Role of Cellular Activation and Tumor Necrosis Factor–α in the Early Expression of *Mycobacterium tuberculosis* 85B mRNA in Human Alveolar Macrophages

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**Background.** Infection of alveolar macrophages (AMs), which constitute the first line of defense against *Mycobacterium tuberculosis*, initiates an intense interaction between the host's innate immune response and mycobacteria that may assist in the successful intracellular parasitism of *M. tuberculosis*.

**Methods.** Expression of tumor necrosis factor (TNF)–α and *M. tuberculosis* 85B mRNA was studied in *M. tuberculosis*–infected AMs, to better delineate the role of macrophages in the early events in initiation of infection.

**Results.** Both TNF-α mRNA and *M. tuberculosis* 85B were induced in AMs; at 24 h, the time point of maximum TNF-α induction, the mRNA levels for TNF-α and *M. tuberculosis* 85B correlated with one another, and induction of either gene correlated strongly with their protein levels. Inhibition of endogenous TNF-α by soluble (s) TNF receptor (R) I and sTNFRII reduced expression of both TNF-α and *M. tuberculosis* 85B. The activation of nuclear factor–κB was found to underlie expression of both TNF-α and *M. tuberculosis* 85B. Exogenous TNF-α was slightly more potent than interleukin (IL)–6 and granulocyte-macrophage colony-stimulating factor and was significantly stronger than IL-1 in inducing expression of *M. tuberculosis* 85B. Interestingly, inhibition of bactericidal mediators, reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs), reduced expression of TNF-α and *M. tuberculosis* 85B genes in *M. tuberculosis*–infected AMs.

**Conclusion.** Activation of AMs by *M. tuberculosis* initiates a cascade of events whereby TNF-α, ROI, and RNI enhance the expression of the *M. tuberculosis* 85B gene.

Although *Mycobacterium tuberculosis* infects at least one-third of the world’s population, a complete understanding of the mechanisms of its pathogenicity in humans is still lacking. The virulence of *M. tuberculosis* likely entails both its ability to survive the intracellular environment of host alveolar macrophages (AMs), which constitute the first line of defense against the pathogen, and its capacity to withstand the intense cytokine microenvironment generated after infection. Knowledge of the role of the host’s innate immune responses in the pathogenesis of *M. tuberculosis* infection may allow better targeting of therapies and vaccines to eradicate tuberculosis.

The initial interaction of *M. tuberculosis* with mononuclear phagocytes gives rise to a cytokine profile that is dominated by the proinflammatory macrophage-activating molecule tumor necrosis factor (TNF)–α. TNF-α is induced not only by phagocytic and nonphagocytic interaction of *M. tuberculosis* bacilli with mononuclear phagocytes [1, 2] but also by mycobacterial protein and nonprotein components [3]. TNF-α is present at sites of active *M. tuberculosis* infection in humans, regardless of the stage of mycobacterial infection [4–7]. In mice infected with mycobacteria, TNF-α is involved in the development of microbicidal granulomas [8], and disruption of TNF-α responses is associated with myco-
bacterial overgrowth [9, 10]. In subjects with latent *M. tuberculosis* infection, therapies that neutralize TNF-α have been associated with reactivation of tuberculosis [11]. However, it is not clear whether TNF-α contributes to protective immunity during all stages of *M. tuberculosis* infection, because it has been ascribed an immunopathological role in both animal models of *M. tuberculosis* infection and recent human studies [12, 13]. In human macrophages, TNF-α is only modest in its anti-*M. tuberculosis* activity [2], and its role in the formation of granulomas is unclear. In vitro studies indicate that, although AMs are initially more susceptible to *M. tuberculosis* infection and sustain higher *M. tuberculosis* growth, they ultimately contain *M. tuberculosis* better than do their blood precursors, monocytes [14]. The superiority of AMs in containment of growth of *M. tuberculosis* has been ascribed to their expanded capacity to produce TNF-α [14]. Nonetheless, other studies have shown that production of TNF-α either promotes [15] or is associated with [16] the growth of *M. tuberculosis* in human monocytes/macrophages.

Cellular signaling by TNF-α is mediated mainly through activation of NF-κB [17]. However, NF-κB-independent pathways of cellular activation by TNF-α have been described [18, 19]. In turn, activation of NF-κB and other pathways sustains TNF-α activity [17]. Downstream effector pathways activated by TNF-α in host tissues include activation of reactive oxygen intermediate (ROI) and reactive nitrogen intermediates (RNI) [20]. Phagocytosis of microbes and bacterial components, such as lipopolysaccharide (LPS), also activates ROIs [21] and indirectly, through oxygen radicals, activates inducible nitric oxide synthase (iNOS) and, therefore, RNIs in macrophages [22, 23].

