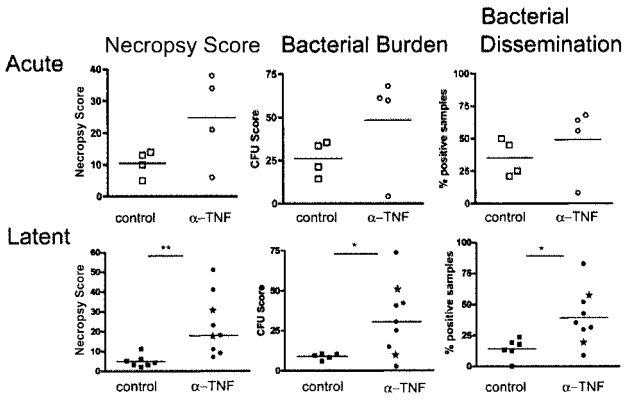

Figure 2. Gross pathologic evaluation of disease and bacterial burden in control monkeys with acute infection, monkeys with acute infection treated with anti–tumor necrosis factor (anti-TNF) agents, control monkeys with latent infection, and monkeys with latent infection treated with anti-TNF agents. **Top,** Necropsy score, bacterial burden, and bacterial dissemination in control monkeys with acute infection and in anti-TNF–treated monkeys with acute infection. Necropsy score was higher in monkeys treated with anti-TNF agents than in control monkeys, although this difference did not reach statistical significance. Overall bacterial burden (colony-forming unit [CFU] score) and distribution of bacterial burden (percent positive samples) were higher in anti-TNF–treated monkeys, but the differences were not statistically different due to variability in the groups. **Bottom,** Necropsy score, bacterial burden, and bacterial dissemination in control monkeys with latent infection and in anti-TNF–treated monkeys with latent infection. Monkeys with latent infection that were treated with TNF-neutralizing agents had higher necropsy scores, bacterial burden, and bacterial dissemination (percent positive samples) compared with control monkeys with latent infection. For necropsy score and bacterial burden, bars show the median; for bacterial dissemination, bars show the mean. Circles indicate monkeys treated with adalimumab; stars indicate monkeys treated with p55-TNFRI. $* = P < 0.05$; $** = P < 0.01$, by Mann-Whitney test or Student's *t*-test.

RESULTS

Importance of TNF in control of initial *M tuberculosis* infection. *Clinical parameters.* To address whether TNF was necessary for control of initial infection, control monkeys with acute infection (n = 4) and monkeys with acute infection treated with TNF-neutralizing agents (n = 4) were killed 8 weeks after infection. During infection, early indications of disease progression among the anti-TNF group included persistent growth of *M tuberculosis* evident on BAL or gastric aspirate (monkeys 2107 and 2807), elevated ESR (monkeys 2807 and 17107), and development of bilateral lower lobe pneumonia (monkey 2107). Control monkeys had no microbiologic or radiographic evidence of disease. Elevated ESR was noted at necropsy in 2 monkeys treated with anti-TNF agents (30.5 mm/hour in monkey 2107 and 16 mm/hour in monkey 2807 [normal range 0–2.0 mm]).

TNF neutralization results in disseminated disease during acute infection with intact granulomas. At necropsy, 3 of 4 adalimumab-treated monkeys with acute infection had more severe disease compared with control monkeys (Figure 1). Average necropsy scores were higher in adalimumab-treated monkeys compared with controls, reflecting an increased frequency of lesions in lung lobes and spread of disease to liver and spleen, although this did not reach statistical significance because 1 monkey was apparently unaffected by anti-TNF treatment (Figures 1 and 2). Gross evidence of invasive tuberculous pneumonia was noted in monkeys 2807 and 2107. Control monkeys had enlarged, granulomatous lymph nodes and fewer grossly visible lung granulomas.

