Tumor-Necrosis-Factor Blockers: Differential Effects on Mycobacterial Immunity

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Background. Tumor necrosis factor (TNF) plays a pathogenic role in rheumatoid arthritis but is essential for antimycobacterial host defenses. The risk of reactivation of latent Mycobacterium tuberculosis infection is greater with the TNF monoclonal antibody infliximab than with the soluble TNF receptor etanercept. The basis of this difference is not known.

Methods. The effects that the monoclonal antibodies infliximab and adalimumab and the receptor etanercept have on antimycobacterial immune functions were studied by use of therapeutic drug concentrations in whole-blood culture.

Results. Infliximab and adalimumab reduced the proportion of tuberculosis-responsive (CD69+) CD4 cells by 70% and 49%, respectively (P < .05), and suppressed antigen-induced interferon (IFN)–γ production by 70% and 64% (P < .05), respectively; in contrast, etanercept produced no significant effect. Interleukin-10 production was equally suppressed by all 3 drugs. Adalimumab and etanercept had divergent, concentration-dependent effects on control of intracellular growth of M. tuberculosis. None of the drugs induced significant levels of apoptosis or necrosis, in either monocytes or T cells.

Conclusions. The tuberculosis risk posed by infliximab may reflect its combined effects on TNF and IFN-γ.

Tumor necrosis factor (TNF) drives the early cytokine cascade at sites of inflammation [1]. It is produced by activated macrophages and lymphocytes as a transmembrane protein, which then undergoes cleavage and aggregation to form biologically active trimeric TNF [2]. TNF acts by binding to 1 of 2 types of receptors—p55 and p75—the trimeric structures of which mimic those of the active cytokine.

At present, 3 TNF antagonists—infliximab, adalimumab, and etanercept—are approved for the treatment of chronic inflammatory conditions such as rheumatoid arthritis (RA). All are parenterally administered 150-kD proteins composed of 2 TNF-binding domains linked to human IgG, Fc. Infliximab and adalimumab are monoclonal antibodies (MAbs)—with murine and human Fv regions, respectively—that recognize both monomeric and trimeric TNF. In contrast, TNF binding by etanercept is mediated by 2 human p75 TNF receptors linked to Fc; it recognizes only trimeric TNF. Nearly 1 million patients have been treated worldwide with either infliximab or etanercept; to date, 10,000 patients have been treated with adalimumab [3]. Although these agents have not been formally compared in clinical trials, they appear to be similarly highly effective for conditions, such as RA, for which all are indicated.

TNF is essential for host defenses against Mycobacterium tuberculosis. Animals lacking the gene for TNF, treated with neutralizing anti-TNF antibody, or genetically engineered to overexpress soluble TNF receptor show increased susceptibility to granulomatous pathogens such as M. tuberculosis, Listeria monocytogenes, and Histoplasma capsulatum, as well as to attenuated organisms such as M. bovis bacille Calmette-Guérin [4–10]. These infections occur with increased frequency in patients treated with TNF blockers. However, several studies indicate that these risks are 2–10-fold greater...
in patients treated with infliximab than in those treated with etanercept [11–13]. The basis of this difference is not known. In addition to their structural differences, infliximab and etanercept differ with respect to dosing, pharmacokinetics, and binding avidity [14, 15]. Infliximab, but not etanercept, has also been reported to induce apoptosis in activated T cells, both in vitro and in vivo in patients with Crohn disease (CD), apparently by binding to and cross-linking membrane-associated TNF [16–19]. This process, which has been attributed to reverse signaling through transmembrane TNF [20, 21], has been implicated as a therapeutic mechanism of infliximab in the treatment of CD [16], a granulomatous inflammatory condition for which etanercept is ineffective [22].

The present study was undertaken to examine the differential effects that therapeutic concentrations of the TNF blockers have on antimycobacterial immune functions, with the goal of elucidating the mechanisms underlying their differential tuberculosis risk.

SUBJECTS, MATERIALS, AND METHODS

Subjects. Heparinized blood was collected from healthy volunteers. Experiments in which either control of intracellular growth of *M. tuberculosis* or T cell responses to *M. tuberculosis* culture filtrate (CF) were assessed were conducted in tuberculin skin test–positive donors. Other experiments were conducted without regard to skin-test status.