Of the many *M. tuberculosis* products that induce production of TNF-α, antigen 85B is of note. Along with the other 2 proteins in the *M. tuberculosis* 85 complex (85A and 85C), with which it has 70%–80% homology [24], 85B is abundantly secreted by *M. tuberculosis* [25], binds fibronectin (fn) [26], and is involved in cell-wall biogenesis [27]. Interestingly, *M. tuberculosis* 85B is immunodominant [26] and potently induces TNF-α when complexed to fn in mononuclear phagocytes [28]. It is possible that, through sustaining TNF-α activity, the abundant release of *M. tuberculosis* 85B in situ contributes to the pathogenesis of *M. tuberculosis* infection. Recently, the expression of *M. tuberculosis* 85B mRNA has been found to be increased early during infection of monocytes [29] and cultured macrophages [30]. The expression of 85B in *M. tuberculosis*-infected monocytes correlates positively with both the amount of secreted TNF-α and subsequent intracellular mycobacterial growth [29]. Therefore, the interaction of *M. tuberculosis* 85B and host TNF-α may create a vicious circle in which each maintains the production of the other.

The present study was conducted to examine the interaction of *M. tuberculosis* 85B and TNF-α early after infection of AMs with *M. tuberculosis*. To fully delineate the contribution of the innate immune response to the pathogenesis of *M. tuberculosis* infection within the cellular context of the lungs, the roles that ROIs and RNIs, induction of TNF-α, and macrophage activation play in expression of *M. tuberculosis* 85B were examined.

**MATERIALS AND METHODS**

**Reagents.** Recombinant (r) cytokines, soluble (s) TNF receptor (R) I (sTNFRI), and sTNFRII were purchased from R&D Systems. NMMA (N⁵-monomethyl-L-arginine-monoacetate), which specifically inhibits the iNOS required for production of RNI, and NAC (N-acetyl cysteine), which scavenges oxygen radicals, were purchased from Calbiochem and Sigma, respectively. In some experiments, oxidized ATP (oATP; Sigma), which inhibits the purinergic receptor P2X, and reduces both RNI and ROI [31], was used. NADPH and H₂O₂ (Sigma), nonoate-9 (NOC-9) (Calbiochem), sodium nitroprusside (SNP; Sigma), and SN50 or its analogue SN50/M (Biomol) were used in some experiments. The endotoxin content of all reagents, as assessed by use of a lymulus amebocyte lysate assay (BioWhittaker), was ≤0.01 ng/mL. In preliminary dose-response experiments, the optimal amount of each reagent was determined. The cytotoxicity of all reagents was assessed by use of Trypan blue exclusion. Reagents were added to cells and assessed either immediately or after 24 h of culture. None of the reagents was toxic to AMs.

**Preparation of mycobacteria.** Avirulent and virulent laboratory-adapted *M. tuberculosis* (H37Ra and H37Rv) were grown in Middlebrook 7H9 broth (Difco Laboratories) at 37°C in 5% CO₂, Midlogarithmic mycobacterial cultures (14 days) were harvested and quantified by use of a colony-forming unit assay, as described elsewhere [2]. Aliquots of the stock were kept at −70°C. The viability of the stock remained >99% at 1 year.

**Study subjects.** Healthy, nonsmoking volunteers, 20–45 years of age, were recruited for bronchoscopy and bronchoalveolar lavage (BAL). All study subjects fulfilled the following criteria: not receiving medication, no history of heart or lung disease, and no upper respiratory tract infection within 6 months before the study. Furthermore, all subjects were tuberculin skin test negative, HIV uninfected, and had not received vaccination with bacille Calmette-Guérin. Informed, written consent was obtained from all participants before undergoing bronchoscopy. The protocol for this study was reviewed and approved by the Institutional Review Board for Human Investigation at University Hospitals of Cleveland.

**Preparation of bronchoalveolar cells (BACs).** BAL was performed as described elsewhere [14]. In brief, after anesthetizing the upper airway with topical 2% lidocaine, a flexible bronchoscope (BF type 4B2 bronchoscope; Olympus Optical) was wedged into the right middle lobe. Then, 360 mL of sterile 0.9% saline was instilled into 2 segments of the middle lobe, and BAL fluid was harvested. BACs were suspended in complete
Table 1. Sequences of primers and probes for quantification of tumor necrosis factor (TNF)-α and Mycobacterium tuberculosis 85B mRNA in infected alveolar macrophages.

