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A Regulatory Effect of the Balance between TNF-α and IL-6 in the Granulomatous and Inflammatory Response to *Rhodococcus aurantiacus* Infection in Mice[1]

Yimin[2] and Masashi Kohanawa

After i.v. inoculation with *Rhodococcus aurantiacus*, wild-type (WT) mice develop nonnecrotic, epithelioid granulomas. Because a high level of TNF-α is observed during the initial phase postinfection, we examined the extent to which TNF-α contributes to granulomatous inflammation using TNF-α gene-deficient (TNF-$\alpha^{-/-}$) mice. Despite a lack of *R. aurantiacus* proliferation, TNF-$\alpha^{-/-}$ mice displayed high mortality rates within 5 days postinfection, as well as a high level of IL-6 in their spleens. Histological examination showed an absence of granuloma formation in TNF-$\alpha^{-/-}$ mice. Pretreatment of TNF-$\alpha^{-/-}$ mice with rTNF-α failed to restore this granuloma formation but accelerated bacterial removal and cellular recruitment. This rTNF-α administration also attenuated IL-6 production, resulting in increased survival rates of TNF-$\alpha^{-/-}$ mice. Heat-killed *R. aurantiacus* induced in vitro enhanced mRNA expression and production of IL-6 in macrophages and DCs from TNF-$\alpha^{-/-}$ mice when compared with WT controls, and treatment of TNF-$\alpha^{-/-}$ mouse cells with rTNF-α decreased the IL-6 secretion. Moreover, anti-TNF-α or anti-IL-6 treatment increased IL-6 or TNF-α production by WT mouse cells, respectively. These data suggest that the production of TNF-α and IL-6 can be negatively regulated by each other. Administration of rIFN-γ to TNF-$\alpha^{-/-}$ mice caused immature granulomas in livers, and treatment with both rTNF-α and rIFN-γ led to the formation of mature granulomas. Overall, TNF-α appears crucial for bacterial clearance, cellular recruitment, and granuloma formation. The balance between TNF-α and IL-6 during the early phase of infection controls the development of the inflammatory response to *R. aurantiacus* infection. ***The Journal of Immunology,*** **2006, 177: 642–650.**

Although TNF-α initially came to prominence because of its antitumor activity, most attention is now focused on its functions in the regulation of infectious, inflammatory, and immune phenomena. As a highly potent proinflammatory cytokine, TNF-α has activities ranging from activating macrophages and lymphocytes against bacterial, parasitic, and viral infections to inducing apoptosis (1–4). However, the production of TNF-α by a number of different cell types under a variety of immune and inflammatory conditions and the complex interaction between TNF-α ligands and receptors have greatly complicated attempts to define the precise physiological role of TNF-α. To properly investigate the effects of TNF-α in vivo, a number of study groups have used mice deficient in the genes for TNF-α or TNFR. TNF-α is thought to play an essential role in limiting the extent and duration of the inflammation induced by *Corynebacterium parvum* in TNF-α gene-deficient (TNF-$\alpha^{-/-}$) mice (5). Furthermore, TNF-$\alpha^{-/-}$ mice and TNFR gene-deficient (TNFR$^{-/-}$) mice readily succumb to *Listeria monocytogenes* infection, which wild-type (WT)[3] mice can control (6). TNF-α has not only beneficial but also detrimental activities in organisms. Studies using TNFR$^{-/-}$ mice have revealed that TNF-α is a mediator in the pathogenesis of endotoxic shock (7). Because the TNFR superfamily includes not only TNFR1 and TNFR2 but also other related membrane-anchored and secreted receptors and ligands (5, 8, 9), we chose to study the TNF-$\alpha^{-/-}$ mouse model, which allows the study of TNF-α function regardless of the receptor engaged.

Granuloma formation is normally a protective process by which noxious substances and infectious agents are sequestered and prevented from damaging the surrounding tissue. This process is dependent on macrophages and T lymphocytes, given that macrophages activated by irritants secret chemotactic cytokines, and T lymphocytes are recruited by these cytokines to the site of infection. The recruited T lymphocytes release cytokines that further activate the macrophages and drive epithelioid cell differentiation, eventually resulting in granuloma formation (10–12). Recent studies showed that functionally mature dendritic cells (DCs), which possess potent Ag-presenting function and can activate T lymphocytes, participate in the granulomatous reaction (13–15). However, an excessive granulomatous response results in tissue injury and fails to localize infection (16). Understanding the immunological mechanisms of protection and pathogenesis in granuloma disease remains problematic. On the basis of cytokine profiles, granulomatous inflammation can be classified into two types. Type 1 is characterized by the predominance of IFN-γ, TNF-α, and IL-12, whereas type 2 is typified by the production of IL-4, IL-5, and IL-10 (1, 17). In short, the type of granulomatous and inflammatory reaction is modulated by the Th1/Th2 immune response. Although high levels of TNF-α have been detected in type 1 granulomatous inflammation induced by *Propionibacterium acnes* and *Mycobacterium tuberculosis* (16, 18) and type 2 induced by *Schistosoma mansoni* (10, 19), there are conflicting data with respect to the role of TNF-α in granuloma formation, and its regulatory role in granuloma inflammation is still unknown.

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[3] Abbreviations used in this paper: p.i., post-infection; WT, wild type; DC, dendritic cell; BM-DC, bone marrow-derived DC; TNF-α/IL-6 balance, balance between TNF-α and IL-6; MOI, multiplicity of infection; p.i., postinfection.

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Current evidence suggests that IL-6, which has been classified as both a pro- and anti-inflammatory cytokine, is rapidly induced during the initiation of inflammation when early TNF-α production is also induced and plays an important regulatory role in various immune responses and inflammatory conditions (12, 20–23). However, whether the balance between TNF-α and IL-6 production (TNF-α/IL-6 balance) affects the development of the immune response remains unclear. In a previous study of *Rhodococcus aurantiacus* infection in IL-6 gene-deficient (IL-6$^{-/-}$) mice, we concluded that IL-6 has a suppressive effect on TNF-α production and type 1 granulomatous inflammation (12). It was also reported that IL-6 deficiency induced overproduction of TNF-α in *Streptococcus pyogenes*-infected mice (24). Thus, we postulated that TNF-α and IL-6 production in an innate immune response may be negatively regulated by each other and that the TNF-α/IL-6 balance may be a key factor in regulating immune responses.

To determine whether there are critical functions for TNF-α in multicytokine-mediated granuloma formation, we infected TNF-$\alpha^{-/-}$ mice with *R. aurantiacus*, which is a psychrophilic acid-fast bacterium closely related to members of the genera *Corynebacterium*, *Mycobacterium* and *Nocardia*. In previous studies, we illustrated that *R. aurantiacus* induces IFN-γ-dependent nonnecrotic and epithelioid type 1 granuloma formation in WT mice (12, 25). The findings of this study establish a critical requirement for TNF-α in bacteria elimination, cellular recruitment, granuloma formation, and immunoregulation by suppressing IL-6 production in a *R. aurantiacus*-infected mouse model.

Materials and Methods

Mice and experimental infections

C57BL/6 WT and TNF-$\alpha^{-/-}$ mice were purchased from SLC and The Jackson Laboratory, respectively. TNF-$\alpha^{-/-}$ mice were generated as previously described (5). Briefly, 129Sv × C57BL/6 mice with a disrupted TNF-α gene were backcrossed onto the C57BL/6 background for 10 generations before being intercrossed to produce homozygotes. Female WT and TNF-$\alpha^{-/-}$ mice, 6–8 wk old, were used.

WT and TNF-$\alpha^{-/-}$ mice were administered via a lateral tail vein with an inoculum of 1×10^8 CFU of viable *R. aurantiacus* (strain 80005) suspended in 0.2 ml of PBS. The survival of the mice was recorded for 28 days postinfection (p.i.), and survival curves were plotted. The organs from infected mice were retrieved at appropriate times p.i.