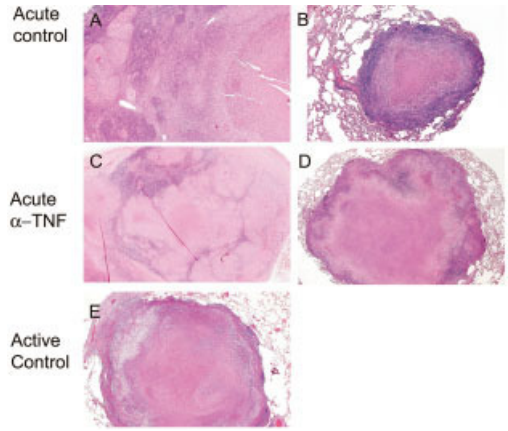

Figure 3. Photomicrographs of granulomas in the lymph nodes and lungs of control monkeys and monkeys treated with anti–tumor necrosis factor (anti-TNF) agents, showing more aggressive disease in monkeys treated with anti-TNF agents with normal granuloma structure. **A,** Caseous granuloma with adjacent non-necrotizing granulomas (left side) in the hilar lymph node of a control monkey 8 weeks after infection (monkey 18007). **B,** Caseous granuloma in the accessory lobe of a control monkey with acute infection (monkey 9703), consisting of a central area of eosinophilic proteinaceous material (caseum) surrounded by epithelioid macrophages and lymphocytes along the outermost periphery. **C,** Massive effacement of carinal lymph node with both caseous and non-necrotizing granulomas in an anti-TNF–treated monkey 8 weeks after infection. **D,** Large caseous granuloma in the lung of an anti-TNF–treated monkey (monkey 2807) with normal architecture. **E,** Large caseous granuloma in the lung lobe of a monkey with active tuberculosis. The architectural structure of the granuloma appears similar to granulomas observed in monkeys treated with TNF-neutralizing agents. (Hematoxylin and eosin stained; original magnification × 5 in **A** and **B**; × 2 in **C, D,** and **E**.)

The architecture of individual granulomas (caseous and non-necrotizing) in mediastinal lymph nodes and lungs was indistinguishable between control groups (Figures 3A and B) and groups treated with TNF-neutralizing agents (Figures 3C and D) by microscopic histopathologic analysis. However, overall the monkeys treated with TNF-neutralizing agents had a more aggressive and invasive disease pattern with granuloma invasion into nearby vessels and airways. This pattern was not observed among control monkeys 5–8 weeks after infection, but has been observed in monkeys with active tuberculosis, which usually takes >3 months to develop. Despite normal granuloma formation with TNF neutralization, function of the granulomas was apparently impaired, since disease was much more aggressive and disseminated.

Bacterial burden. Adalimumab-treated monkeys had slightly higher bacterial burden (CFU score) and bacterial dissemination (percent of tissue with growth) compared with control monkeys (Figure 2). One adalimumab-treated monkey (monkey 17107) did not have exacerbated disease, leading to a large standard deviation in both measurements.

Immunologic responses. Percentages of CD4 and CD8 T cells within PBMCs, lung, lymph node, and BAL specimens obtained at necropsy were similar in control monkeys and monkeys with acute infection treated with TNF-neutralizing agents, as was chemokine receptor (CXCR3 or CCR5) and activation marker (CD69 and CD29) expression. The absolute number of CD4 or CD8 T cells in tissues was similar between groups. Mycobacteria-induced production of IFNγ, determined by ELISpot of PBMCs, BAL specimens, mediastinal lymph nodes, and lungs was also similar between groups. At necropsy, all monkeys treated with adalimumab had detectable levels of macaque-generated antibody against adalimumab, although this did not abrogate the ability

Table 1. Macaque-generated antibody against adalimumab, percent expected TNF activity, and disease outcome in monkeys with acute or latent *Mycobacterium tuberculosis* infection treated with adalimumab*

Monkey	Anti-adalimumab titer†	% expected TNF activity‡	Disseminated disease
Monkeys with acute infection			
17107	1:30,000	0	No
2107	1:20,000	0	Yes
2807	1:15,000	NA	Yes
11307	1:20,000	0	Yes
Monkeys with latent infection			
9605	1:40,000	0	No
10605	1:40,000	0	No
16605	1:40,000	0	No
17905	1:20,000	0.24	No§
23802	1:40,000	0	Yes
12102	1:40,000	0	Yes
25503	1:40,000	0	Yes

* To determine whether the production of macaque-derived antibody against adalimumab abrogated the effects of adalimumab-induced tumor necrosis factor (TNF) neutralization, TNF bioactivity was measured with recombinant human TNF, adalimumab, and serum obtained from control monkeys and from adalimumab-treated monkeys. NA = not applicable.

