Recombinant Guinea Pig Tumor Necrosis Factor Alpha Stimulates the Expression of Interleukin-12 and the Inhibition of Mycobacterium tuberculosis Growth in Macrophages

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Tumor necrosis factor alpha (TNF-α) plays an important role in the host immune response to infection with the intracellular pathogen Mycobacterium tuberculosis. It is essential for the formation of protective tuberculous granulomas and regulates the expression of other cytokines which contribute to a protective immune response. Interleukin-12 (IL-12) is known to promote a Th1 response, which is essential for antimiycobacterial resistance. Recombinant guinea pig TNF-α (rgpTNF-α) protein (17 kDa) was purified, and its bioactivity was confirmed by its cytotoxicity for L929 fibroblasts. High titers of polyclonal anti-gpTNF-α antibody were obtained by immunization of rabbits. Resident alveolar and peritoneal macrophages were isolated from guinea pigs and infected with either the H37Ra or H37Rv strain of M. tuberculosis. The mRNA levels for TNF-α and IL-12 p40 were measured using real-time PCR. IL-12 p40 mRNA was up-regulated in a dose-dependent manner by rgpTNF-α alone. In infected macrophages, a lower dose of rgpTNF-α intensified the mRNA levels of TNF-α and IL-12 p40. However, higher doses of rgpTNF-α suppressed TNF-α and IL-12 p40 mRNA. The antimiycobacterial activity of macrophages was assessed by metabolic labeling of M. tuberculosis with [3H]uracil. Resident alveolar and peritoneal macrophages treated with anti-gpTNF-α antibody to block endogenous TNF-α exhibited increased intracellular mycobacterial growth. These data suggest that the dose of TNF-α is crucial to the stimulation of optimal expression of protective cytokines and that TNF-α contributes to the control of mycobacterial replication to promote host resistance against M. tuberculosis.

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Tuberculosis (TB) remains a global health crisis because Mycobacterium tuberculosis kills approximately 2 million people per year, more than any other single bacterial pathogen. Also, over 2 billion people may carry the organism in a dormant or latent state that could result in reactivation disease (29). The human immune response against M. tuberculosis is highly effective in controlling the primary infection, but the organism is almost never eradicated (1, 42). Reactivation of the latent M. tuberculosis infection occurs frequently in people who are immunosuppressed as a result of diseases such as AIDS (21). Vaccination against M. tuberculosis has been shown to require activated Th1 cells that induce the expression of Th1-type cytokines, e.g., interleukin-12 (IL-12) and gamma interferon (IFN-γ) (45). In contrast, Th2 cytokines, e.g., IL-4 and IL-10, have been shown to be associated with progressive disease (51). Th1 cells secrete IL-2, their main T-cell growth factor, and IFN-γ, an important activating signal for macrophages which is also required for the formation of protective granulomas along with tumor necrosis factor alpha (TNF-α) (20). The production of IFN-γ is stimulated by IL-12 (5) and suppressed by IL-10 (16) and transforming growth factor β (12), all of which are released from activated macrophages in mice.

TNF-α is a pleiotropic cytokine produced primarily by monocytes and macrophages. The activities of TNF-α are mediated by specific cell surface receptors. Ligand-mediated homo- or heterotrimersiation causes recruitment of several intracellular adaptor proteins, which subsequently activate multiple signal transduction pathways. The recruitment of a death domain results in apoptosis. However, the recruitment of TRAF family proteins leads to the activation of transcriptional factors, such as NF-κB, which promote cell survival and differentiation as well as immune and inflammatory responses (10).

TNF-α also plays an important role in the host immune response to infection with M. tuberculosis. TNF-α is known to be essential for the formation of protective tuberculous granulomas, which serve to control bacterial infection (47). TNF-α-receptor-knockout mice showed decreased survival and disrupted granuloma formation compared to wild-type mice following infection with virulent M. tuberculosis (19). In addition, TNF-α-deficient mice became highly susceptible to reactivation of TB infection (6, 41).