Reconstitution of TNF-$\alpha^{-/-}$ mice with exogenous cytokines

For a therapeutic experiment, 1 μg of rTNF-α (PeproTech) in 0.2 ml of PBS was administered i.v. to each TNF-$\alpha^{-/-}$ mouse 1 day before *R. aurantiacus* infection. Compared with WT mice, there was a remarkable decrease in endogenous IFN-γ production at 2 wk p.i. in the TNF-$\alpha^{-/-}$ mice. To supplement this decrease, 5 μg of rIFN-γ (PeproTech) in 0.2 ml of PBS was administered i.v. to each TNF-$\alpha^{-/-}$ mouse on days 5, 8, and 11 p.i.; 0.2 ml of PBS alone was injected as a control.

In vivo depletion of endogenous IL-6

To deplete the large amount of endogenous IL-6 produced in the early stage of *R. aurantiacus* infection in the TNF-$\alpha^{-/-}$ mice, 10 μg of anti-murine IL-6 mAb (BioSource International) in 0.2 ml of PBS was injected i.v. into each TNF-$\alpha^{-/-}$ mouse 1 day before infection; 0.2 ml of PBS alone was injected as a control.

Determination of the number of bacteria in the organs

The retrieved spleens and livers from infected mice were homogenized with RPMI 1640 (Sigma-Aldrich) (0.1 g/10 ml). Then, 100 μl of the organ homogenates and their serial 10-fold dilutions were plated onto nutrient agar plates (Nissui Pharmaceutical). The colonies of viable *R. aurantiacus* in the spleens and livers were counted at 48 h after culture. With this method, $>10^3$ bacteria/g organ were detectable.

Preparation of organ extracts

Spleen and liver extracts for cytokine assays were prepared as follows. Spleens and livers were aseptically removed from *R. aurantiacus*-infected mice at the indicated times p.i. and suspended in RPMI 1640 containing 1% (w/v) CHAPS (Wako Pure Chemical). Ten percent (w/v) homogenates were prepared with a Dounce grinder. The homogenates were clarified by centrifugation at 2000 × *g* for 25 min, and the supernatants were stored at −80°C until cytokine protein assays.

Primary cell cultures

Bone marrow-derived DCs (BM-DCs) and peritoneal macrophages were generated from uninfected TNF-$\alpha^{-/-}$ and WT mice. BM-DCs were prepared as described by Faure et al. (26), with minor modifications. Briefly, BM-DCs from femurs were cultivated with IMDM (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 600 μg/ml L-glutamine, 50 mM 2-ME, 30% NIH-3T3 cultured supernatant, and 10 ng/ml mouse GM-CSF (PeproTech). At day 6, loosely adherent cells were harvested, and > 80% of the cells were DCs as determined by flow cytometric analysis of CD11c$^+$.

Murine peritoneal macrophages were isolated by i.p. injection of PBS into mice (27). After culture with RPMI 1640 containing 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin in plastic dishes for 2 h, floating cells were removed from dishes, and attached cells were maintained in RPMI 1640 for 3 days before use. The resulting cell population was >90% CD11b$^+$.

Stimulation of BM-DCs and macrophages

BM-DCs and macrophages from uninfected TNF-$\alpha^{-/-}$ and WT mice were plated at density of 1×10^6 cells/well in 12-well plates. After 12 h of culture, these cells were stimulated with heat-killed *R. aurantiacus* (multiplicity of infection (MOI) 2). The cells and the culture supernatants were harvested at the indicated times poststimulation for total RNA extraction and cytokine protein assays, respectively.

BM-DCs and macrophages from the TNF-$\alpha^{-/-}$ mice were pretreated with rTNF-α at final concentrations of 0.1, 1, 10, and 100 ng/ml for 2 h and then stimulated with heat-killed *R. aurantiacus* (MOI 2). Controls were pretreated with medium alone. Those from the WT mice were pretreated with either anti-murine TNF-α mAb (Genzyme) or anti-murine IL-6 mAb at 10 μg/ml final concentration 2 h before the stimulation. Isotype hamster IgG and rat IgG (Santa Cruz Biotechnology) were used as control mAbs, respectively. The cell culture supernatants were harvested at 24 and 48 h poststimulation for TNF-α and IL-6 assays, respectively.

Semiquantitative RT-PCR analysis

Total RNA was isolated from BM-DCs or macrophages using the RNeasy Mini Kit (Qiagen), and 4 μg of total RNA were reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Toyobo). The cDNA was amplified by PCR using TaKaRa Ex *Taq* (Takara) and the following primer pairs: sense primer 5′-CAT CCA GTT GCC TTC TTG GGA-3′ and antisense primer 5′-CAT TGG GAA ATT GGG GTA GGA AG-3′ for IL-6 (40 cycles); sense primer 5′-ATG AGC ACA GAA AGC ATG ATC-3′ and antisense primer 5′-TAC AGG CTT GTC ACT CGA ATT-3′ for TNF-α (27 cycles); and sense primer 5′-TGG AAT CCT GTG GCA TCC ATG AAA-3′ and antisense primer 5′-TAA AAC GCA GCT CAG TAA CAG TCCG-3′ for β-actin (30 cycles). Amplified PCR products for IL-6, TNF-α, and β-actin genes were 463, 276, and 349 bp, respectively, and were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and photographed under UV light.

Measurement of cytokines

Cytokine concentrations in organ extracts and culture supernatants were determined by ELISAs. To measure TNF-α, plates were coated with purified hamster anti-murine TNF-α mAb and incubated with culture supernatants or organ extracts. TNF-α was detected with purified rabbit anti-mouse TNF-α polyclonal Ab (Endogen) followed by peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and *o*-phenylenediamine (Wako Pure Chemical) substrate. A standard curve was constructed for each experiment by serially diluting rTNF-α (Genzyme). IFN-γ was determined as described previously (12). Purified rat anti-murine IFN-γ mAb produced by hybridoma R4–6A2 and rabbit anti-mouse IFN-γ polyclonal Ab (PeproTech) were used as capture and detecting Abs, respectively. All ELISAs were run with rIFN-γ. IL-6 concentration was also measured by ELISA. Briefly, 100 μl of samples and rIL-6 (BD Pharmingen) were respectively added to a 96-well microtiter plate coated with the purified rat anti-murine IL-6 mAb (MP5-20F3; BD Pharmingen), and IL-6 was detected with a biotinylated rat anti-murine IL-6 mAb (MP5-32C11; BD Pharmingen). The sensitivities of the ELISA were 50 pg/ml for TNF-α, 20 pg/ml for IFN-γ, and 50 pg/ml for IL-6.

Histological analyses

The mice were sacrificed, and their liver tissue samples were removed, fixed in 10% Formalin, and embedded in paraffin blocks. Sections (3 μm) were stained with H&E. In each experiment, sections were made from three different areas of each organ. The size of the granuloma was calculated by the diameter of each granuloma measured with an ocular micrometer. The mean size of a granuloma and the mean number of granulomas per field were measured from six random optical fields within each section. Granuloma area was determined from the size of granuloma multiplied by the number of granulomas.

Statistical analysis

All data are expressed as mean ± SD. Significant differences between the values in experimental and control groups were calculated by Student's *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

Differential survival between TNF-$\alpha^{-/-}$ mice and WT mice infected with R. aurantiacus

After TNF-$\alpha^{-/-}$ and WT mice were inoculated i.v. with 1×10^8 CFU of viable *R. aurantiacus*, survival was monitored for 28 days p.i. The TNF-$\alpha^{-/-}$ mice were significantly more susceptible to *R. aurantiacus* infection and displayed higher mortality rates than their WT counterparts. Death of TNF-$\alpha^{-/-}$ mice began as early as day 1 p.i., and 70% of these mice had succumbed to infection by 5 days. Treatment with exogenous rTNF-α 1 day before infection markedly improved the survival of TNF-$\alpha^{-/-}$ mice to an equivalent level to that of WT mice (Fig. 1). A similar result was obtained for TNF-$\alpha^{-/-}$ mice pretreated with anti-murine IL-6 mAb 1 day before *R. aurantiacus* infection (Fig. 1). These data indicate that pretreatment of infected TNF-$\alpha^{-/-}$ mice with rTNF-α or anti-IL-6 Ab enhanced survival rates.