† Determined by enzyme-linked immunosorbent assay. The minimum detectable antibody titer is shown.

‡ Percent expected TNF activity was based on the amount of biologically active TNF when adalimumab, monkey serum, and recombinant TNF in the presence of WEHI var 13 cells (numerator) were compared with recombinant TNF and serum alone (denominator). We hypothesized that macaque-derived functionally neutralizing antibody against adalimumab would result in bioavailable TNF, but this was not seen.

§ There was histopathologic evidence of microscopic reactivation of disease in this monkey.

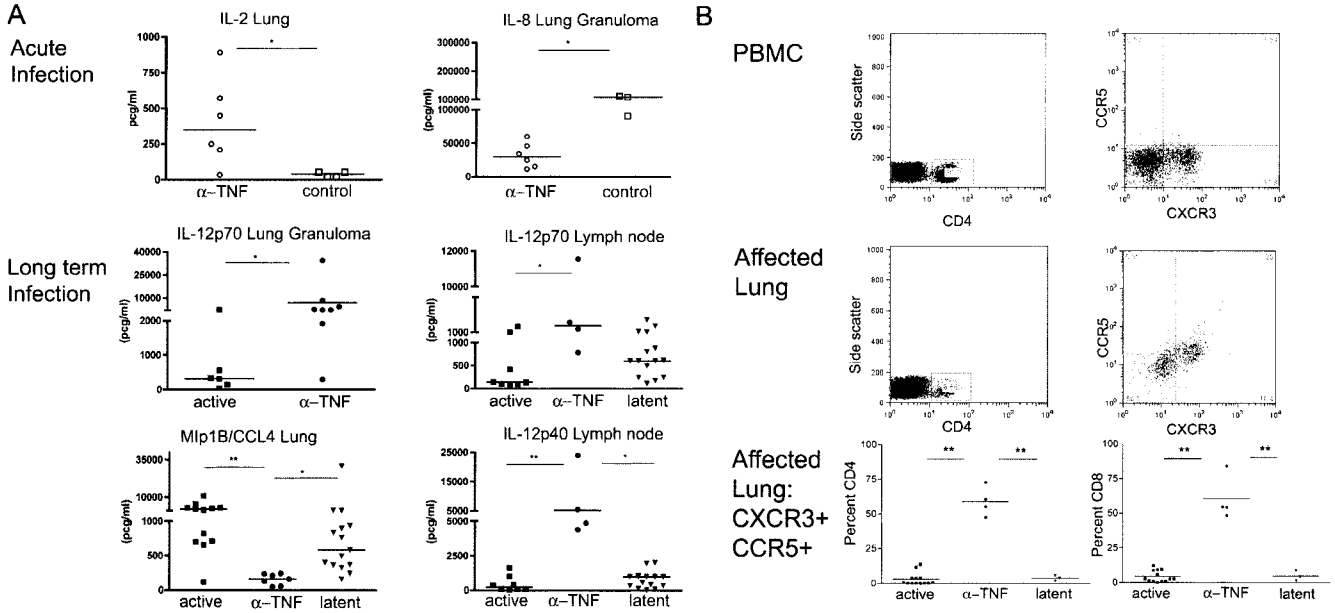

Figure 4. Cytokine and chemokine receptor expression in monkeys with untreated acute and latent infection and in anti–tumor necrosis factor (anti-TNF)–treated monkeys with acute and latent infection. **A,** Cytokine production in lung and lymph node homogenates. During acute infection, TNF neutralization resulted in higher levels of interleukin-2 (IL-2) in the lungs and lower levels of IL-8 in lung granuloma compared with control groups. Increased IL-12p70 was observed in lung and hilar lymph nodes among monkeys with latent infection that were treated with TNF-neutralizing agents compared with controls with active and latent infection. CCL4 was reduced in monkeys treated with TNF-neutralizing agents compared with controls with active and latent infection. Bars show the mean. $* = P < 0.05$; $** = P < 0.001$, by Kruskal-Wallis test with Dunn's multiple comparison. MIP-1B = macrophage inflammatory protein 1B. **B,** Flow cytometry dot plots of CD4 T cell gate and CXCR3+CCR5+ dual-positive cells in peripheral blood mononuclear cells (PBMCs) and affected lung samples. Significantly greater percentages of CXCR3+CCR5+ CD4 and CD8 T cells were observed in monkeys treated with anti-TNF agents compared with control monkeys with active and latent infection. Bars show the mean. $* = P < 0.05$; $** = P < 0.001$, by analysis of variance with Bonferroni post hoc analysis.

of adalimumab to neutralize TNF in vitro and was not correlated with disease outcome (Table 1).