IL-12 is a disulfide-linked heterodimer, IL-12 p70, which consists of IL-12 p35 and IL-12 p40. IL-12 is released from antigen-presenting cells (e.g., macrophages and dendritic cells) by various microbial stimuli including mycobacteria. One chain, p35, is ubiquitously produced, whereas p40 is inducible and detected only in cell types producing biologically active IL-12 (32). IL-12 has been shown to be crucial to the devel-
opment of protective immunity against TB in mouse and human studies. IL-12-deficient mice showed an increased susceptibility to *M. tuberculosis* infection and a decrease in IFN-γ production (7). IL-12 injection into susceptible mice enhanced the survival rate and reduced bacterial numbers following infection with *M. tuberculosis* (44). In a clinical study, IL-12 has been shown to act as an effective adjuvant in patients with TB (22).

In previous studies, we found that *Mycobacterium bovis* BCG vaccination increased bioactive TNF-α responses in guinea pig leukocyte populations (36). To further elucidate the role of TNF-α, we produced recombinant guinea pig TNF-α (rgTNF-α) in an *Escherichia coli* expression system. Polyclonal anti-gpTNF-α antiserum was obtained by immunization of rabbits with rgpTNF-α and showed a neutralizing effect on the bioactivity of rgpTNF-α. Treatment of splenocytes with rgpTNF-α suppressed purified protein derivative-induced lymphoproliferation, while anti-rgpTNF-α treatment resulted in hyperproliferation (35).

In this study, we investigated the effect of rgpTNF-α on mRNA levels of TNF-α and IL-12 p40 in resident alveolar and peritoneal guinea pig macrophages either uninfected or infected with *M. tuberculosis*. Also, the effect of anti-gpTNF-α antibody on intracellular bacterial growth was examined in both macrophage populations.

**MATERIALS AND METHODS**

Production of rgpTNF-α. The cloning of gpTNF-α cDNA was previously described (52). The subcloning and expression of the gpTNF-α gene followed a previously published protocol from our laboratory (35). The amplified gpTNF-α product was ligated into the plasmid pQE-30 (Qiagen, Valencia, Calif.) expression vector. *E. coli* strain M15 transformed with pQE-30/gpTNF-α was grown to mid-log phase and induced with 1 mM IPTG (isopropyl-

weight. To isolate alveolar macrophages, bronchoalveolar lavage (BAL) was performed by cannulating the trachea and washing the lungs five times with 10 ml of 12 mM lidocaine in RPMI 1640 with 2% heat-inactivated fetal bovine serum (FBS). To isolate resident peritoneal macrophages, the peritoneal cavity was washed twice with 30 ml of RPMI 1640 containing 10 U of heparin/ml and 2% FBS. Cells from BAL fluid were resuspended at 10^6 cells/ml and peritoneal cells were resuspended at 1.5 × 10^6 cells/ml in RPMI 1640 with 10% FBS medium.

**Macrophage stimulation and infection.** Five hundred microliters of each cell suspension from BAL fluid and the peritoneal cavity was plated on 48-well tissue culture plates and allowed to adhere for 1.5 h at 37°C in a 5% CO₂ incubator. Nonadherent cells were washed off, and adherent macrophages were stimulated with 10, 50, or 200 ng of rgpTNF-α/ml alone or in combination with live attenuated *M. tuberculosis* H37Rv or live virulent *M. tuberculosis* H37Rv (multiplicity of infection [MOI], 1:100). Another set of macrophages was infected without the addition of exogenous rgpTNF-α. At 3, 6, and 12 h after stimulation or infection, supernatants were obtained from each of the culture wells, centrifuged at 12,000 × g, and stored at −80°C until analyzed by the L929 bioassay. Cells were lysed with buffer RTL (Qiagen) and frozen at −80°C.

To investigate intracellular mycobacterial growth, each macrophage population was infected with live attenuated *M. tuberculosis* H37Rv or live virulent *M. tuberculosis* H37Rv (MOIs of 1:1 and 1:10) in antibiotic-free RPMI 1640 with 10% FBS. After 3 h of infection, extracellular mycobacteria were washed off with medium containing 200 μg of gentamicin/ml and macrophage cultures were incubated with or without anti-TNF-α polyclonal antibody (1:1,000) and medium containing gentamicin and 10% FBS for 4 to 7 days. The cultures were pulsed with 1 μCi of [³H]uracil for 24 h. Before harvesting, mycobacteria were killed by incubating the plate at 80°C for 30 min and harvested using a FiterMate Cell Harvester. [³H]Uracil incorporation was measured in a scintillation counter (Beckman LS-1801).