In vivo kinetics of bacterial growth and clearance in infected mice

Because in numerous infectious diseases mice are often unable to control bacterial replication and quickly succumb to infections, we asked whether the high mortality rates displayed in TNF-$\alpha^{-/-}$ mice resulted from *R. aurantiacus* growth in vivo. To investigate the kinetics of bacterial proliferation and clearance in TNF-$\alpha^{-/-}$ and WT mice, the number of viable bacteria in their spleens and livers was assessed at appropriate time points after these mice were challenged with 1×10^8 CFU of *R. aurantiacus*. As shown in Fig. 2, no bacterial proliferation was observed p.i. in the two mouse groups. Bacterial counts in WT mouse organs markedly decreased and fell to an undetectable level ($<10^3$ bacteria) at day 10 p.i., whereas *R. aurantiacus* was still detectable at 2 wk p.i. in the TNF-$\alpha^{-/-}$ mice (Fig. 2). Pretreatment of TNF-$\alpha^{-/-}$ mice with anti-murine IL-6 mAb 1 day before infection had no effect on bacterial clearance in organs but improved the mortality rate of the TNF-$\alpha^{-/-}$ mice (Figs. 1 and 2). These data suggest that the high mortality rate observed in the TNF-$\alpha^{-/-}$ mice is not associated with a bacterial burden in various target organs but that an abnormal production of IL-6 maybe contribute to this increased mortality. Pretreatment of TNF-$\alpha^{-/-}$ mice with exogenous rTNF-α 1 day before infection induced an increased rate of bacterial elimination in the early phase of infection (days 1–5 p.i.). Administration of exogenous rIFN-γ to TNF-$\alpha^{-/-}$ mice on days 5, 8, and

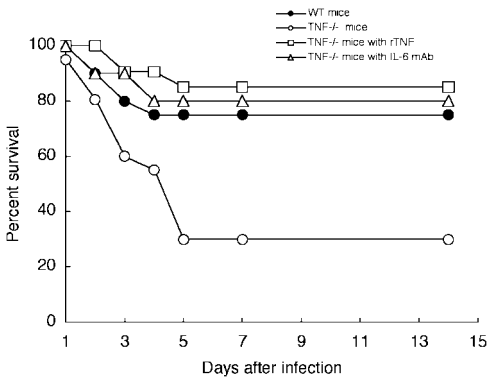

FIGURE 1. Survival rate in mice after i.v. infection with 1×10^8 CFU of *R. aurantiacus*. WT mice (●) showed 75% survival rate and served as controls. TNF-$\alpha^{-/-}$ mice (○) showed increased mortality as early as day 1 p.i., and 70% of these mice were dead by 5 days p.i. Pretreatment with either rTNF-α (□) or anti-murine IL-6 mAb (△) 1 day before infection improved survival for TNF-$\alpha^{-/-}$ mice to 85 and 80%, respectively. Survival curves were generated from two independent experiments in which 20 WT mice and 30 TNF-$\alpha^{-/-}$ mice were used.

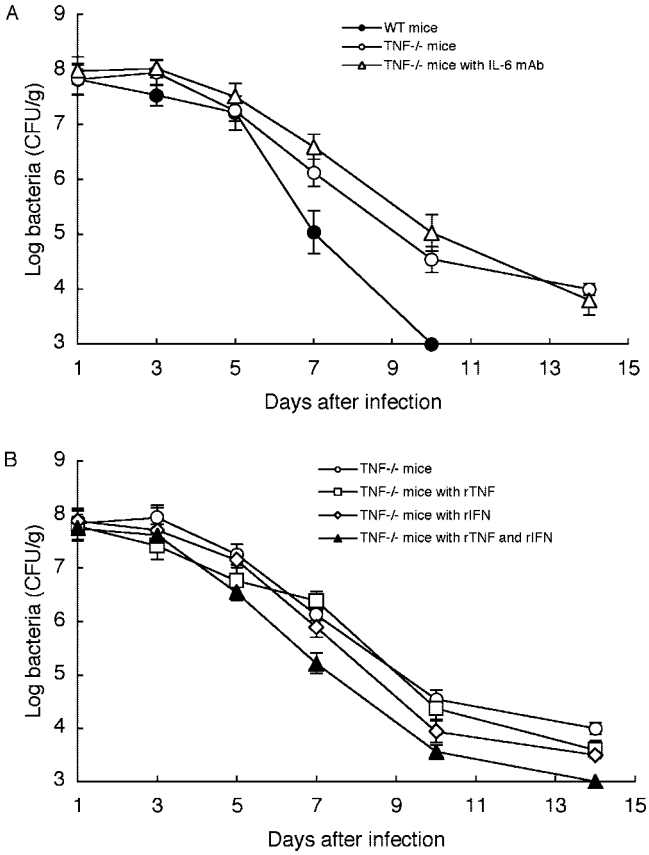

FIGURE 2. Kinetics of bacterial load in mice after i.v. *R. aurantiacus* infection. After TNF-$\alpha^{-/-}$ mice and WT mice were infected i.v. with 1×10^8 CFU of *R. aurantiacus*, the numbers of viable bacteria present in liver were determined from days 1 to 14 p.i. *A*, *R. aurantiacus* did not proliferate in the organs of either TNF-$\alpha^{-/-}$ or WT mice, but there was a delayed clearance of bacteria in the TNF-$\alpha^{-/-}$ mice (○) compared with the control WT mice (●) ($p < 0.01$ on day 10). Pretreatment of TNF-$\alpha^{-/-}$ mice with anti-murine IL-6 mAb (△) 1 day before infection had no effect on *R. aurantiacus* clearance. *B*, TNF-$\alpha^{-/-}$ mice treated with rTNF-α (□) 1 day before infection showed an increased bacterial clearance in the early phase of infection compared with untreated TNF-$\alpha^{-/-}$ mice (○) ($p < 0.05$ on day 5). Treatment of TNF-$\alpha^{-/-}$ mice with rIFN-γ (◇) on days 5, 8, and 11 p.i. to supplement the decrease in IFN-γ production seen at 2 wk also displayed an accelerated bacterial clearance at the granuloma formation phase ($p < 0.01$ on day 14). Moreover, treatment of TNF-$\alpha^{-/-}$ mice with both rTNF-α and rIFN-γ (▲) resulted in a significant increase in bacterial elimination rates, and the numbers of bacteria were undetectable at 2 wk p.i. ($p < 0.001$ on day 14). Each point represented the mean ± SD of the *R. aurantiacus* CFU from two independent experiments with a total of 20–30 mice per group.

11 p.i. to supplement the decrease in IFN-γ production seen at 2 wk also led to an accelerated bacterial clearance during the granuloma formation phase. Moreover, treatment with both rTNF-α and rIFN-γ considerably accelerated *R. aurantiacus* elimination rates in these animals, resulting in undetectable levels of bacteria at 2 wk p.i. (Fig. 2). (The spleen data were similar to those of the liver and are not shown.) These results suggest that TNF-α and IFN-γ, but not IL-6, contributed to *R. aurantiacus* clearance in TNF-$\alpha^{-/-}$ mice, which was delayed in these mice compared with WT controls.