TNF neutralization alters interleukin-2 (IL-2) and IL-8 production in the lungs during acute infection. Production of cytokines was measured in tissue homogenates of lung and hilar lymph nodes (Figure 4). Lung samples from adalimumab-treated monkeys showed an ~10-fold higher level of IL-2 than did lung samples from controls ($P = 0.04$). Within granulomatous lung, IL-8 levels were 30% lower among adalimumab-treated monkeys compared with controls ($P = 0.02$). This cytokine plays a role in recruitment of neutrophils and has been identified within granulomas (specifically fibroblasts) (23,24). TNF neutralization reduces IL-8 secretion in response to *M tuberculosis*–infected fibroblasts in vitro (24). However, histopathologic analysis showed more neutrophils in lung tissue from adalimumab-treated monkeys than in lung tissue from controls. The paucity of detectable IL-8 in the lungs of monkeys treated with anti-TNF agents may be a direct result of TNF neutralization or may be the result of increased neutrophils binding to free IL-8. Levels of CCL2, CCL3, CCL4, CCL5, IL-12, IL-6, and granulocyte–macrophage colony-stimulating factor were similar in anti-TNF and control groups during acute infection.

Reactivation of tuberculosis in monkeys with latent infection after treatment with TNF-neutralizing agents. Monkeys can remain latently infected for many months to years (15). The control group of monkeys with latent infection in the current study (monkeys 7804, 24602, 17706, 17705, 12903, 18905, and 9005) remained clinically stable, with no evidence of reactivation, until necropsy (10–15 months after infection). Monkeys with latent infection were infected 13–32 months prior to adalimumab administration (n = 7) or 12–14 months prior to p55-TNFRI treatment (n = 2). No monkeys developed cough, anorexia, or respiratory distress. However, after 14 weeks of p55-TNFRI treatment, monkey 6604 had gastric aspirates positive for *M tuberculosis*, and an elevated ESR at necropsy. Of those treated with

adalimumab, monkey 23802 had 10% weight loss and elevated ESR (35 mm/hour) after 4 weeks of treatment, and monkey 25503 had a positive gastric aspirate after 2 weeks of treatment.

Humoral response against TNF agents. Monkeys did not develop detectable antibodies against soluble p55-TNFRI. All monkeys treated with adalimumab had developed anti-adalimumab antibodies by the time of necropsy (Table 1). Again, the presence of anti-adalimumab antibodies did not abrogate the ability of adalimumab to neutralize TNF activity in vitro, and did not correlate with disease outcome (Table 1).