**Total RNA isolation and real-time PCR.** Total RNA was isolated using the RNeasy kit from Qiagen. Reverse transcription was performed using Taqman reverse transcription reagents (Applied Biosystems). Real-time primers for gpTNF-α, IL-12 p40, and hypoxanthine phosphoribosyltransferase (HPRT) were designed using Primer Express software and are described in Table 1. Quantitative real-time PCR was performed using SYBR Green PCR Supermix and the ABI Prism 7700 sequence detector from Applied Biosystems according to our previously published protocol (38). Fold induction levels of mRNA were obtained by analyzing cycle threshold (Ct) levels normalized to HPRT Ct values.

**Bioassay of TNF-α.** The bioassay for TNF-α was performed according to our published procedure (36). Briefly, purified rgpTNF-α or culture supernatants were assessed for TNF bioactivity by measuring their cytotoxicity on L929 cells. L929 cells were suspended in RPMI 1640 without phenol red supplemented with 2 μM l-glutamine (Gibco Life Technologies), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2% FBS at 4 × 10^5 cells/ml. One hundred microliters of L929 cell suspension (American Type Culture Collection) was seeded into 96-well flat-bottomed plates and incubated overnight in a CO₂ incubator at 37°C. On the following day, 50 μl of serially diluted samples and 50 μl of an 8 μg/ml actinomycin D solution (final concentration, 2 μg/ml) were added to each well and incubated for an additional 20 h. A tetrazolium reagent, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2-H-tetrazolium salt (WST-1; Dojindo, Kamakura, Japan), and 1-methoxyethyl phenazin methysulfate (Dojindo) were dissolved at 0.6 and 0.4 mM, respectively, in phosphate-buffered saline. These were mixed at a ratio of 1:1, and 20 μl was added to each well. The cells were incubated for 2 h in a CO₂ incubator at 37°C to allow the color to develop, and 25 μl of 1 N H₂SO₄ was added to stop further development. The optical density at both 450 nm (OD₄₅₀) and 630 nm (OD₆₃₀) in each well was measured with a microplate reader for the test and reference wavelengths. The net change (net OD₄₅₀ − OD₆₃₀) for each well was calculated by the following equation: net

<table>
<thead>
<tr>
<th>Primer*</th>
<th>mRNA</th>
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<td>F, forward; R, reverse.</td>
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<table>
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<tr>
<th>primer</th>
<th>F primer</th>
<th>R primer</th>
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<tr>
<td>TNF-α</td>
<td>5′ CTAACCTGCTTCTCACCCATACCC 3′</td>
<td>5′ R GATGCGAGAAAGTGTGA 3′</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>5′ GGTCTAGATGTTGATTTCCACACAAA 3′</td>
<td>5′ TCCTCAGACGTTGCAGGT 3′</td>
</tr>
<tr>
<td>HPRT</td>
<td>5′ AGGTGGTTATACCCCTATCGACTAATT 3′</td>
<td>5′ CTCCTCCTCTCCTTCATCAT 3′</td>
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*Table 1. Primer sequences used for quantitative PCR.
Bioactivity of rgpTNF-α and neutralization of the bioactivity by anti-gpTNF-α antibody. rgpTNF-α was expressed and purified as described in Materials and Methods. The biological activity of rgpTNF-α was measured by its cytotoxic effect on L929 fibroblasts. Figure 1 shows the dose-dependent cytotoxicity of rgpTNF-α on L929 cells. Equivalent concentrations of recombinant human TNF-α and rgpTNF-α showed almost the same level of bioactivity. Since the reactivity of polyclonal rabbit anti-gpTNF-α antisera with rgpTNF-α has been previously demonstrated (35), we decided to determine its neutralization potential by testing various dilutions of antisera in the presence of either rgpTNF-α or recombinant human TNF-α as a specificity control. Figure 2 shows that increasing concentrations of antisera decreased residual bioactivity of rgpTNF-α. Finally, the bioactivity of 50 ng of rgpTNF-α/ml was completely neutralized by the range of 1:5,000 to 1:2,500 dilutions of antisera. In contrast, this antisera failed to neutralize the bioactivity of recombinant human TNF-α.