Mycobacteria. *M. tuberculosis* H₃₇Rv CF was prepared as described elsewhere [23]. *M. tuberculosis* H₃₇Ra was propagated in BACTEC 13A medium (Becton Dickinson) and was frozen in aliquots, as described elsewhere [24]. A standard curve relating inoculum size to days to positivity (DTP) in BACTEC 12B was generated by use of inoculum volumes of 0.01–1000 μL. Cultures were scored as positive when the growth index, with interpolation, was 30. The inoculum was selected as that volume calculated to have a DTP of 4.5.

Cytokine production. Cytokine production was assessed in whole-blood culture, as described elsewhere [25]. In brief, blood was diluted 1:4 with tissue-culture medium (RPMI 1640 [Invitrogen] with HEPES at 25 mmol/L). Cultures were stimulated with either *M. tuberculosis* CF at 5 μg/mL or phytohemagglutinin A (PHA) (Sigma-Aldrich) at 5 μg/mL. TNF blockers were added at trough and peak drug concentrations (etanercept, 1 and 2 μg/mL; infliximab, 5 and 80 μg/mL; and adalimumab, 5 and 10 μg/mL) reached in blood during therapy, on the basis of information provided by either the manufacturer [26, 27] or published studies [28]; etanercept was also tested at a supratherapeutic concentration of 5 μg/mL. Blood was cultured in 24-well culture plates at 37°C in 5% CO₂. Supernatants were collected after 5 days and were stored at −70°C. Interferon (IFN)–γ and interleukin (IL)–10 were analyzed by ELISA (R&D Systems), according to the manufacturer’s instructions.

Control of intracellular *M. tuberculosis* growth. Control of intracellular *M. tuberculosis* growth was assessed in whole-blood culture, as described elsewhere [29]. In brief, blood was mixed 1:1 with tissue-culture medium in 2-mL vials and was inoculated with *M. tuberculosis* H₃₇Ra. TNF blockers were added at the concentrations indicated above. The vials were sealed and were incubated at 37°C, with slow constant mixing. After either 24 or 96 h, blood cells were pelleted by centrifugation, and the supernatant was discarded. The pelleted cells were lysed, and recovered bacilli were inoculated into BACTEC 12B medium. Growth indices were monitored daily. The extent of bacillary growth or killing was determined by comparing DTP values of the completed cultures with those of the inoculum. Data management and calculation of bacillary survival were performed by use of software written by one of the authors of the present report (R.S.W.); this software is available from that author by request.

T cell activation, apoptosis, and necrosis. In polypolypropylene tubes, 600 μL of heparinized blood was mixed with an equal volume of tissue-culture medium and was stimulated with either PHA at 5 μg/mL or *M. tuberculosis* CF at 5 μg/mL. TNF blockers were added at the concentrations indicated above. Cultures were incubated, for either 24 or 72 h, at 37°C. Red blood

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cell type</th>
<th>No treatment</th>
<th>Etanercept at 2 μg/mL</th>
<th>Adalimumab at 5 μg/mL</th>
<th>Infliximab at 80 μg/mL</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>MB-CF</td>
<td>CD4</td>
<td>0.73</td>
<td>0.60</td>
<td>0.37*</td>
<td>0.22*</td>
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<tr>
<td>PHA</td>
<td>CD4</td>
<td>14.4</td>
<td>15.1</td>
<td>10.5</td>
<td>10.1*</td>
<td>.015</td>
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<tr>
<td>PHA</td>
<td>CD8</td>
<td>11.1</td>
<td>9.1</td>
<td>7.8</td>
<td>5.9</td>
<td>.064</td>
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</table>

NOTE. Data are median values for 10 subjects. An asterisk (*) indicates significant difference, compared with values in untreated control cultures, as determined by repeated-measures analysis of variance on ranks followed by post-hoc testing by Dunn’s method. Adalimumab and infliximab both significantly inhibited antigen-induced CD4 activation, and similar effects of smaller magnitude were also observed in mitogen-stimulated cells; etanercept had no significant effect. PHA, phytohemagglutinin; MB-CF, Mycobacterium tuberculosis culture filtrate.
Figure 1. Effects that tumor-necrosis-factor (TNF) blockade has on antigen-induced interferon (IFN)-γ (left) and interleukin (IL)-10 (right) production in whole-blood cultures stimulated with Mycobacterium tuberculosis culture filtrate. Median and interquartile-range values for 15 subjects are shown. Median IFN-γ and IL-10 levels when TNF blockers were absent were 1028 and 875 pg/mL, respectively. An asterisk (*) indicates statistical significance, in comparison with control cultures ( ), on the basis of repeated-measures analysis of variance on ranks followed by post-hoc testing by Dunn’s method. Both trough and peak concentrations of adalimumab and infliximab inhibited IFN-γ production, whereas etanercept, even at a supratherapeutic concentration, did not. All 3 drugs inhibited IL-10 production, at all concentrations tested.