Reactivation and disseminated disease in monkeys treated with TNF-neutralizing agents. Among control monkeys with latent infection, limited gross pathologic findings were observed at necropsy, including no to few granulomas in a single lung lobe, thoracic lymph node enlargement with or without granulomas (19) (Figure 1B), and no extrapulmonary lesions. Both monkeys that received p55-TNFRI (monkeys 7104 and 6604) had gross evidence of reactivation characterized by multiple granulomas in liver and spleen or other organs with limited lung involvement. Three of the 7 adalimumab-treated monkeys developed gross evidence of reactivation that included miliary disease throughout the lungs, liver, and spleen (in monkey 23802), multiple granulomas in the liver, spleen, and extrapulmonary lymph nodes (in monkey 12102), and granulomas in multiple lung lobes with dissemination along the pleura of the chest, liver, spleen, and kidney (in monkey 25503). Two monkeys (9605 and 10605) had multiple granulomas in one or more lung lobes (more than in control monkeys with latent infection), suggesting early reactivation. Necropsy scores for monkeys treated with anti-TNF agents were significantly higher than those for control monkeys with latent infection (Figure 2).

Intact granuloma structure and formation in monkeys with latent infection treated with TNF-neutralizing agents. Monkeys with latent infection demonstrated lung granulomas with typical cellular architecture consisting of scarce peripherally located lymphoplasmacytic cells surrounding a zone of epithelioid macrophages with a core of caseous necrosis (Figure 5D). Some granulomas had peripheral fibrosis and centrally located mineralization (Figure 5B). One or several isolated caseous granulomas with or without mineralization could be seen within normal lymph node architecture (Figure 5A).

Microscopic histopathologic analysis of granulomas in adalimumab-treated monkeys demonstrated both non-necrotizing and caseous granulomas (Figures 5E–H), with typical cellular architecture observed in monkeys with active disease and natural reactivation (Figures 5C and D) (19). Even the extrapulmonary granulomas, likely to be newly formed as a result of dissemination, had normal cellular architecture. A miliary pattern of disease was observed, both grossly and microscopically, in some monkeys. Although granulomas in anti-TNF–treated monkeys were clearly not identical to latent granulomas, since these monkeys

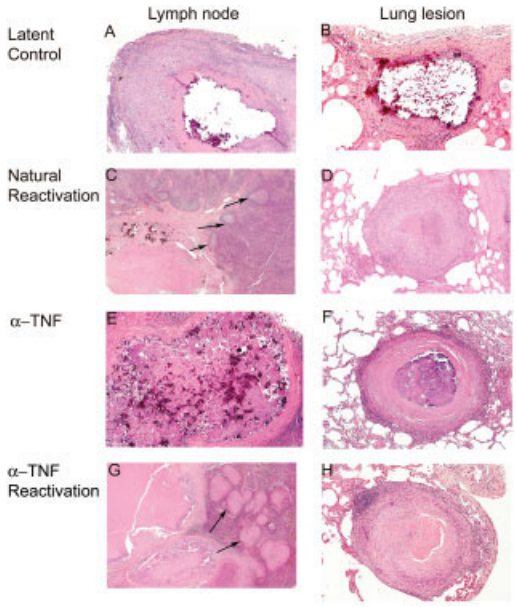

Figure 5. Normal granuloma structure despite reactivation tuberculosis in monkeys that were treated with anti–tumor necrosis factor (anti-TNF) agents. Monkeys with latent infection had caseous and/or mineralized granulomas in lungs and mediastinal lymph nodes. **A,** Mineralized granuloma (central core of mineral has artifactually dropped out of section) surrounded by a fibrotic rim within a mediastinal lymph node. **B,** Focal mineralized granuloma (mineral shattered during cutting) in the lung of a monkey with latent infection. **C,** Non-necrotizing granulomas (**arrows**) emanating from caseous granuloma (left) with mineralization, suggesting that reactivation is occurring from the carinal lymph node in a monkey with natural reactivation. **D,** Classic caseous granuloma in the lung with a proteinaceous central area of necrosis surrounded by epithelioid macrophages and lymphocytes in a monkey with natural reactivation. **E,** Mineralized granulomas (and caseous granulomas) in the carinal lymph node of a monkey treated with TNF-neutralizing agents. **F,** Caseous granuloma with fibrocalcific changes in a monkey treated with TNF-neutralizing agents, indistinguishable from a granuloma in a control monkey with latent infection. **G,** Non-necrotizing granulomas (**arrows**) emerging from caseous granulomas (with mineralization) in a monkey treated with TNF-neutralizing agents, suggesting reactivation from the carinal lymph node, similar to that shown in **C**. **H,** Classic caseous granuloma structure in the lung of a monkey with disseminated disease throughout the lung, liver, and spleen (monkey 25503). (Hematoxylin and eosin stained; original magnification × 5 in **A, B, D, E, F,** and **H**; × 2 in **C** and **G**.)