<table>
<thead>
<tr>
<th>Target, strain, primer</th>
<th>PCR primer sequence, 5'–3'</th>
<th>Taqman probe sequence, 5'–3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>GGGTTCTACAACA</td>
<td>CCAAGGGGAAGAGGAGTCCCAGGAGCC</td>
</tr>
<tr>
<td>RT</td>
<td>AGGCGGTGCTTGTCTTCA</td>
<td>GTCGAAGAAGATGATCTGACTGCCC</td>
</tr>
<tr>
<td>Forward</td>
<td>GACGGTTATCTGATC</td>
<td>ACCGGCGCAAGACGAGACCAGA</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGCCGCTAGAGGGCTAAATTC</td>
<td>R18 ACCGGCGCAAGACGAGACCAGA</td>
</tr>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td>TCGAGTGACCCGGCATGGGAGCG</td>
<td>TCGAGTGACCCGGCATGGGAGCG</td>
</tr>
<tr>
<td>85B</td>
<td>GCTGGGATCTGCTGCTGTA</td>
<td>AGCACCAGGCACTACGTGCCAG</td>
</tr>
<tr>
<td>RT</td>
<td>TCAGGGGATGGGGCCTAGCC</td>
<td>AGCACCAGGCACTACGTGCCAG</td>
</tr>
<tr>
<td>Forward</td>
<td>GCTGGGATCTGCTGCTGTA</td>
<td>AGCACCAGGCACTACGTGCCAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTCTCTGAGATGGGTAGGT</td>
<td>CGCTCGACACCTAGTTTAC</td>
</tr>
<tr>
<td><strong>16S</strong></td>
<td>R18 CGCCGCTAGAGGTGAAATTC</td>
<td>R18 ACCGGCGCAAGACGAGACCAGA</td>
</tr>
<tr>
<td>RT</td>
<td>ACCGGCGCAAGACGAGACCAGA</td>
<td>R18 ACCGGCGCAAGACGAGACCAGA</td>
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<tr>
<td>Forward</td>
<td>R18 ACCGGCGCAAGACGAGACCAGA</td>
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</tr>
<tr>
<td>Reverse</td>
<td>R18 ACCGGCGCAAGACGAGACCAGA</td>
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</tbody>
</table>

**NOTE.** PCR, polymerase chain reaction; RT, reverse transcription.

medium (RPMI 1640 medium containing 2 mol/L l-glutamine, 25 mmol/L HEPES [Gibco Laboratories], and no antibiotics) and kept on ice. BACs were 90%–95% nonspecific-esterase positive and, therefore, comprised predominantly AMs and contained 5%–10% lymphocytes and <1% granulocytes, as assessed by Wright staining.

**Cell culture.** The α-chymotryptic fragment (120 kDa) of human fn (Life Sciences) was used to obtain adherent cells. In brief, 0.5 mL of complete medium with fn (1 µg/mL) was placed in 12-well tissue-culture plates (Costar) and incubated for 20 min at 37°C in 5% CO₂; then the plates were washed extensively to remove unbound fn. BACs were added to wells (60–105 × 10⁶ cells/mL), and the plates were incubated for 1–2 h at 37°C in 5% CO₂ and then were washed 4 times with medium to remove nonadherent cells. In preliminary experiments, we found that the purity of adherent AMs, as determined by cytostaining, was 100%. To maintain cell attachment, adherent AMs were cultured in RPMI 1640 medium supplemented with 1% fn. Then the cells were rested overnight at 37°C in 5% CO₂. Before infection, the plates were washed twice with RPMI 1640 medium.

**Infection of AMs.** Using conditions that maximize the opsonic uptake of M. tuberculosis [14] in preliminary experiments, we found an MOI of 1:1 (bacteria:cell) to be necessary for quantification of intracellular M. tuberculosis mRNA by use of real-time polymerase chain reaction (PCR). Thus, AMs were infected with M. tuberculosis at an MOI of 1:1 in 30% autologous unheated serum for 90 min at 37°C in 5% CO₂. Thereafter, the infected monolayers were washed 4 times with complete medium. Cells harvested at this time point were considered as time zero (t₀) after infection. Other cultures received RPMI 1640 medium with 2% autologous serum. At different time points, cultures were harvested, and cells were lysed in 0.5 mL of TRIZOL Reagent (Invitrogen). The cell-free culture supernatants were kept at −70°C.

**RNA extraction and quantitative reverse-transcription (RT)–PCR.** Cell lysates were transferred to FastRNA blue tubes and agitated in a Fast Prep FP120 BIO 101 SAVANT vibrator/cell-wall disrupter (Bio 101) at full speed, as described elsewhere [29]. RNA preparation and DNAse treatment were as before.