Figure 2. Effects that tumor-necrosis-factor blockade has on control of growth of colony-forming units (cfu) of Mycobacterium tuberculosis during the first 24 h of whole-blood culture. Median and interquartile-range values for 20 subjects are shown. Divergent effects at peak levels approached statistical significance ( , by repeated-measures analysis of variance on ranks) at 24 h, although none of the treatments had effects that differed significantly from those in untreated control cultures. Note that overlapping error bars do not preclude differences that might be revealed by paired- or repeated-measures testing.
Table 2. Tumor-necrosis-factor (TNF) blockade: effect on apoptosis and necrosis, in antigen- and mitogen-stimulated T cells.

<table>
<thead>
<tr>
<th>Gating</th>
<th>Stimulus</th>
<th>Cell type</th>
<th>Culture duration, h</th>
<th>Effect induced</th>
<th>Treatment result, % of cells ( ^a )</th>
<th>Treatment result, % of cells ( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No treatment at 2 ( \mu g/mL )</td>
<td>Etanercept at 2 ( \mu g/mL )</td>
</tr>
<tr>
<td>All cells</td>
<td>MB-CF</td>
<td>CD4</td>
<td>24</td>
<td>Apoptosis</td>
<td>2.9</td>
<td>3.2</td>
</tr>
<tr>
<td>PHA</td>
<td>CD4</td>
<td>24</td>
<td>Apoptosis</td>
<td>7.7</td>
<td>4.7*</td>
<td>5.8*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Necrosis</td>
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<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
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<td></td>
<td>72</td>
<td>Apoptosis</td>
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<td>15.0</td>
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</tr>
<tr>
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<tr>
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<td></td>
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<td></td>
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<tr>
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<td>CD4</td>
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<td>Apoptosis</td>
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<td>7.9</td>
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<td>72</td>
<td>Apoptosis</td>
<td>23.0</td>
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<td>Apoptosis</td>
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<tr>
<td></td>
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<td>72</td>
<td>Necrosis</td>
<td>11.0</td>
<td>9.8</td>
<td>11.0</td>
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</table>

**NOTE.** Apoptosis was inhibited by TNF blockade in 24-h cultures of unselected PHA-stimulated CD4 cells, but this effect was abolished when analysis was restricted to activated (CD69+) cells; apoptosis was not induced by TNF blockade under any conditions tested. PHA, phytohemagglutinin; MB-CF, Mycobacterium tuberculosis culture filtrate.

\( ^a \) Values are median percentage of either annexin V+/7AAD- cells (in the case of apoptosis) or 7AAD+ cells (in the case of necrosis) for 9 subjects. An asterisk (*) indicates significant difference, compared with values in untreated control cultures, as determined by repeated-measures analysis of variance on ranks and subsequent post-hoc testing by Dunn’s method. 

**RESULTS**

**Antigen- and mitogen-induced T cell activation.** The effects that TNF blockade has on expression of CD69 (an early marker of activation) by T cells were studied in antigen (i.e., M. tuberculosis CF)–stimulated and mitogen (i.e., PHA)–stimulated whole-blood cultures of 24 h duration. Resting cells failed to show detectable expression of CD69 (data not shown). As indicated in table 1, both adalimumab and infliximab inhibited T cell activation. The greatest effect was seen with infliximab, which reduced antigen-induced CD4 activation to 30% of baseline values; in contrast, etanercept caused no significant effect.