developed reactivation, in fact we found no evidence of disorganized or abnormal granuloma structure compared with monkeys with active disease and natural reactivation.

Histolopathologic evidence of reactivation in thoracic lymph nodes of monkeys treated with TNF-neutralizing agents. Mineralized granulomas were observed in the lungs and lymph nodes of monkeys from both treated and untreated groups, consistent with latent infection. In contrast, early reactivation lesions adjacent to established granulomas were observed in monkeys treated with anti-TNF agents. Analysis of tissue specimens from monkeys with latent or active tuberculosis suggested that non-necrotizing granulomas are found only during early or active disease (19). This pattern of "satellite" non-necrotizing granulomas in mediastinal lymph nodes of anti-TNF–treated monkeys (Figure 5G) was similar to lesions identified in mediastinal lymph nodes in monkeys that developed spontaneous reactivation of latent infection (19) (Figure 5C). This pattern suggests that early reactivation in response to TNF neutralization originates from latent lesions in thoracic lymph nodes, and this may lead to rapid dissemination. Based on this, monkey 17905 (treated with adalimumab) had evidence of microscopic reactivation in lymph nodes without gross evidence of reactivation.

Increased bacterial burden in monkeys with latent infection treated with TNF-neutralizing agents. Bacterial burden and dissemination were greater in monkeys treated with TNF-neutralizing agents than in control monkeys with latent infection (Figure 2B). Taken together, the gross pathologic, histologic, and microbiologic results demonstrate that neutralization of TNF can lead to overwhelming reactivation and dissemination of latent *M tuberculosis* infection, while maintaining apparently normal granuloma architecture.

IFNγ in hilar lymph nodes is not altered by TNF neutralization. Greater bacterial burden and production of mycobacteria-induced IFNγ was observed among monkeys with active disease compared with control monkeys with latent infection (19). In hilar lymph nodes, monkeys treated with TNF-neutralizing agents had greater IFNγ production than control monkeys with latent infection ($P < 0.01$) and responses that were similar to those of control monkeys with active disease, indicating appropriate IFNγ responses. Given the limited lung disease, too few samples were available for meaningful comparison. PBMC production of IFNγ, however, was similar between control monkeys with latent disease and monkeys with latent disease treated with anti-TNF agents. This is in contrast to the results of our previously published study, which showed that monkeys with active disease had greater PBMC IFNγ production than did control monkeys with latent disease (19). Taken together, these data suggest that IFNγ production is not altered by TNF neutralization at the site of disease, in contrast to blood (23,25).

TNF neutralization impairs cellular recruitment and alters chemokine receptor expression. Despite similar bacterial burden in monkeys treated with anti-TNF agents and controls with active infection, the absolute numbers of CD4 and CD8 T cells per gram of tissue in the hilar lymph nodes and lungs of monkeys treated with anti-TNF agents were similar to those in control monkeys with latent infection, as determined by flow cytometry (data not shown). We previously reported that increased T cell numbers in the lungs are associated with increased bacterial burden (19). These data suggest that despite similar bacterial burdens between monkeys with active disease and those treated with anti-TNF agents, TNF neutralization may impair cellular recruitment of T cells to the lungs. However, such results may also be explained by the disproportionate degree of extrapulmonary disease in monkeys treated with TNF-neutralizing agents.