We used quantitative real-time RT-PCR with internal fluorescent hybridization probes in ABI Prism 7700 Detection System (ABI/PerkinElmer Biosystems), which allows the sensitive and specific quantification of individual host, as well as M. tuberculosis RNA, transcripts [29]. A target-specific RT primer, PCR primers, and probes for each assay were either designed according to specifications recommended by ABI/PerkinElmer Biosystems using Primer Express software or were as described elsewhere [29, 32] (table 1). All probes were dually labelled with FAM (5-carbofluorescein) at the 5’ end and TAMRA (N,N,N',N'-tetramethyl-6-carborhodamine) at the 3’ end. The proximity of the dye (FAM) and the quencher (TAMRA) on the intact probe prevents detection of any fluorescence. However, degradation of the probe during the course of PCR allows the release and detection of FAM [33]. The PCRs for all amplifications were similar: 5 µL of each cDNA, 20 µL of Taqman Universal PCR Master Mix (PE Biosystems), which contains optimal amounts of AmpliTaq Gold DNA polymerase (which protects against amplicon carryover) and of dNTPs, and optimal amounts of probe and primers calibrated to allow measurement of the targets. First, cDNA was synthesized in the
presence of 0.5 μL of murine leukemia virus enzyme (Invitrogen)/reaction and 10 μmol/L each RT primer, dNTPs, and other substrate. Conditions for PCR were similar for all products (1 cycle of 2 min at 50°C and 1 cycle of 10 min at 95°C and then 40 cycles of 15 s at 95°C and 1 min at 60°C). The cycle threshold for each sample was compared with the cycle threshold values of known amounts of a standard DNA constructed for each target and amplified simultaneously. To assure lack of DNA contamination in the RNA samples, in some experiments, a duplicate tube of sample with no RT enzyme was included as control. DNA contamination remained negligible. Expression of TNF-α mRNA was corrected to host 18S rRNA in the same sample and was expressed as copies of TNF-α in 10^10 copies of R18 (equivalent to 1 × 10^6 cells). M. tuberculosis 85B mRNA was corrected to mycobacterial 16S rRNA and expressed as 85B:16S. Data are mean + SEM of 5 experiments.

Measurement of TNF-α and M. tuberculosis 85 complex protein by ELISA. The TNF-α content of culture supernatants was determined by use of a commercial ELISA (R&D Systems), according to the manufacturer’s specifications. The lower limit of sensitivity was 4.4 pg/mL.

An ELISA for detection of the mycobacterial 85 complex (includes M. tuberculosis 85A, B, and C proteins) was developed using the monoclonal antibody to mycobacterial 85 complex (CS-90; available from Colorado State University, Fort Collins) as coating and a rabbit anti–Mycobacterium bovis antibody (DAKO) as detection reagent [34]. Purified 85 complex antigen (also available from Colorado State University) was used as a standard. This assay has a lower limit of sensitivity of 50 pg/mL and does not detect other M. tuberculosis products (purified M. tuberculosis 19-kDa and 45-kDa antigens, or lipoarabinomannan).

Statistical analysis. Results were analyzed by use of Mann-Whitney rank sum and paired t tests, and linear correlation and regression analysis were performed where needed. P < .05 was considered to be significant.

RESULTS

Expression of TNF-α and M. tuberculosis mRNA in M. tuberculosis–infected AMs. First, we investigated the kinetics (0–120 h) of expression of TNF-α and M. tuberculosis 85B mRNA in M. tuberculosis H37Ra–infected AMs. TNF-α mRNA was corrected to host 18S rRNA in the same sample, and M. tuberculosis 85B mRNA was expressed as 85B:16S. Expression of TNF-α was maximal at 24 h (P < .05) and thereafter decreased (figure 1A). Expression of M. tuberculosis 85B continued to increase up to 120 h (figure 1B); at 4 h, M. tuberculosis 85B:16S was 3-fold higher (P < .05), and, at 24 h, it was 5-fold higher (P < .01), compared with levels in t₀ cultures. Furthermore, between 4 and 24 h, M. tuberculosis 85B:16S increased significantly (P < .01). In contrast to the increase in intracellular expression of M. tuberculosis 85B:16S, it did not increase when bacteria (10^4–10^7 cfu) were cultured for up to 120 h in medium alone (in the absence of cells). Of importance, the early levels of expression of TNF-α mRNA and M. tuberculosis 85B:16S (at t₀ and 4 h) correlated with one another (r² = 0.6, P < .001). In some experiments (n = 4), the intracellular growth of M. tuberculosis was assessed in duplicate wells of M. tuberculosis–infected AMs (by the colony-forming unit assay) at t₀ and at 4 days. The increase in intracellular growth of M. tuberculosis at 4 days correlated with intracellular M. tuberculosis 85B:16S at 24 h (r² = 0.93; P < .05).

To ascertain that the intracellular changes in expression of M. tuberculosis 85B were not attributable to any extracellular bacteria left after extensive washing of cultures or released from
cells during 24 h, in some experiments, culture supernatants of *M. tuberculosis* H37Ra-infected AMs (at t₀ and 4 and 24 h) also were collected and centrifuged in the presence of 10% polyethylene glycol 8000 (Sigma), and *M. tuberculosis* RNA was extracted in the presence of excess yeast RNA (1 M). The concentration of 85 complex in 24-h culture supernatants was 592 ± 182 pg/mL (n = 12). As noted elsewhere [34], *M. tuberculosis* 85B is a component of *M. tuberculosis* antigen 85 complex, and changes in the 85 complex immunoreactivity in supernatants likely reflect changes in 85B.