Effect of rgpTNF-α on cytokine mRNA expression in uninfected guinea pig alveolar and peritoneal macrophages. Both alveolar and peritoneal macrophages were stimulated with 10, 50, and 200 ng of rgpTNF-α/ml. The expression of TNF-α and IL-12 p40 mRNA was examined by real-time qualitative reverse transcription-PCR at 3, 6, and 12 h after stimulation, and the results are shown in Fig. 3. In alveolar macrophages, the induction of TNF-α mRNA (Fig. 3A) by various concentrations of rgpTNF-α was moderate during the poststimulatory phase but increased significantly at 6 h of culture with 200 ng of rgpTNF-α/ml. IL-12 p40 mRNA expression in alveolar macrophages (Fig. 3B) increased dramatically in a dose-dependent manner and also peaked at 6 h. Levels of IL-12 p40 mRNA were significantly higher in cultures stimulated with 200 ng of rgpTNF-α/ml compared to the 10-ng/ml dose. In peritoneal macrophages, TNF-α mRNA expression (Fig. 3C) peaked at 3 h with levels very similar to that from alveolar macrophages with significantly higher levels induced by the 200-ng/ml dose of rgpTNF-α compared to the lower doses. As with the alveolar macrophages, IL-12 p40 mRNA expression in peritoneal macrophages (Fig. 3D) started at 3 h, was clearly dose dependent, and reached levels approximately 10-fold higher than that from alveolar macrophages treated with the same concentrations of rgpTNF-α. Again, significantly higher levels of IL-12 p40 mRNA were induced by the highest dose of rgpTNF-α.

Effect of rgpTNF-α on cytokine mRNA expression in infected alveolar and peritoneal macrophages. Each macrophage population was infected with live M. tuberculosis H37Ra or H37Rv (MOI of 1:100) alone or with rgpTNF-α (50 and 200 ng/ml) for 3, 6, and 12 h. As before, total RNA from both macrophage populations was collected, and real-time PCR was performed to detect cytokine mRNA levels. Figure 4 shows the expression of TNF-α and IL-12 p40 mRNAs in both alveolar (Fig. 4A and B) and peritoneal (Fig. 4C and D) macrophages infected with the attenuated strain of M. tuberculosis (H37Ra). While infection with H37Ra induced the expression of TNF-α mRNA from infected alveolar macrophages (Fig. 4A), the addition of rgpTNF-α did not significantly alter its expression. Although the levels of IL-12 p40 mRNA were significantly elevated in alveolar macrophage cultures at 12 h, there was no significant difference between infected control cultures and the cultures with additional rgpTNF-α (Fig. 4B). By comparison, infected peritoneal macrophages expressed significantly higher levels of TNF-α mRNA at 6 h in the presence of 50 ng of rgpTNF-α/ml (Fig. 4C), and IL-12 p40 mRNA expression was intensified significantly by 50 ng of rgpTNF-α/ml at both 6 and 12 h of culture (Fig. 4D).

In separate experiments, we also investigated mRNA expression in macrophage cultures infected with live virulent M. tuberculosis H37Rv (MOI of 1:100) alone or in the presence of rgpTNF-α (50 and 200 ng/ml). These results are illustrated.
in Fig. 5. TNF-α mRNA expression from both alveolar (Fig. 5A) and peritoneal (Fig. 5C) macrophages showed similar patterns compared to those from each macrophage population infected with attenuated *M. tuberculosis* H37Ra. However, adding exogenous rgpTNF-α to the cultures significantly increased TNF-α mRNA expression in both cell types (Fig. 5A and C). The IL-12 p40 mRNA expression in alveolar macrophages infected with virulent mycobacteria was not affected by adding rgpTNF-α (Fig. 5B). However, expression of IL-12 p40 mRNA in peritoneal macrophages was significantly enhanced by the addition of 50 ng of rgpTNF-α/ml after 6 h of culture (Fig. 5D).