Within granulomatous lung, a greater percentage of CD4 and CD8 T cells expressing both CXCR3 and CCR5 were present in monkeys treated with TNF-neutralizing agents compared with both control monkeys with latent infection and control monkeys with active infection ($P < 0.01$) (Figure 4B). These data suggest that TNF neutralization is associated with altered chemokine receptor expression, perhaps contributing to extrapulmonary spread.

TNF neutralization alters IL-12 and CCL4 levels in the lungs and lymph nodes. Within the lymph nodes, IL-12p70 levels were significantly greater in monkeys treated with TNF-neutralizing agents compared with control monkeys with active disease ($P < 0.05$) but not compared with control monkeys with latent infection (Figure 4). IL-12p40 in hilar lymph nodes was higher in anti-TNF–treated monkeys compared with both control monkeys with latent infection and control monkeys with active infection. Within granulomatous lung, IL-12p70 levels (Figure 4) and IL-12p40 levels (data not shown) in monkeys receiving anti-TNF agents were significantly higher than those in controls with active infection ($P = 0.02$). CCL4 in lung tissue was significantly reduced in monkeys treated with TNF-neutralizing agents compared with controls with active or latent infection ($P < 0.05$). This finding may be associated with the disproportionate degree of extrapulmonary disease seen.

There were insufficient samples of granulomatous lung tissue available from monkeys with latent infection for this comparison.

DISCUSSION

Humans treated with TNF-neutralizing agents are at a substantially higher risk of developing tuberculosis. To date, this appears to be due to reactivation of latent infection. However, as these immunomodulating agents begin to be used in developing countries with higher endemic tuberculosis rates, the risk of primary tuberculosis in these patients is likely to increase. This study investigated the host response to *M tuberculosis* following anti-TNF treatment in macaques, an established model of latent tuberculosis that provides a spectrum of granuloma histopathology that is most similar to that in humans. We demonstrated that TNF neutralization leads to exacerbation of primary disease and reactivation of latent infection with increased bacterial burden, disease, and extrapulmonary spread.

Importantly, normal granuloma structure was observed in the present study, despite TNF neutralization during both primary infection (granuloma formation) and latent infection (granuloma maintenance). The overall granuloma architecture in monkeys treated with TNF-neutralizing agents was similar to that in monkeys with active tuberculosis. This is consistent with data from the zebrafish model of *Mycobacterium marinum* infection, in which normal and accelerated granuloma formation was observed in the absence of TNF despite increased bacterial growth, suggesting that early granuloma formation is independent of TNF signaling (26). These data challenge the current presumption that tuberculosis associated with TNF neutralization in humans is due to impaired granuloma organization. We and others have demonstrated that granuloma structure is disrupted during both primary and chronic disease without functional TNF in murine models (4–9,16), suggesting that TNF was necessary not only for macrophage activation but also for signals leading to formation and maintenance of granulomas. This disparity in findings may be due to the inherent nature of murine granulomas, which are loosely organized aggregates of inflammatory cells, compared with the distinct architecture of tightly localized inflammatory cells seen in zebrafish and primates (1,2).

Simulations of a granuloma in a mathematical model and dissection of the various functions of TNF in control of *M tuberculosis* predicted that granuloma formation or maintenance was not dependent on TNF, but instead that the primary role of TNF in the granuloma was macrophage activation and stimulation of chemokine production. That model also demonstrated that granuloma size, and to some degree structure, depended primarily on bacterial numbers, consistent with the findings of the present study (27).

Previous studies of murine models resulted in speculation regarding granuloma structure among patients with anti-TNF–associated tuberculosis. Iliopoulos et al (28) reviewed reports of anti-TNF–associated tuberculosis and found only 2 cases in which tuberculosis was diagnosed by culture with negative biopsy evidence (2,29). The authors contend that this notion of "granuloma-free tuberculosis" may have dissuaded physicians from performing diagnostic biopsy. Likewise, the majority of published reports describe a range of disease involvement (pulmonary, extrapulmonary [2], or miliary disease [28,30]), yet no cases of aberrant granuloma structure. A high proportion of disseminated and/or extrapulmonary disease, as seen in our model, occurs in humans treated with anti-TNF agents compared with reactivation tuberculosis in the general population (2,31), in which mediastinal disease is the most common manifestation (13). The unusual presentation of tuberculosis in these cases likely contributes to difficulty in diagnosis but should not dissuade physicians from obtaining diagnostic biopsy or culture.