To ascertain that expression of the *M. tuberculosis* 85B gene was not divergent in H37Rv- and H37Ra-infected AMs, we compared expression of TNF-α and *M. tuberculosis* 85B in the same experiment, using AMs infected with either strain of *M. tuberculosis*, from 3 donors. Again, despite differences in levels of expression of TNF-α and *M. tuberculosis* 85B, the patterns of gene expression were similar. At 4 h, the level of TNF-α mRNA induced by H37Rv was 12–20 times higher than that induced by H37Ra, and the level of *M. tuberculosis* 85B induced by H37Rv was >6 times higher than that induced by H37Ra. At 24 h, levels of both TNF-α and *M. tuberculosis* 85B induced by H37Rv were higher than those induced by H37Ra. Thus, virulent *M. tuberculosis* strains induce higher levels of both TNF-α and *M. tuberculosis* 85B in AMs.

**Modulation by sTNFRs of expression of TNF-α and *M. tuberculosis* 85B gene in *M. tuberculosis*-infected AMs.** On the basis of results presented in the previous section, we hypothesized that endogenous *M. tuberculosis*-induced TNF-α in cultures of AMs may contribute to modulation of expression of the *M. tuberculosis* 85B gene during the initial 24 h after infection. To assess the effect of TNF-α on expression of *M. tuberculosis* 85B in AMs early after *M. tuberculosis* infection, we inhibited TNF-α signaling by use of sTNFRI and sTNFRII. In preliminary experiments, we defined the optimal dose of each reagent to inhibit TNF-α. H37Ra-infected AMs received either sTNFRI (10 ng/mL), sTNFRII (10 ng/mL), or medium alone for 4 and 24 h. *M. tuberculosis*-induced TNF-α mRNA was down-regulated significantly in AMs by sTNFRI and sTNFRII at 4 h (P < .001 for both) and at 24 h (P < .01 for both) (figure 2). Both soluble receptors also significantly decreased expression of *M. tuberculosis* 85B in AMs (P < .05 at 4 h and P < .02 at 24 h, for both). Despite the fact that sTNFRI appeared to be stronger than sTNFRII in inhibition of expression of TNF-α and *M. tuberculosis* 85B gene in AMs, these differences were not significant. In addition, in a few experiments, we found that the co-presence of the 2 sTNFRs was not synergistic in inhibition of expression of TNF-α or 85B:16S (data not shown).

Because the results presented in the previous section indicated that H37Rv was stronger in induction of both TNF-α and 85B mRNA, we also examined the effect of neutralization of TNF-α (by sTNFRI or sTNFRII) on expression of either in AMs infected with H37Rv. Neutralization of TNF-α (by s-
TNFRI or sTNFRII) reduced expression of TNF-α and M. tuberculosis 85B in H37Rv-infected AMs (data not shown).

Effect of exogenous TNF-α on activation of TNF-α and expression of M. tuberculosis 85B gene in M. tuberculosis–infected AMs. The above data suggested a role for endogenous TNF-α in induction of both its own gene and that of M. tuberculosis. To further confirm the role of TNF-α in early induction of the M. tuberculosis 85B gene, exogenous TNF-α was added to H37Ra-infected AMs, and then TNF-α mRNA and M. tuberculosis 85B were assessed. In these experiments, rTNF (0.2, 2, and 20 ng/mL) or medium alone was added to M. tuberculosis–infected AMs. At 24 h, rTNF (at 2 and 20 ng/mL) increased expression of the TNF-α gene by 2–4-fold (P < .05) and increased expression of the M. tuberculosis 85B gene by 3–8-fold (P < .01 and P < .001 for both) (figure 3). Therefore, cellular activation by exogenous TNF-α induces expression of both TNF-α and M. tuberculosis 85B genes in AMs.

TNF-α is only 1 of a multitude of proinflammatory cytokines released by mononuclear phagocytes after mycobacterial infection. Other macrophage-activating cytokines include interleukin (IL)—1β, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF); however, their release follows different kinetics [35]. Therefore, we compared induction of M. tuberculosis 85B by these cytokines (IL-1β, IL-6, and GM-CSF) with that by TNF-α. M. tuberculosis 85B was induced by rGM-CSF and rIL-6 but not by rIL-1β (figure 4). Induction of M. tuberculosis 85B by rIL-6 and rGM-CSF was not significantly different from that by TNF-α. When 24-h culture supernatants of M. tuberculosis–infected AMs were assessed for these cytokines, the immunoreactivity of TNF-α was 4-fold higher than that of GM-CSF and 1.7-fold higher than that of IL-6. Thus, a number of proinflammatory cytokines may induce expression of M. tuberculosis 85B in M. tuberculosis–infected AMs; however, TNF-α appears to be the leading cytokine during the initial 24 h of infection.