**Effect of neutralizing endogenous TNF-α on *M. tuberculosis* H37Ra or H37Rv growth in guinea pig alveolar and peritoneal macrophages.** Both alveolar and peritoneal macrophages were infected with live *M. tuberculosis* H37Ra (MOI of 1:1 or 1:10) for 3 h, extracellular mycobacteria were washed off, and infected macrophages were cultured for 4 to 7 days with or without a 1:1,000 dilution of polyclonal anti-gpTNF-α antibody. The antibody was added every other day to neutralize endogenous TNF-α. We confirmed that endogenous TNF-α production in these macrophage cultures was completely neutralized by our anti-gpTNF-α antibody using the L929 bioassay to test the treated cell culture supernatant fluids (data not shown). [3H]uracil incorporation by mycobacteria was measured on days 1, 4, and 7. Figure 6 illustrates the effect of anti-gpTNF-α on bacterial accumulation in alveolar (Fig. 6A and B) and peritoneal (Fig. 6C and D) macrophages. Figure 6A shows that the growth of attenuated *M. tuberculosis* H37Ra in alveolar macrophages was not influenced by anti-gpTNF-α antibody at an MOI of 1:1. However, Fig. 6B shows that anti-gpTNF-α antibody significantly increased the growth of attenuated *M. tuberculosis* H37Ra at an MOI of 1:10 on day 7. In peritoneal macrophages, seen as early as day 4, we detected a dramatic increase of mycobacterial growth at both infection ratios (MOI of 1:1 and MOI of 1:10) in the presence of anti-gpTNF-α antibody (Fig. 6C and D).

In Fig. 7, we examined the effect of anti-gpTNF-α antibody on virulent *M. tuberculosis* H37Rv growth in alveolar (Fig. 7A and B) and peritoneal (Fig. 7C and D) macrophage populations. Anti-gpTNF-α antibody exerted no effect on the growth of virulent mycobacteria at an MOI of 1:1 in either macrophage culture (Fig. 7A and C). However, at a lower MOI (1:10), mycobacterial growth was significantly increased by anti-gpTNF-α antibody at day 7 in alveolar macrophages (Fig. 7B). Accordingly, this significant increase in mycobacterial...
growth was also observed at both day 4 and day 7 in peritoneal macrophages (Fig. 7D).

DISCUSSION

TNF-α has been suggested to function as a regulatory factor in the host immune response to viral, parasitic, fungal, and bacterial infections. It has been shown to stimulate macrophage activation including the up-regulation of major histocompatibility complex II expression, cytokine expression (e.g., IL-1, IL-6, transforming growth factor β, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor), and chemokine expression (e.g., IL-8, MCP-1, RANTES, MIP-1β, and MIP-2) in human and mouse studies (8, 9, 27, 34). TNF-α and IL-12 act as two major mediators of proinflammatory responses by bridging the innate and adaptive immune responses. Therefore, their interactions could significantly influence macrophage activation and the development of antigen-specific immune responses. There are few studies that suggest a direct relationship between these two cytokines. TNF-α has been shown to inhibit IL-12 expression induced by lipopolysaccharide (LPS) and IFN-γ at the level of transcription of the p40 gene (39). IL-12 protein production in thiglycolate-elicited mouse macrophages was suppressed by TNF-α, and TNF-α-deficient mice showed a vigorous inflammatory response to heat-killed Corynebacterium parvum with high levels of IL-12 production (24, 40). In contrast, TNF receptor 1-deficient mice infected with M. bovis BCG were found to have a significant depression of IL-12 p40 mRNA in vivo and an inability of macrophages to produce IL-12 p40 protein in vitro (53). In another study, IL-12 production was blocked by anti-TNF-α antiserum (17). Therefore, the relationship between TNF-α and IL-12 is complex.