Histological features of mice infected with R. aurantiacus

After i.v. injection of *R. aurantiacus*, histological changes in livers were investigated at appropriate time points in WT and TNF-$\alpha^{-/-}$ mice. A 2-wk period was sufficient to allow evaluation of granuloma containing inflammatory macrophages and infiltrating T lymphocytes. In the control WT mice, nonnecrotic and epithelioid granulomas were observed in the liver (mean area of granulomas per field, 38%), however, cellular infiltrates were less well formed, and no granulomas were observed in the surviving TNF-$\alpha^{-/-}$ mice (Figs. 3, *A* and *B*, and 4). Next, we examined the effects of anti-murine IL-6 mAb, exogenous rTNF-α and rIFN-γ on the granulomatous response induced by *R. aurantiacus* infection in TNF-$\alpha^{-/-}$ mice. Treatment of TNF-$\alpha^{-/-}$ mice with anti-murine IL-6 mAb 1 day before infection had no effect on granuloma formation and cellular recruitment. (The histological features of these mice were similar to those of infected TNF-$\alpha^{-/-}$ mice and are not shown herein.) Administration of rTNF-α to TNF-$\alpha^{-/-}$ mice 1 day before *R. aurantiacus* challenge failed to restore granuloma formation, but it significantly increased inflammatory cell recruitment to the sites of inflammation (Figs. 3*C* and 4). Treatment of TNF-$\alpha^{-/-}$ mice with rIFN-γ on days 5, 8, and 11 p.i. to supplement the decrease in IFN-γ production seen at 2 wk induced a small number of nontypical granulomas (mean area of granulomas per field, 5%) (Figs. 3*D* and 4). Furthermore, administration of both rTNF-α (on day −1) and rIFN-γ (on days 5, 8, and 11) resulted in the formation of many mature granulomas in *R. aurantiacus*-inoculated TNF-$\alpha^{-/-}$ mice (mean area of granulomas per field, 18%), although the size and the number of granulomas were less than in WT mice (Figs. 3*E* and 4). These results show that administration of rTNF-α to TNF-$\alpha^{-/-}$ mice increased cellular recruitment but restore granuloma formation only when combination with rIFN-γ.

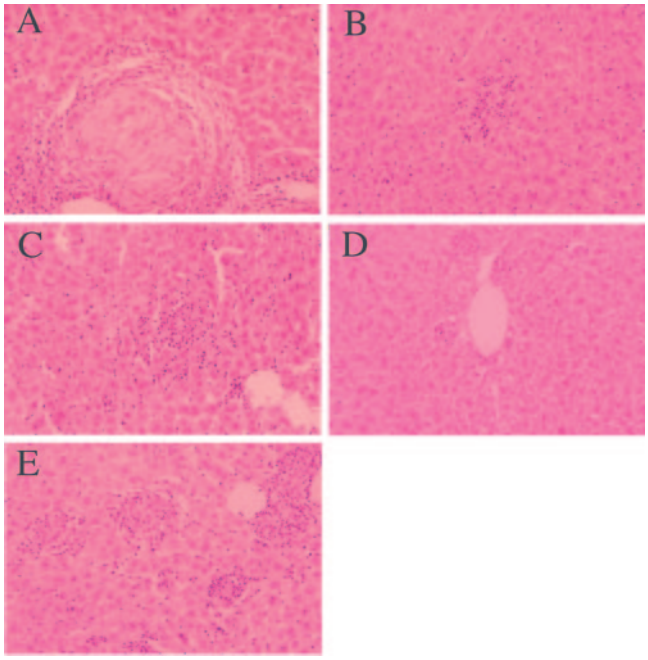

FIGURE 3. Liver histological response to *R. aurantiacus* infection at 2 wk p.i. Mice were sacrificed at 2 wk p.i., and formalin-fixed sections were stained with H&E. WT mice showed nonnecrotic and epithelioid granulomas at the sites of infection (*A*). Clusters of mononuclear cells and lymphocytes appeared, but no granulomas developed in TNF-$\alpha^{-/-}$ mice (*B*). Pretreatment of TNF-$\alpha^{-/-}$ mice with rTNF-α 1 day before infection caused increased cellular infiltration into inflamed tissues, but failed to restore granuloma formation (*C*). TNF-$\alpha^{-/-}$ mice treated with rIFN-γ on days 5, 8, and 11 p.i. developed small numbers of immature granulomas (*D*). Administration of both rTNF-α (1 day before infection) and rIFN-γ (on days 5, 8, and 11 p.i.) resulted in mature epithelioid granuloma formation in TNF-$\alpha^{-/-}$ mice, although the size and the number of granulomas were less than those in the infected WT mice (*E*). Original magnification: *A*, ×400; *B–E*, ×200.

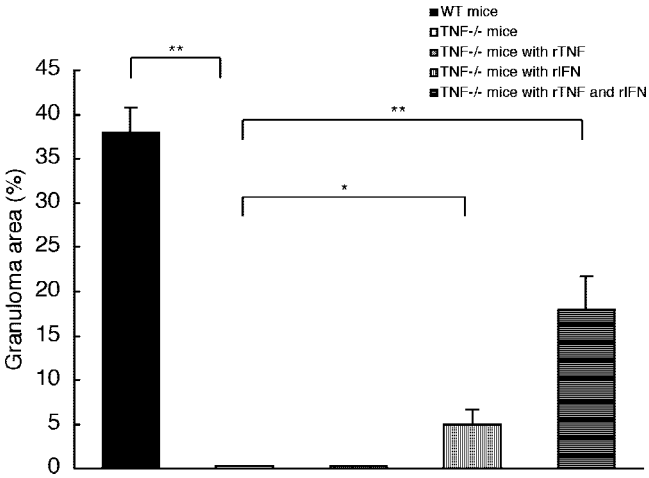

FIGURE 4. The percentage of granuloma area per field at 2 wk p.i. in the livers of mice. WT mice (■) showed a 38 ± 2.74% coverage of granuloma area per field, whereas no granuloma developed in TNF-$\alpha^{-/-}$ mice (□) and TNF-$\alpha^{-/-}$ mice pretreated with rTNF-α 1 day before infection (▨). TNF-$\alpha^{-/-}$ mice that received rIFN-γ on days 5, 8, and 11 p.i. (▥) showed only a 5 ± 1.67% coverage of granuloma area per field. Furthermore, TNF-$\alpha^{-/-}$ mice treated with both rTNF-α (1 day before infection) and rIFN-γ (on days 5, 8, and 11 p.i.) (▤) showed 18 ± 3.74% coverage of granuloma per field. The mean size of granulomas and the mean number of granulomas per field were measured in six optical fields within each tissue section. Granuloma area was determined from the size of granuloma multiplied by the number of granulomas. Data are generated by a blind analysis and presented as the mean ± SD. *, $p < 0.01$. **, $p < 0.001$.

Secretion of endogenous cytokines in TNF-$\alpha^{-/-}$ mice and WT mice

To determine whether the absence of TNF-α might influence the induction of other cytokines involved in granuloma formation, the levels of several cytokines from spleen and liver extracts were measured. We first examined IFN-γ production, which is an essential element in *R. aurantiacus*-induced granuloma formation. In WT mice infected with *R. aurantiacus*, biphasic IFN-γ production was observed in the spleen, with an early peak phase detected between days 1 and 3, and a later phase observed at 2 wk (Fig. 5*A*). In the liver, the elevated IFN-γ level only peaked at 2 wk in WT mice (Fig. 5*B*). Similarly, on days 1 through 3 after *R. aurantiacus* infection, a large amount of IFN-γ was produced by the spleen in TNF-$\alpha^{-/-}$ mice; however, the high level of IFN-γ seen in WT mice at 2 wk was not observed in either the spleen or liver of the