**Antigen-induced IFN-\( \gamma \) and IL-10 expression.** Effects of TNF blockade on M. tuberculosis CF–induced IFN-\( \gamma \) and IL-10 production were examined in whole-blood cultures of 5 days duration. Without TNF blockade, median IFN-\( \gamma \) production was 1082 pg/mL (interquartile range [IQR], 651–1621). Infliximab and adalimumab suppressed IFN-\( \gamma \) production to approximately one-third of baseline values (\( P<.05 \)) (figure 1, left); significant effects were observed even at trough drug concentrations (5 \( \mu g/mL \)), whether comparison was with control cultures without drug or with corresponding cultures with etanercept. In contrast, etanercept showed no statistically significant effect on IFN-\( \gamma \), in comparison with control cultures, even when tested at a supratherapeutic concentration (5 \( \mu g/mL \)). To ensure that these findings were not due to a nonspecific effect of antibody, an additional experiment was performed in 6 subjects by adding human immunoglobulin for intravenous administration (IVIG; Gamunex) to antigen-stimulated cultures. Addition of IVIG at 80 \( \mu g/mL \) tended to increase IFN-\( \gamma \) production (1161 pg/mL vs. 1097 pg/mL in paired control cultures; \( P = .065 \)), an effect that could not account for the observed TNF MAb–induced inhibition of IFN-\( \gamma \).

Median IL-10 production by cells stimulated with M. tuberculosis CF was 875 pg/mL (IQR, 399–1295). Infliximab, adalimumab, and etanercept all suppressed IL-10 production to 20%–30% of baseline values, regardless of drug concentration (figure 1, right).

**Control of intracellular M. tuberculosis growth.** The effect that TNF blockade has on control of intracellular mycobacterial growth was assessed in whole-blood cultures infected with M.
Figure 3. Effect that tumor-necrosis-factor blockade has on apoptosis in 48-h cultures of Mycobacterium tuberculosis culture filtrate–stimulated monocytes. Median and interquartile-range values for 20 subjects are shown. Apoptosis was measured, by ELISA, as histone-associated cytoplasmic DNA. Apoptosis in cultures treated with peak therapeutic concentrations of infliximab was significantly greater than that in control cultures, by repeated-measures analysis of variance, although it reached only one-tenth the level reported in cultures with other proapoptotic stimuli [30].

Tuberculosis H₃Ra. Mycobacterial viability declined by 70% (from 1100 to 344 cfu) during the first 24 h and did not change further during the subsequent 72 h, a result that is consistent with what has been reported by previous studies using this model [29]. Adalimumab and etanercept had divergent, concentration-dependent effects on control of intracellular growth during the first 24 h (figure 2); at peak drug levels, this difference approached statistical significance (P = .05). Mycobacterial viability was not further affected by TNF blockade during the subsequent 72 h (not shown).

T cell apoptosis and necrosis. The effects that TNF blockade has on T cell apoptosis and necrosis were studied in antigen- and mitogen-stimulated whole-blood cultures of 24 and 72 h duration (table 2). Induction of apoptosis was not observed under any of the conditions tested; indeed, the only significant effect observed was inhibition of apoptosis, by all 3 TNF blockers, in PHA-stimulated cultures. Other researchers have reported that induction of apoptosis by infliximab occurs only in activated T cells [18]. Because we had observed inhibition of T cell activation by TNF blockade (table 1), we repeated the analysis in PHA-stimulated cultures after gating on activated (CD69⁺) cells. Higher levels of apoptosis and necrosis were indeed observed in this cell population; however, no effects of TNF blockade were identified.

Monocyte apoptosis and necrosis. The effects that TNF blockade has on apoptosis in monocytes activated with M. tuberculosis CF were studied after 24 and 48 h incubation, by ELISA-based detection of histone-associated cytoplasmic DNA. This method permitted the analysis of drug effects without requiring the removal of cell monolayers by mechanical scraping or enzymatic digestion. It also avoided monocyte loss due to adherence or clumping in activated mixed-cell suspensions. No significant effects were observed in 24-h cultures (not shown); at 48 h, peak concentrations of infliximab resulted in a 40% increase in histone-associated DNA, compared with levels in tuberculosis-stimulated control cultures, whereas neither etanercept nor adalimumab had a significant effect (P < .05, compared to results in control cultures) (figure 3). However, the biological significance of this observation is uncertain, because other apoptotic stimuli (e.g., serum starvation) have been reported to produce up to 10-fold-greater effects when this method is used [30]. Necrosis was assessed, in the treated monolayers, in terms of trypan-blue permeability; <3% of cells were trypan-blue positive, regardless of anti-TNF treatment (data not shown).