Increased expression of TNF-α and M. tuberculosis 85B gene by activation of NF-κB in AMs by M. tuberculosis. Activation of cells by bacterial LPS [36], mycobacteria [37, 38], and cytokines, including TNF-α [20], is mediated via NF-κB. To assess the role of NF-κB in activation of gene expression in M. tuberculosis–infected AMs, the NF-κB inhibitor, SN50 [39], was used. This hybrid peptide contains the nuclear localization sequence of the p50 subunit of the NF-κB heterodimer and has been shown to completely inhibit the translocation of NF-κB in human cell lines at 100 μg/mL [39]. SN50 (100 μg/mL) was added to AM cultures 3 min before infection with M. tuberculosis H37Ra. Control cultures did not receive SN50. After infection, cells were washed rigorously and cultured in medium...

Figure 3. Effect of recombinant (r) tumor necrosis factor (TNF)–α on expression of TNF-α and Mycobacterium tuberculosis 85B. Alveolar macrophages were infected with M. tuberculosis H37Ra (1:1 bacteria/cell) and cultured with rTNF-α (0.2–20 ng/mL) for 4 and 24 h. Total RNA was extracted and assessed for TNF-α mRNA (A) and M. tuberculosis 85B:16S (B). Data are mean + SEM of 4 experiments. *P < .05, vs. medium; **P < .01, vs. medium.

Figure 4. Effect of proinflammatory cytokines on Mycobacterium tuberculosis 85B. Alveolar macrophages were infected with M. tuberculosis H37Ra (1:1 bacteria/cell) and cultured with recombinant tumor necrosis factor–α (●), recombinant interleukin (IL)–6 (▲), recombinant granulocyte-macrophage colony-stimulating factor (▼), or recombinant IL-1 (○) (0.2–20 ng/mL) for 24 h. Total RNA was extracted and assessed for M. tuberculosis 85B:16S. Results of 1 of 3 similar experiments are shown.
alone for 4 h; SN50 reduced expression of both TNF-α mRNA (P < .005) and *M. tuberculosis* 85B:16S (P < .05) in AMs (figure 5). To assure that cellular inhibition was not nonspecific, in some experiments, we compared the effect of SN50 (100 μg/mL) with its inactive analogue, SN50/M (100 μg/mL). SN50/M did not affect expression of either TNF-α or *M. tuberculosis* 85B (data not shown). Therefore, the increased expression of 85B and TNF-α mRNA in *M. tuberculosis*-infected AMs is mediated via activation of NF-κB.

**Role of RNI and ROI in expression of TNF-α and *M. tuberculosis* mRNA in *M. tuberculosis*-infected AMs.** After stimulation of mononuclear phagocytes by a variety of substances, including microbes and their products, ROI and RNI pathways are produced. The activation of RNI and ROI pathways are further potentiated by macrophage-activating cytokines [40, 41]. To assess the effect of activation of ROI and RNI on expression of the *M. tuberculosis* 85B gene, we used specific inhibitors of each pathway, NAC and NMMA. In some experiments, oATP, which inhibits both pathways [31], was used. After infection of AMs with *M. tuberculosis* H37Ra, NAC (10 nmol/L), NMMA (10 nmol/L), or oATP (10 nmol/L) was added to cultures. Control cultures received medium alone. NAC, NMMA, and oATP significantly reduced expression of both TNF-α mRNA and *M. tuberculosis* 85B mRNA at both 4 and 24 h (figure 6). At 4 h, expression of TNF-α was reduced by 1–2 logs (P < .001). At 24 h, reduction in expression of TNF-α by NAC, NMMA, or oATP was comparable (∼3 logs; P < .001 for all). NAC, NMMA, and oATP also reduced expression of *M. tuberculosis* 85B at both 4 (P < .05 for all) and 24 h (P < .01 for NAC; P < .001 for NMMA and oATP). In a single experiment, we ascertained that the reduction in expression of TNF-α and *M. tuberculosis* 85B by these inhibitors was also demonstrable in AMs infected with H37Rv.

To confirm the above observations, in some experiments (n = 3), classic agents that increase formation of oxygen radicals (NADPH or H₂O₂), or the nitric oxide (NO) donors (SNP or NOC-9), were added to H37Ra-infected AMs during and after infection. Control AM cultures received oxygen radicals or NO donors but were not infected. The oxygen radicals and NO donors significantly increased the expression of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected AMs (figure 7). Oxygen radicals and NO donors also increased TNF-α mRNA in uninfected AMs, but to low levels (10⁵–10⁶ copies/sample) (data not shown).