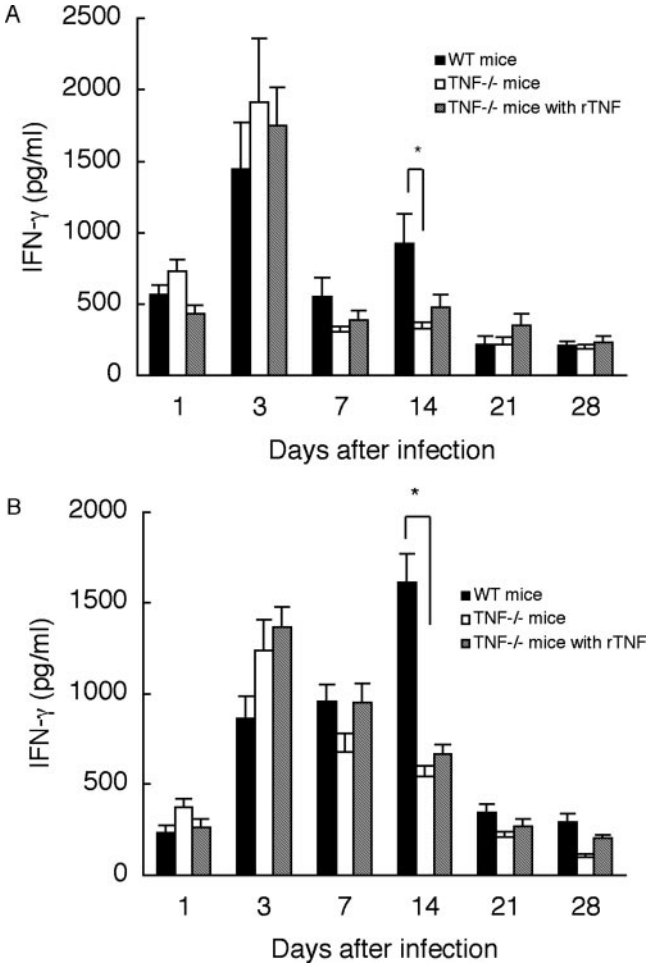

FIGURE 5. Endogenous IFN-γ production in infected mice. After infection with 1×10^8 CFU of *R. aurantiacus*, IFN-γ levels in spleen extracts (*A*) and liver extracts (*B*) from WT mice (■), TNF-$\alpha^{-/-}$ mice (□), and TNF-$\alpha^{-/-}$ mice pretreated with exogenous rTNF-α (▨) 1 day before infection were measured by ELISA. Compared with control WT mice, a markedly lower level of IFN-γ was observed at 2 wk p.i. in TNF-$\alpha^{-/-}$ mice, although there was no significant difference in IFN-γ production between the two groups during the early phase of infection. Pretreatment with rTNF-α did not affect IFN-γ production by TNF-$\alpha^{-/-}$ mice. The results shown here are the mean ± SD from three independent experiments in which 2–3 mice per group were used at each time point. *, $p < 0.001$.

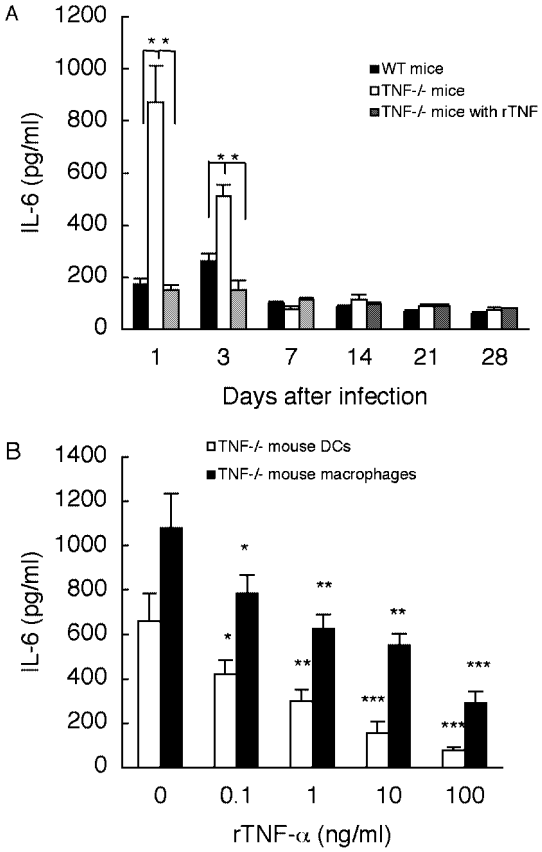

FIGURE 6. Effect of exogenous TNF-α on IL-6 production in TNF-$\alpha^{-/-}$ mice and TNF-$\alpha^{-/-}$ mouse cells. *A*, After infection with 1×10^8 CFU of *R. aurantiacus*, IL-6 concentrations in spleen extracts from WT mice (■), TNF-$\alpha^{-/-}$ mice (□), and TNF-$\alpha^{-/-}$ mice pretreated with exogenous rTNF-α (▨) at different time intervals were assayed by ELISA. Compared with WT mice, significantly higher levels of IL-6 were detected during the early phase of infection in TNF-$\alpha^{-/-}$ mice. Pretreatment of TNF-$\alpha^{-/-}$ mice with rTNF-α markedly prevented this IL-6 production. Data are the mean ± SD from three independent experiments in which 2–3 mice per group were used at each time point. *, $p < 0.001$. *B*, BM-DCs (□) and macrophages (■) from TNF-$\alpha^{-/-}$ mice were stimulated with heat-killed *R. aurantiacus* (MOI 2) in the presence of rTNF-α (0.1, 1, 10, 100 ng/ml), then culture supernatants were harvested at 48 h poststimulation and analyzed by ELISA for IL-6. IL-6 levels in the BM-DCs and the macrophages from TNF-$\alpha^{-/-}$ mice tended to be lower with increasing dose of rTNF-α. Data are the mean ± SD from three independent experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

TNF-$\alpha^{-/-}$ mice (Fig. 5, *A* and *B*). Pretreatment with rTNF-α did not effect IFN-γ production in TNF-$\alpha^{-/-}$ mice (Fig. 5). After administration of rTNF-α (1 day before infection) and rIFN-γ (on day 5, 8, and 11 p.i.) to TNF-$\alpha^{-/-}$ mice, the level of TNF-α or IFN-γ in these mice was similar to that in TNF-$\alpha^{-/-}$ mice without these cytokine treatments on day 1, 3, 7, 14, 21, and 28. This was thought to be due to a short half-life of either rIFN-γ or rTNF-α.

There was a marked increase in IL-6 production in the spleen of the TNF-$\alpha^{-/-}$ mice between days 1 and 3 p.i. To determine whether the increased production of IL-6 is associated with TNF-α deficiency, we administered exogenous rTNF-α i.v. to TNF-$\alpha^{-/-}$ mice 1 day before infection. Similar to administration of anti-murine IL-6 mAb to TNF-$\alpha^{-/-}$ mice, pretreatment with rTNF-α attenuated the elevated IL-6 level observed at the early phase of infection (Fig. 6*A*). These data show that excessive IL-6 production during the early phase of infection is associated with absence of TNF-α.