The presence of normal granuloma structure suggests that the mechanism of disease exacerbation in humans may differ from that in the murine model. No differences in T cell number, cellular phenotype, or mycobacteria-induced production of IFNγ were identified in monkeys with acute infection, despite the disseminated disease seen in monkeys treated with TNF-neutralizing agents. The greater production of IL-2 in the lungs of anti-TNF–treated monkeys is likely to be the result of more severe disease. Lower levels of IL-8 were detected in lung granulomas of monkeys treated with anti-TNF agents during acute infection compared with controls, although more neutrophils were present in granulomas in the anti-TNF–treated monkeys.

Anti-TNF–treated monkeys with latent infection had a greater disease burden and bacterial load compared with control monkeys with latent infection. Human studies of PBMCs have suggested that TNF-neutralizing agents decrease mycobacteria-induced IFNγ production (32,33) and proliferative responses (25). In contrast, our data suggest that TNF neutralization does not alter IFNγ responses at the site of disease. While differences in chemokine expression due to TNF neutralization in mice have been described (9,10,34), in

the present study CCL4 was the only chemokine altered during TNF neutralization in monkeys. Substantially more T cells expressing both CXCR3 and CCR5 were observed in the lungs of anti-TNF–treated monkeys compared with controls. Increased expression of chemokine receptors may reflect changes in cytokine or chemokine concentrations, and affect appropriate cell trafficking within tissues or granulomas. IL-12 levels were higher in TNF-neutralized hilar lymph nodes and lungs, supporting the notion that TNF plays a role in the regulation of IL-12 expression as in in vitro models (35,36). Overall, it appears that TNF neutralization results (whether directly or indirectly) in dysregulation of critical cytokines and chemotactic factors that ultimately contributes to disseminated disease. Thus, TNF may still affect localization of cells (26,37) and the integrity of cellular interactions within the granuloma, rather than overall granuloma structure.

TNF has multiple effector mechanisms that may be important in the control of tuberculosis, including influencing cytokine (6) and chemokine (9,10) responses, macrophage activation (6), and apoptosis (7,8). Macrophage function is likely altered by TNF neutralization, as has been reported previously (26,27,37,38). Anti-TNF antibody has been associated with reduction in a subset of CD8 memory effector cells that have tuberculocidal activity (39). Our current efforts are focused on these aspects of the role of TNF in tuberculosis.

These findings have important implications in studying the complexity of reactivation of tuberculosis. The presence of non-necrotizing granulomas (seen exclusively in active disease) in the hilar lymph nodes of both monkeys with natural reactivation and those with reactivation as a result of TNF neutralization suggests that this process begins in hilar lymph nodes. While the rate of reactivation among monkeys treated with TNF-neutralizing agents was not 100%, it was certainly very high, as described in the literature regarding humans (1,2). In our model, a spectrum of latent infection can be observed (19), suggesting that the risk of reactivation likely depends on a complex combination of factors, including state of infection and severity and duration of immunosuppression. Currently, it is recommended that patients with latent tuberculosis take anti-tuberculosis medications prior to starting a TNF inhibitor. Despite this, tuberculosis cases still occur during TNF inhibitor treatment (40), demonstrating that TNF plays a critical role in the control of tuberculosis for some patients.

In summary, the high rate and rapid onset of reactivation that can occur during TNF neutralization and the presence of normal granuloma structure should remind clinicians to maintain a high level of clinical suspicion and low threshold for culture and biopsy in patients receiving such treatments. Our data strongly support the notion that the use of TNF-neutralizing agents in areas of endemic tuberculosis should be carefully monitored, as the incidence of both primary and reactivation tuberculosis is likely to increase dramatically and may be difficult to diagnose, due to unusual presentation.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Flynn had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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