**DISCUSSION**

The initial interaction of *M. tuberculosis* and host mononuclear phagocytes at sites of *M. tuberculosis* infection is critical to intracellular parasitism of *M. tuberculosis*. Intense cellular activation and proinflammatory cytokine release, predominated by TNF-α, occurs and may affect the pathogenesis of *M. tuberculosis*. Of the ~12 *M. tuberculosis* genes shown to be up-regulated after *M. tuberculosis* infection of human macrophages, most of which are essential components of bacterial metabolism and stress adaptation, *M. tuberculosis* 85B was expressed most frequently [30]. However, the role of 85B in the pathogenesis of *M. tuberculosis* in humans is not clear. Here, the relationship between expression of TNF-α and *M. tuberculosis* 85B mRNA during the first 24 h of infection of AMs with *M. tuberculosis* has been examined.

On the basis of results of previous studies examining kinetics of induction of expression of TNF-α mRNA and levels of TNF-α [2, 14], we hypothesized that this cytokine may play a major role in the innate immune response early after *M. tuberculosis* infection of human macrophages. In support of this hypothe-

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**Figure 5.** Effect of inhibition of NF-κB on expression of tumor necrosis factor (TNF)-α and *Mycobacterium tuberculosis* 85B. Alveolar macrophages were infected with *M. tuberculosis* H37Ra (1:1 bacteria/cell) in the presence or absence (−) of SN50 (100 μg/mL). Total RNA was extracted at 4 h and was assessed for expression of TNF-α (A) and *M. tuberculosis* 85B:16S (B). Data are mean ± SEM of 3 experiments. **P < .05, vs. medium; **P < .01, vs. medium.
sis, we have found that induction of TNF-α and *M. tuberculosis* 85B mRNA expression corresponded with each other at $t_0$ and 4 h, which represent time points of initial mononuclear cell activation and release of TNF-α by *M. tuberculosis* infection. The more-virulent *M. tuberculosis* strain (H37Rv) induced greater expression of TNF-α and mycobacterial 85B mRNA than did the less-virulent *M. tuberculosis* strain (H37Ra). Thus, stronger cell activation (as reflected by induction of TNF-α) was associated with a higher induction of expression of the *M. tuberculosis* 85B gene. We found that *M. tuberculosis* infection induced expression of both TNF-α and *M. tuberculosis* 85B and that neutralization of TNF-α by sTNFRI and sTNFRII reduced expression of either gene. Furthermore, exogenous TNF-α significantly induced expression of both TNF-α and *M. tuberculosis* 85B at both 4 and 24 h in AMs. Some other proinflammatory cytokines (IL-6 and GM-CSF, but not IL-1β) produced early after *M. tuberculosis* stimulation by macrophages also induced *M. tuberculosis* 85B:16S. However, after immunoassay, they were less abundant than TNF-α in *M. tuberculosis*-infected AM cultures. Cumulatively, these data suggest a strong association between the proinflammatory cytokine response and expression of the *M. tuberculosis* 85B gene during the initial 24 h of infection of human AMs. The maintenance of expression of *M. tuberculosis* 85B at time points after 24 h (figure 1), when TNF-α activity is reduced but expression of *M. tuberculosis* 85B is high, suggests the involvement of alternative mechanisms.

![Figure 6](image1.png)  
**Figure 6.** Inhibition of expression of tumor necrosis factor (TNF)-α and *Mycobacterium tuberculosis* 85B by inhibition of reactive oxygen and reactive nitrogen pathways. Alveolar macrophages were infected with *M. tuberculosis* H37Ra (1:1 bacteria/cell) and cultured with 10 mmol/L N-acetyl cysteine (striped bars), N⁵-monomethyl-L-arginine monoaacetate (hatched bars), or oxidized ATP (gray bars) or medium alone (black bars) for 4 and 24 h. Total RNA was extracted and assessed for expression of TNF-α (A) and 85B:16S (B). Data are mean ± SEM of 4 experiments. *P < .05, vs. *M. tuberculosis* alone; **P < .01, vs. *M. tuberculosis* alone; ***P < .001, vs. *M. tuberculosis* alone.

![Figure 7](image2.png)  
**Figure 7.** Induction of expression of tumor necrosis factor (TNF)-α and *Mycobacterium tuberculosis* 85B by oxygen radicals or nitric oxide donors. Alveolar macrophages were infected with *M. tuberculosis* H37Ra (1:1 bacteria/cell) in the absence (−) or presence of H₂O₂ (10 nmol/L), NADPH (5 μmol/L), nitroate-9 (NOC-9; 300 μmol/L), or sodium nitroprusside (SNP; 0.5 μmol/L). Cultures were washed at 90 min and maintained in medium alone for 4 h. Total RNA was extracted and assessed for expression of TNF-α (left) and 85B:16S (right). Data are mean ± SEM of 3 experiments. *P < .05, vs. *M. tuberculosis* alone; **P < .01, vs. *M. tuberculosis* alone. HP, hydrogen peroxide.
has to be noted that expression of the TNF-α gene and production of its protein are under strong intracellular control mechanisms. Expression of the *M. tuberculosis* 85B gene likely follows the unrestrained intracellular growth of *M. tuberculosis*. However, an effect of cytokines released by *M. tuberculosis*-infected AMs on intracellular or even extracellular (as macrophages undergo lysis) expression of *M. tuberculosis* gene throughout infection of mononuclear phagocytes needs to be considered.