DISCUSSION

Recognition of the central role that TNF plays in the pathogenesis of chronic inflammatory disease such as RA has profoundly changed the treatment of these conditions, with the introduction, into clinical use, of infliximab in 1998, etanercept in 1999, and adalimumab in 2004. As experience with these drugs has grown, important differences between them have emerged. Despite sharing a common therapeutic target, infliximab poses a 9-times-greater risk of reactivation of latent M. tuberculosis infection during the first 3 months of treatment than does etanercept [11]; and histoplasmosis, listeriosis, and coccidioidomycosis also are increased when infliximab is used [12, 13]. The present study was undertaken to elucidate the basis of this difference. The experiments were conducted to reflect, as accurately as possible, conditions in vivo during therapeutic TNF blockade. Trough and peak drug concentrations were selected to reflect those during treatment. M. tuberculosis CF was selected on the basis of its ability to stimulate TNF expression in monocytes as well as in T cells [31]. Culturing was performed with fresh autologous plasma, to permit complement activation by TNF MAb immune complexes. Whole-blood cultures were used to permit expression of antibody-dependent cell-mediated cytotoxicity in mixed cell populations. To our knowledge, no other published studies of the mechanism of action of TNF blockers have adopted this approach.

The main finding of the present study is that infliximab and adalimumab inhibit T cell activation and IFN-γ production, whereas etanercept does not. IFN-γ, like TNF, is essential for protection against tuberculosis. The inability to appropriately produce or respond to IFN-γ strongly predisposes to tuberculosis, often resulting in disseminated infection even when the mycobacterium is attenuated [32, 33]. Etanercept and infliximab were found to have differing effects on IFN-γ production, at both equal and peak therapeutic concentrations. Adalimumab...
mab shared a profile similar to that of infliximab. These findings may indicate a fundamental relationship to the drug mechanism of action, rather than other factors. Although clinical experience with adalimumab is limited, recent postmarketing surveillance in Europe and North America has revealed rates of tuberculosis that are greater than those which had been anticipated [3]. These clinical and experimental findings together indicate that adalimumab and infliximab may share similar risks of reactivation of latent *M. tuberculosis* infection, despite their differences in route of administration, dosing, and pharmacokinetics.

The mechanism of inhibition of IFN-γ by infliximab and adalimumab otherwise remains uncertain; it could not be attributed to excess production of IL-10, which was inhibited equally by all 3 drugs at all tested concentrations. IL-10 production is increased in tuberculosis, in which it inhibits expression of IFN-γ and costimulatory molecules [34, 35]. The findings of the present study indicate that inhibition of IL-10 by TNF blockade does not protect against reactivation of latent *M. tuberculosis* infection by infliximab. Other cytokines, such as transforming growth factor-β and IFN-α, have also been implicated in the regulation of IFN-γ production in tuberculosis [36, 37]. Further studies will be necessary to determine the role that these cytokines play in the TNF MAb-dependent regulation of IFN-γ production.

In the present study, the TNF blockers had divergent effects on control of mycobacterial growth in whole-blood culture. This intracellular infection model has been advocated as a tool for the study of new tuberculosis vaccines [24, 38–40]. Impaired control of mycobacterial growth in whole-blood culture is evident in persons with HIV infection and in healthy tuberculosis reactors after either depletion of CD4 or CD8 T cells or addition of methylprednisolone or the TNF inhibitor pentoxifylline [24, 41, 42]. Host effector mechanisms expressed during the first 24 h of whole-blood culture may include innate bactericidal mechanisms of phagocytic cells, cellular cytotoxicity, and release of antibacterial peptides. Additional studies will be necessary to identify which of these mechanisms is/are differentially affected by TNF blockade.

The present study’s experiments failed to detect biologically significant apoptosis due to any of the TNF blockers. Induction of apoptosis in lamina propria T cells has been proposed as a therapeutic mechanism in CD, because T cell apoptosis increases to normal after treatment with infliximab [16, 18]; and some, but not all, investigators have reported that infliximab and adalimumab have similar effects on activated blood lymphocytes and monocytes of healthy donors [17, 19–21, 43]. This variation in results may reflect methodological differences, because levels of apoptosis appear to be highest in reports of studies in which monocytes have been cultured under conditions of low serum concentrations. In the present study, the lack of significant apoptosis or necrosis indicates that IFN-γ inhibition can occur in the absence of cell death. Further studies to examine the mechanism of this apparent cellular dysfunction are warranted.

In summary, the present study has found that the anti-TNF antibodies infliximab and adalimumab inhibit T cell activation and IFN-γ production in vitro, whereas the soluble TNF receptor etanercept has no effect. The risk that infliximab poses with regard to reactivation of latent *M. tuberculosis* infection may reflect its ability both to block the effects of TNF and to inhibit IFN-γ production.

References