In vitro kinetics of cytokines production by WT and TNF-$\alpha^{-/-}$ mouse cells

Because IL-6 is principally produced by DCs and macrophages in response to inflammation signals (21, 28), we made a comparison of the IL-6 secretion by BM-DCs and peritoneal macrophages from unchallenged TNF-$\alpha^{-/-}$ mice with those from WT mice. The cells were stimulated with heat-killed *R. aurantiacus,* and then IL-6 concentrations in culture supernatants were measured by ELISA. As shown in Fig. 7*A,* although IL-6 cellular secretion from both the TNF-$\alpha^{-/-}$ and WT mice increased in a time-dependent manner, at least until 48 h poststimulation, there was a greater production of IL-6 in the cell culture supernatant from the TNF-$\alpha^{-/-}$ mice than in those from the WT mice. At 48 h poststimulation, 1.8- and 1.9-fold increases (TNF-$\alpha^{-/-}$ mice vs WT mice) were evaluated in IL-6 protein in BM-DCs and macrophages, respectively (Fig. 7*A*). Thus, in vitro increased production of IL-6 by

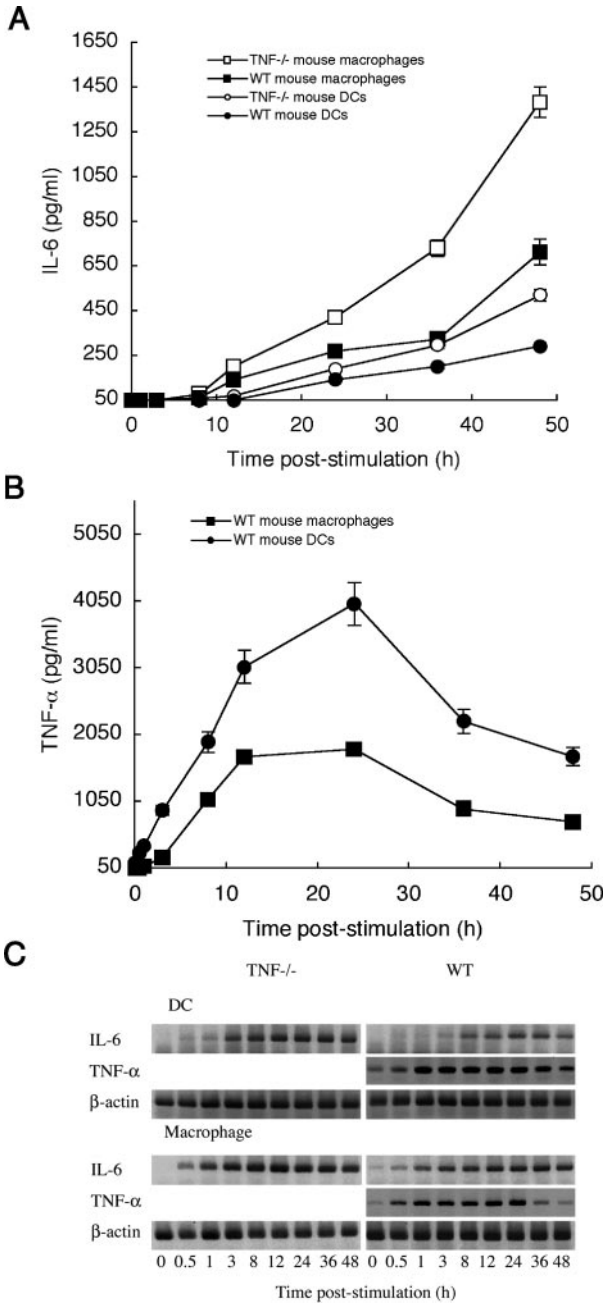

FIGURE 7. Time course of IL-6 (*A*) and TNF-α (*B*) production and their mRNA expression (*C*) in WT and TNF-$\alpha^{-/-}$ mouse cells. Macrophages from WT mice (■) and TNF-$\alpha^{-/-}$ mice (□) and BM-DCs from WT mice (●) and TNF-$\alpha^{-/-}$ mice (○) were stimulated with heat-killed *R. aurantiacus* (MOI 2); then, culture supernatants were harvested at different time intervals and assayed by ELISA for IL-6 and TNF-α. At the same time, total RNA in these cells were isolated and analyzed using RT-PCR for IL-6 and TNF-α mRNA expression. IL-6 cellular secretion increased in a time-dependent manner, and the cells from TNF-$\alpha^{-/-}$ mice released a greater level of IL-6 than those from WT mice. TNF-α levels in WT mouse cells peaked at 24 h poststimulation and thereafter decreased. The cytokine production corresponded with their mRNA expression. Data shown in *A* and *B* are the mean ± SD from three independent experiments. Each lane in *C* was representative of one of three independent experiments for hours 0, 0.5, 1, 3, 8, 12, 24, 36, and 48 h poststimulation.

TNF-$\alpha^{-/-}$ mouse cells coincided with those observed in TNF-$\alpha^{-/-}$ mice.

We also measured, by ELISA, TNF-α secretion in the culture supernatants of BM-DCs and macrophages isolated from WT mice. The cells released abundant TNF-α after heat-killed *R. aurantiacus* stimulation. The amount of TNF-α from the BM-DCs and the macrophages peaked at 24 h poststimulation (mean TNF-α concentration, 4009 pg/ml for BM-DCs and 1827 pg/ml for macrophages) and thereafter decreased (Fig. 7*B*), suggesting that IL-6 appeared predominant to inhibit TNF-α release after 24 h of stimulation. Moreover, the data in Fig. 7, *A* and *B*, also reveal that IL-6 and TNF-α production by WT mouse cells were inversely regulated by each other.

In vitro kinetics of cytokine mRNA expression in by WT and TNF-$\alpha^{-/-}$ mouse cells

Cytokine production is regulated at both the transcriptional and translational levels. To determine whether production of IL-6 and TNF-α was associated with their respective mRNA expression, we evaluated cytokine mRNA synthesis using semiquantitative RT-PCR. As shown in Fig. 7*C,* IL-6 mRNA was undetectable in unstimulated BM-DCs and macrophages from either TNF-$\alpha^{-/-}$ or WT mice. After stimulation with heat-killed *R. aurantiacus*, earlier and greater induction of IL-6 mRNA occurred in the cells from TNF-$\alpha^{-/-}$ mice than from WT mice. Twofold and 1.6-fold increases in IL-6 transcripts were observed at 12 h in macrophages and BM-DCs, respectively (TNF-$\alpha^{-/-}$ mice vs WT mice). These results indicated that enhanced production of IL-6 by BM-DCs and macrophages from TNF-$\alpha^{-/-}$ mice is associated with a concomitant increase in transcribed IL-6 mRNA.

TNF-α mRNA was barely detected in BM-DCs and macrophages from WT mice without stimulation, but there was an increase in the expression of TNF-α mRNA in these cells, especially in BM-DCs, after heat-killed *R. aurantiacus* stimulation (Fig. 7*C*). This stimulation did not affect the expression of β-actin mRNA.

Effect of TNF-α on IL-6 production by TNF-$\alpha^{-/-}$ mouse cells

To further test the inhibitory effect of TNF-α on IL-6 production observed in vivo, different concentrations of rTNF-α were administered to the cultured BM-DCs and macrophages from uninfected TNF-$\alpha^{-/-}$ mice 2 h before stimulation with heat-killed *R. aurantiacus*. At 48 h poststimulation, the production of IL-6 in culture supernatants were measured by ELISA. As shown in Fig. 6*B*, addition of rTNF-α inhibited IL-6 production in a dose-dependent manner. Treatment with 100-ng/ml concentrations of rTNF-α resulted in an 88% decrease for BM-DCs and a 74% decrease for macrophages in IL-6 secretion compared with untreated control cells. These data show that TNF-α had an inhibitory effect on IL-6 secretion agreeing with those obtained by our in vivo (Fig. 6*A*).