Induction of expression of TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected AMs was mediated through activation of NF-κB, because both mRNAs were suppressed when SN50, an inhibitor of NF-κB, was present in cultures. The analogue of SN50 did not have any effect. Thus, cellular activation is associated with augmentation of expression of both TNF-α and *M. tuberculosis* 85B in *M. tuberculosis*-infected AMs.

After phagocytosis of microbes and cellular activation, ROIs are activated [21]. One product of the ROI pathway, H₂O₂, activates expression of iNOS and production of NO [22]. On the other hand, both ROI and RNI are downstream mediators of macrophage-activating cytokines and are thought to be microbicidal. Activation of iNOS and production of NO may be important in the final containment of *M. tuberculosis* by macrophages [42]. However, *M. tuberculosis* has evolved resistance mechanisms against both ROI [43, 44] and RNI [45, 46]. Here, when the activation of RNI and ROI was inhibited by NMMA, NAC, or oATP, the expression of both TNF-α and *M. tuberculosis* 85B was significantly lowered in *M. tuberculosis*-infected AMs at 24 h. We had previously found that these treatments had no effect on *M. tuberculosis*-infected monocytes [29]. We confirmed these observations by using agents that release oxygen radicals (NADPH and H₂O₂) or NO (SNP or NOC-9).

In *M. tuberculosis*-infected AMs, these reagents increased expression of both TNF-α and *M. tuberculosis* 85B. Thus, both RNI and ROI, induced early after *M. tuberculosis* infection of AMs, activate expression of *M. tuberculosis* gene in turn. Moderate-to-high production of NO has been reported in AMs from 2–3 healthy donors after stimulation with *M. tuberculosis* [47], and ROI activates NF-κB [22].

*M. tuberculosis* 85B is a predominant protein [25] during human *M. tuberculosis* infection; however, its role in the pathogenesis of *M. tuberculosis* infection is not clear. At least with regard to mycolyl transferase activity, which underlies cell-wall biosynthesis, it appears that both *M. tuberculosis* 85A and 85B are redundant [48]. In sputum from patients with tuberculosis, levels of *M. tuberculosis* 85B protein and mRNA correlate with *M. tuberculosis* growth, and maintenance of 85B levels correlates with a lack of response to therapy [34, 49]. Studies using the human myelo-monocytic cell line, THP-1, have shown that 85B may be dispensable for intracellular growth of *M. tuberculosis* [50]. However, the activation state of primary macrophages is different from tumor cell lines [51], and, despite the fact that THP-1 cells have been used in studies of macrophage cytokine expression [52], their secretory capacities are different from primary macrophages (Z.T., unpublished data). Therefore, studies of relevance of 85B to pathogenesis of *M. tuberculosis* need to be extended to primary human macrophages. Interestingly, although many mycobacterial components induce TNF-α in mononuclear phagocytes [53–55], only the members of 85 complex interact with host fn [26]. Moreover, binding of 85B to fn enhances the expression of TNF-α in monocytes [28]. Therefore, the role of *M. tuberculosis* 85B in intracellular infection may be the maintenance of an inflammatory response. It is likely that other fn-binding proteins of *M. tuberculosis* increase proinflammatory cytokines as well. In addition, *M. tuberculosis* 85 complex may act as an intermediary to synthesis of trehalose dimycolate, which enhances the host inflammatory response [56]. A vicious circle may exist in which expression of host inflammation and mycobacterial products amplify one another.

In summary, *M. tuberculosis* infection of AMs leads to a concomitant activation of TNF-α and expression of *M. tuberculosis* 85B gene. Although the intracellular induction of expression of *M. tuberculosis* 85B may be merely a marker for a set of *M. tuberculosis* genes associated with initial mycobacterial survival, an intense interaction of *M. tuberculosis* with host AMs is suggested by our data. *M. tuberculosis* infection of lung macrophages initiates a cascade of events whereby cellular activation by TNF-α, RNI, and ROI enhances the expression of *M. tuberculosis* 85B in AMs. Further studies on the initial interplay of human macrophages and *M. tuberculosis* will help delineate the contribution of this early host-pathogen interaction to the pathogenesis of tuberculosis.

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References