We observed that rgpTNF-α could induce IL-12 p40 expression directly at the mRNA level in guinea pig alveolar (Fig. 3B) as well as peritoneal (Fig. 3D) macrophages. Expression of TNF-α mRNA appeared a bit earlier than IL-12 p40 mRNA in peritoneal macrophages (Fig. 3C), which is consistent with the findings in murine listeriosis (37). In addition, bioactive TNF-α protein was also produced in macrophages stimulated with rgpTNF-α and showed a pattern similar to IL-12 p40 mRNA.
expression (data not shown). This suggests that TNF-α may play an important role in the induction of a protective Th1-cell-mediated immune response through enhancing IL-12 production. In another study (35), when vaccinated or naïve guinea pigs were challenged with virulent mycobacteria, BAL fluid cells and presenting cells (60% macrophages and 3% lymphocytes) produced much more bioactive TNF-α than did splenocytes (SPC) (50% lymphocytes and 10% macrophages). This suggests that macrophage populations are likely the main source of TNF-α in the guinea pig model. Therefore, as Zhan and Cheers (53) and others (17, 33) have suggested, it is possible that TNF-α released by macrophages may up-regulate IL-12 expression in an autocrine fashion, and TNF-α together with bacterial products may promote IL-12 induction. The increase of IL-12 p40 mRNA expression by 50 ng of rgpTNF-α/ml in infected peritoneal macrophages (Fig. 4D and 5D) clearly supports this hypothesis.

Actually, many biologic activities of TNF-α (e.g., induction of TNF-α and IL-12) are also properties of TLR ligands such as LPS. As the rgpTNF-α was made in gram-negative bacteria, it is critical to confirm the absence of LPS contamination. In this study, the 10 pg/μg endotoxin level in the rgpTNF-α is below the acceptable level in commercial recombinant cytokines (including TNF-α), which is 0.1 ng/μg. In addition, the highest concentration of rgpTNF-α used in our experiments was 200 ng/ml, which theoretically has 2 pg of LPS/ml. Other investigators have reported that less than 1 ng of LPS/ml is not biologically active. For example, Kaufmann et al. (28) showed that there is no production of cytokines (TNF-α and IL-6) or chemokines (MCP-1, IL-8, etc.) when human monocytes are exposed to low concentrations of LPS (0.01 to 1 ng/ml). Medvedev et al. (43) also reported that 1 ng of LPS/ml failed to stimulate the activation of extracellular signal-related kinases 1 and 2, Jun-regulated kinases 1 and 2, and p38 in mouse C3H/OUJ macrophages. These molecules are involved in cellular signaling pathways for the expression of various cytokines.

Although TNF-α is crucial to the protective immune response in TB, it also takes part in the pathogenesis of TB and other chronic diseases such as graft-versus-host disease (15), rheumatoid arthritis (30), and Crohn’s disease (46). High levels of TNF-α were associated with worsening of symptoms shortly after the initiation of tuberculostatic therapy in TB patients (4). The in vivo effects of TNF-α appear to be dose dependent, with low levels of the cytokine mediating protection against

FIG. 5. Expression of TNF-α and IL-12 p40 mRNA in guinea pig resident alveolar or peritoneal macrophages following infection with virulent M. tuberculosis H37Rv alone or with rgpTNF-α. Expression of TNF-α (A and C) and IL-12 p40 (B and D) mRNA was quantified in guinea pig alveolar (A and B) or peritoneal (C and D) macrophage cultures at 3, 6, and 12 h after infection with a live virulent (H37Rv) strain of M. tuberculosis alone at an infectivity ratio of 1:100 or in the presence of 50 or 200 ng of rgpTNF-α/ml. Fold induction was determined from the Ct values normalized for HPRT expression and then normalized to the values derived from unstimulated macrophage cultures at 0 h. Results are given as the means ± standard errors of the means of results from three experiments. Differences between the fold inductions from in vitro infected cultures were compared by analysis of variance (*, P < 0.05).
TB, whereas high concentrations provoke tissue damage (3). This double-edged role of TNF-α may explain why 50 ng of rgpTNF-α/ml stimulated IL-12 p40 mRNA expression, while 200 ng of rgpTNF-α/ml suppressed IL-12 expression in infected peritoneal macrophages (Fig. 4D and 5D). There was no significant difference in cell viability between macrophages stimulated with either dose of rgpTNF-α (50 and 200 ng/ml) and unstimulated macrophages by the lactate dehydrogenase assay (25) (data not shown). However, the possibility of apoptosis induced by rgpTNF-α was not ruled out by this assay. Therefore, we conclude that the dose effect observed represents a differential regulation of IL-12 p40 mRNA induction.