Negative relationship between TNF-α and IL-6 production by WT mouse cells

Negatively regulated effects between TNF-α and IL-6 production were observed in TNF-$\alpha^{-/-}$ and IL-6$^{-/-}$ mice infected with *R. aurantiacus* (Ref. 12; Fig. 6*A*). To further determine whether production of TNF-α and IL-6 might be regulated by each other, we investigated the in vitro production of these two cytokines by BM-DCs and macrophages from uninfected WT mice in the presence of TNF-α or IL-6 neutralizing Ab. Pretreatment of BM-DCs and macrophages with 10 μg/ml anti-murine TNF-α mAb 2 h before stimulation with heat-killed *R. aurantiacus* significantly augmented IL-6 production compared with controls without any pretreatment. At 48 h poststimulation, IL-6 secretion by the BM-DCs and macrophages, which were pretreated with anti-TNF-α Ab, were 1.8- and 1.6-fold higher than the controls, respectively (Fig. 8*A*). Conversely, BM-DCs and macrophages pretreated with 10 μg/ml anti-murine IL-6 Ab 2 h before stimulation also produced markedly larger amounts of TNF-α than those stimulated alone. At

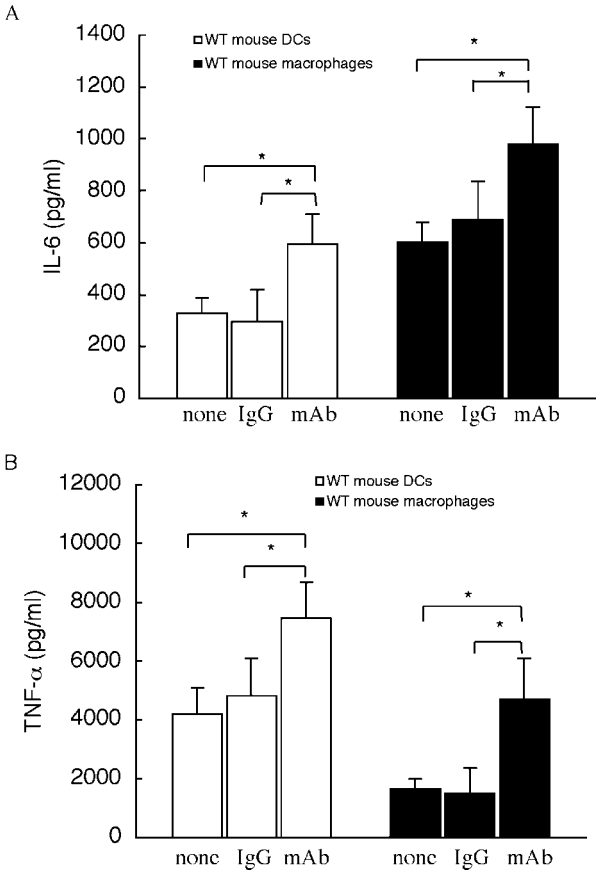

FIGURE 8. Effect of anti-TNF-α or anti-IL-6 neutralizing Ab on production of IL-6 (*A*) and TNF-α (*B*) by WT mouse cells. BM-DCs (□) and macrophages (■) from WT mice were stimulated with heat-killed *R. aurantiacus* (MOI 2) in the presence of 10 μg/ml anti-murine TNF-α Ab or anti-murine IL-6 Ab; then, IL-6, at 48 h poststimulation, and TNF-α, at 24 h poststimulation, in cultured supernatants were assessed by ELISA. The equivalent concentration of isotype IgGs were used for control Abs. Neutralization of TNF-α or IL-6 resulted in increase of IL-6 or TNF-α production by WT mouse cells, respectively. Data are the mean ± SD from three independent experiments. *, $p < 0.05$.

24 h poststimulation, TNF-α was found at 1.8- and 2.8-fold higher concentrations in culture supernatants of BM-DCs and macrophages pretreated with anti-IL-6 Ab in comparison with the controls, respectively (Fig. 8*B*). These data reveal that TNF-α and IL-6 production were negatively regulated by each other after challenge with *R. aurantiacus.*

Discussion

Several studies have shown that the high mortality rate in the type 1 granulomatous response to mycobacteria is caused by a markedly increased bacterial load and widespread tissue necrosis in TNF-$\alpha^{-/-}$ mice (18, 29, 30). In the present study, TNF-$\alpha^{-/-}$ mice also displayed significantly increased mortality within 5 days after i.v. inoculation of *R. aurantiacus* in contrast to WT mice (Fig. 1). However, there was no bacteria increase in vivo (Fig. 2), and examination of tissue sections showed no fatal liver injury in the TNF-$\alpha^{-/-}$ mice in the early phase (data not shown). Surprisingly, excessive production of IL-6 was noted in the TNF-$\alpha^{-/-}$ mice relative to the infected WT mice within 3 days p.i. Also, pretreatment with rTNF-α or anti-murine IL-6 mAb blocked this increased release of IL-6, improving survival of the TNF-$\alpha^{-/-}$ mice (Figs. 1 and 6*A*). These data demonstrate that the primary cytokine responses induced by *R. aurantiacus* are vital for survival of the animals and that the high mortality rate in the infected TNF-$\alpha^{-/-}$ mice was associated with the abnormal IL-6 secretion in the TNF-α deficiency state. The mechanism by which IL-6 increases the mortality rates of TNF-$\alpha^{-/-}$ mice is not well understood. A similar result has been reported that excessive secretion of IL-6 can contribute to the death of mice as a marker of severe infection in *Staphylococcus aureus* sepsis (31). *R. aurantiacus* is a Gram-positive bacterium as is *S. aureus*; it therefore is thought that a high level of IL-6 produced during the early phase of *R. aurantiacus* infection in TNF-$\alpha^{-/-}$ mice might induce sepsis that is different from that induced by Gram-negative bacteria and is associated with increased mortality.

R. aurantiacus, which is an intracellular bacterium, did not proliferate in vivo in either WT or TNF-$\alpha^{-/-}$ mice (Fig. 2). A rapid clearance of bacteria during 10 days p.i. was observed in the WT mice. In the absence of TNF-α, bacterial elimination was delayed, taking >2 wk. The exogenous administration of rTNF-α or rIFN-γ to the TNF-$\alpha^{-/-}$ mice resulted in a relative acceleration in the bacterial clearance in the initial and late phases, respectively (Fig. 2). Moreover, the administration of both rTNF-α and rIFN-γ to TNF-$\alpha^{-/-}$ mice led to increased bacterial clearance during the infection, whereas there was no significant difference between untreated TNF-$\alpha^{-/-}$ mice and those treated with neutralizing anti-murine IL-6 mAb (Fig. 2). Therefore, it is evident that *R. aurantiacus* clearance depends on the existence of TNF-α and IFN-γ, but not IL-6. Our previous study using IL-6$^{-/-}$ mice also confirmed that IL-6 has no effect on the *R. aurantiacus* elimination (12). Similarly, TNF-$\alpha^{-/-}$ mice infected with *M. tuberculosis* showed synergy between TNF-α and IFN-γ on disturbances in bacterial growth (29, 32). Otherwise, the present data also demonstrate that the increased mortality rate in TNF-$\alpha^{-/-}$ mice does not correlate with impaired bacterial clearance.

After inoculation with *R. aurantiacus*, the production of endogenous IFN-γ in WT mice was biphasic with an early peak detected between days 3 and 5 p.i. and a late phase detected during granuloma formation (Fig. 5). Interestingly, in TNF-$\alpha^{-/-}$ mice, IFN-γ production declined at the granuloma formation phase in comparison with that of their counterparts, although a similar level of IFN-γ was observed in the early phase of infection in the two groups of mice (Fig. 5). We previously reported that the early IFN-γ, which is derived from NK cells, contributes to the activation of T lymphocytes and macrophages and that the late IFN-γ, which originates from CD8$^+$ T lymphocytes, plays an important role in the differentiation of macrophages into epithelioid cells in response to *R. aurantiacus* in WT mice (33). Other studies have shown that TNF-α is required as a stimulatory factor for activating T lymphocytes to produce IFN-γ and that the activation of T lymphocytes is defective in *M. tuberculosis*-infected TNF-$\alpha^{-/-}$ mice (29, 34). Thus, it appears that the lack of TNF-α decreased IFN-γ production by T lymphocytes during the granuloma formation phase but did not affect NK cell production of IFN-γ in the early phase in TNF-$\alpha^{-/-}$ mice. In the present study, the administration of rTNF-α to TNF-$\alpha^{-/-}$ mice did not increase the level of IFN-γ to that seen at the late phase in WT mice. Because TNF-$\alpha^{-/-}$ mice have a form of hereditary TNF-α deficiency, it might be difficult to match timing and/or dosage of rTNF-α administration with TNF-α production in WT mice.