In the past, there has been some controversy surrounding the question whether attenuated or virulent mycobacteria induce higher levels of TNF-α (14). One recent study reported higher TNF-α induction by virulent strains in human macrophages (50). We also observed higher levels of TNF-α mRNA in guinea pig macrophages infected with the virulent strain (H37Rv) compared to the attenuated strain (H37Ra) of M. tuberculosis (Fig. 4A and C and 5A and C). In our previous study (36), we reported that virulent mycobacteria induced significantly more bioactive TNF-α from the same guinea pig macrophage populations.

The differential expression of cytokines between alveolar and peritoneal macrophages might be explained by different biological responses of two different cell populations (13, 48). Salez et al. (48) demonstrated that differentiation of mononuclear cells depends on the tissue environment: for example, the pulmonary environment allows alveolar macrophages to have different functions from peritoneal macrophages even though they derive from the same population of circulating monocytes. Our data show that guinea pig alveolar macrophages are less sensitive to stimulation by rgpTNF-α than are peritoneal macrophages. This might be due to tissue-specific characteristics of alveolar macrophages, which play a critical role in homeostatic processes of the lung.

The impact of TNF-α on the intracellular growth of M. tuberculosis has been investigated extensively. Anti-TNF-α treatment increased mycobacterial replication in mice (31). Denis found that recombinant TNF-α injections resulted in a significant reduction in the number of viable bacteria in the lungs and spleens of mice (11). The exposure of infected murine bone marrow-derived macrophages to TNF-α and IFN-γ together has been shown to inhibit M. tuberculosis replication in vitro (18). Recently, several studies showed that neutralization of TNF-α led to reactivation of infection in mice and humans, and bacteria grew dramatically, leading to death (49). In this study, we used [3H]uracil incorporation to estimate mycobac-
The measurement of uracil incorporation has been widely accepted to provide a reliable indication of bacterial replication. In preliminary experiments conducted when the assay was first brought into the lab, we confirmed the relationship between \(^{3}H\)uracil uptake and CFU (unpublished results). Others have reported that there is a very close correlation between CFU and \(^{3}H\)uracil uptake by mycobacteria (2).

Our data also indicate that anti-gpTNF-α antibody significantly increased intracellular growth of both attenuated and virulent mycobacteria in resident alveolar macrophages as well as peritoneal macrophages at a lower MOI (1:10). Engele et al. demonstrated that TNF-α inhibits the growth of attenuated M. tuberculosis but supports the growth of virulent M. tuberculosis in human macrophages (14). However, our results show that virulent mycobacterial growth also could be controlled by TNF-α at a lower MOI (1:10). Interestingly, the dramatic growth of attenuated M. tuberculosis stimulated by anti-gpTNF-α antibody at an MOI of 1:1 was not seen in treated macrophages infected with virulent M. tuberculosis at the same MOI, which may support the finding of Engele et al. (14). Surprisingly, we observed a differential level of control of intracellular growth of M. tuberculosis between guinea pig alveolar and peritoneal macrophages. In alveolar macrophages, the significant increase of mycobacterial growth following treatment with anti-gpTNF-α antibody was shown only at day 7 (Fig. 6B and 7B). However, peritoneal macrophages treated with anti-gpTNF-α antibody showed enhanced mycobacterial accumulation even from day 4 (Fig. 6D and 7D). This may indicate that alveolar macrophages are less permissive for mycobacterial growth or that they have TNF-α-independent mechanisms to control early mycobacterial growth.

In conclusion, this study provides evidence of the direct involvement of TNF-α in enhancing, at least at the mRNA level, IL-12 p40 expression in guinea pig resident macrophages. The results from M. tuberculosis-infected macrophages suggest a possible mechanism for double-edged roles of TNF-α, which is that TNF-α can be beneficial as well as detrimental through modulation of IL-12 expression depending on the dose. In addition, the increase of intracellular M. tuberculosis growth in guinea pig macrophages by anti-gpTNF-α treatment clearly supports a protective role for TNF-α in host resistance against M. tuberculosis infection in this susceptible species.

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