There was a significant difference in granuloma formation between WT and TNF-$\alpha^{-/-}$ mice. In WT mice, *R. aurantiacus* infection is characterized by a marked inflammatory cell infiltration into organs and nonnecrotic epithelioid granuloma formation that peaks at 2 wk p.i. (Fig. 3*A*). However, TNF-$\alpha^{-/-}$ mice challenged with *R. aurantiacus* developed a poor cellular infiltration and failed to form granulomas (Fig. 3*B*). An i.v. administration of

rTNF-α to TNF-$\alpha^{-/-}$ mice was ineffective in restoring granuloma formation, but it increased the cellular infiltration in liver tissue (Fig. 3*C*). This finding suggests that TNF-α is essential for the recruitment of inflammatory cells into inflamed tissues. Smith et al. (35) also confirmed that depletion of TNF-α completely abolished cellular recruitment in response to murine *Mycobacterium avium* infection. Anti-IL-6 treatment failed to restore granuloma formation and did not increase cellular recruitment in TNF-$\alpha^{-/-}$ mice, suggesting that the level of IL-6 was not directly concerned in inflammatory response, although it contributes to the mortality of the mice. Furthermore, after rIFN-γ therapy during the granuloma formation period, a few immature granulomas formed in the livers of TNF-$\alpha^{-/-}$ mice at 2 wk p.i. (Fig. 3*D*). The administration of both rTNF-α and rIFN-γ to TNF-$\alpha^{-/-}$ mice resulted in mature granuloma formation and an increase in the size and number of granulomas (Fig. 3*E*), indicating that the *R. aurantiacus*-induced granulomatous response was dependent not only on IFN-γ but also on TNF-α. Granuloma formation in response to murine mycobacterial infection was also reported to depend on the presence of IFN-γ from T lymphocytes and TNF-α from macrophages (11). TNF-α is essential for the differentiation of macrophages into epithelioid cells, and the neutralization of TNF-α in established mycobacterial infections leads to the disruption of granulomas, with dissemination of *M. tuberculosis* organisms (36–38). In addition to its role in the initial cellular recruitment response to *R. aurantiacus* infection, the central effect of TNF-α appears to be in the regulation of the inflammatory response and, in particular, the generation of mature and organized granulomas.

The most important finding in this paper is the role of the TNF-α/IL-6 balance in immunomodulation. Clearly, cross-regulation occurs between Th1 and Th2 cytokines, and the balance among these cytokines determines the type of T cell-mediated adaptive immune response. For example, in the case of a Th1 response, IFN-γ is the major cytokine produced by Th1 cells and is negatively regulated by Th2 cytokines (39, 40). In the *R. aurantiacus*-induced type 1 granulomatous response, we observed that the TNF-α/IL-6 balance in the extremely early phase of the immune response is similar to the Th1-Th2 balance and is important for controlling the ensuing immune responses. After infection with *R. aurantiacus*, TNF-$\alpha^{-/-}$ mice showed overproduction of IL-6 in the early phase and a decreased type 1 granulomatous response coincident with diminished IFN-γ secretion by T lymphocytes in the late phase (Figs. 3*B,* 5*,* and 6*A*). Administration of rTNF-α to TNF-$\alpha^{-/-}$ mice decreased IL-6 production (Fig. 6*A*). In contrast, *R. aurantiacus*-infected IL-6$^{-/-}$ mice displayed excessive production of TNF-α and IFN-γ in the early stage and a hypertrophic type 1 granulomatous response, and this overproduction of TNF-α was suppressed by administration of rIL-6 (12). These findings suggest that TNF-α and IL-6 production during the initiation of the immune response is mutually counterregulated and that this TNF-α/IL-6 balance influences the development of the type 1 granulomatous response induced by *R. aurantiacus* infection. The negative relationship between TNF-α and IL-6 was also found in the acute stage of the liver abscess induced by *Entamoeba histolytica* infection (41). Although it was reported that IFN-γ plus *Salmonella* Minnesota induced IL-6 production from RAW264.7 cells (42), in this study a higher level of IL-6 was observed during the early phase of infection in TNF-$\alpha^{-/-}$ mice compared with WT mice (Fig. 6*A*), and there was no significant difference in IFN-γ production between the two groups (Fig. 5). It is therefore thought that rIFN-γ may have little or no effect on IL-6 production. Several studies have indicated that APCs, such as macrophages and DCs, are the major sources of TNF-α and IL-6 in the early stage of the immune response (13, 21, 43). Thus, we attempted a detailed comparison of IL-6 production by macrophages and BM-DCs from TNF-$\alpha^{-/-}$ mice and those from WT mice. By stimulation with heat-killed *R. aurantiacus* in vitro, the cells from the TNF-$\alpha^{-/-}$ mice secreted greater amounts of IL-6 than those from the WT mice (Fig. 7, *A* and *C*). Pretreatment of the TNF-$\alpha^{-/-}$ mice with rTNF-α strongly inhibited IL-6 production by both macrophages and BM-DCs (Fig. 6*B*). These results agreed with those obtained in vivo (Fig. 6*A*). Moreover, in the culture of macrophages and BM-DCs from WT mice, IL-6 production showed a continual increase, whereas TNF-α secretion peaked at 24 h and decreased thereafter (Fig. 7), suggesting that an elevated level of IL-6 prevented TNF-α production, at least partially. Other reports have indicated that IL-6 releases persist longer than those of TNF-α in other Gram-positive bacterial infections (44, 45).

The negatively regulated mechanism between TNF-α and IL-6 production in the early innate immune response is currently not well understood. Gonzalez-Amaro et al. (41) have reported that IL-6 production was induced by monocytes through a TNF-α-independent pathway, and the increased level of IL-6 in turn down-regulated the production of TNF-α. Other studies also demonstrated that IL-6-activated STAT3 inhibits TNF-α production by macrophages, whereas pretreatment of macrophages with TNF-α blocks IL-6-induced STAT3 activation in vitro (46, 47). Regarding the pattern of function that macrophages and DCs express, it has been proposed that these cells develop into type 1 inflammatory or type 2 anti-inflammatory subsets and can shift their functional phenotype in response to changes in the cytokine microenvironment (48, 49). As shown herein, neutralization of the cytokine in cultured supernatants with anti-murine IL-6 Ab resulted in increased TNF-α production by the macrophages and DCs from WT mice, and in reverse, treatment with anti-murine TNF-α Ab up-regulated the IL-6 production (Fig. 8). Thus, the TNF-α/IL-6 balance in the early innate immune response may be mediated by an intercellular mechanism.

To the best of our knowledge, no previous studies have described the effect of the TNF-α/IL-6 balance on regulating inflammatory and immune responses, but TNF-α is reported to be required for differentiation and maturation of classical APC and NO production by macrophages, which are important effectors in regulating innate immunity and adaptive immunity (50, 51). TNF-α also initiates the polarization of naive T lymphocytes to the Th1 phenotype (52). In contrast, IL-6 is an effective differentiation factor for Th2 cells and can directly up-regulate Th2 cytokine production and suppress IFN-γ secretion by CD4$^+$ T lymphocytes (43). Therefore, it is tempting to consider that the TNF-α/IL-6 balance is important in modulating the immune response to different pathogens.

In summary, it appears that TNF-α plays an essential role in early bacterial clearance, cellular recruitment, and generation of a structurally effective granulomatous response following *R. aurantiacus* infection. Moreover, the absolute effect of the TNF-α/IL-6 balance on regulating granulomatous inflammation is distinctly revealed in the current study. Thus, as a general concept, the TNF-α/IL-6 balance should receive great attention in the study of immunomodulation.

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Disclosures

The authors have no financial conflict of interest.

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