American Journal of Pathology, Vol. 166, No. 6, June 2005


*Gastrointestinal, Hepatobiliary and Pancreatic Pathology*

# Acute Tumor Necrosis Factor-α-Induced Liver Injury in the Absence of Tumor Necrosis Factor Receptor-Associated Factor 1 Gene Expression

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**Pulmonary and serum levels of tumor necrosis factor-α (TNF-α), are elevated in many lung diseases, causing local inflammation, fever, and multiorgan, including hepatic, dysfunction. Cellular responses to TNF-α are determined by recruitment of specific proteins to intracellular receptor signaling complexes. One of these proteins, TNF receptor-associated factor 1 (TRAF1), is highly regulated in pulmonary cells. To determine the effect of reduced pulmonary TRAF1 expression, TRAF1-null (−/−) and control, BALB/c (wild-type), mice were treated intratracheally, intraperitoneally, or intravenously, with TNF-α. Despite relatively mild lung injury, intratracheal TNF-α-treated TRAF1−/− mice exhibited marked liver injury with an approximate fivefold increase in serum liver enzyme levels as compared to wild-type mice. In addition, serum TNF-α levels were strikingly elevated in TRAF1−/− mice. Pretreatment with neutralizing anti-TNFRI antibody significantly reduced liver injury and serum TNF-α. Cells isolated by bronchoalveolar lavage from intratracheally treated TRAF1−/− mice produced more TNF-α than cells from treated wild-type mice, suggesting that lung cells contributed to elevated serum TNF-α. These studies suggest that TRAF1 provides negative feedback for TNF-α synthesis and limits TNFRI-mediated systemic effects of TNF-α originating in the lung. *(Am J Pathol 2005, 166:1637–1645)***

Acute liver failure is a clinical syndrome characterized by elevated serum levels of liver transaminases including aspartate and alanine aminotransferases (AST and ALT) and pathologically by liver cell death. The syndrome occurs in the setting of viral, fungal, and bacterial infections, alcohol-, drug-, and toxin-induced hepatitis and may ultimately require liver transplantation for survival. The cytokine tumor necrosis factor-α (TNF-α) has been implicated in numerous studies as having a critical role in the pathogenesis of acute liver failure.[1,2]

The effects of TNF-α on the liver, as in many tissues, are diverse and variable. Seemingly within the same cell, TNF-α can signal apoptotic, necrotic, or aponecrotic cell death or, on the other hand, proliferation and secondary cytokine production. It is likely that the availability of specific proteins for interaction with the TNF receptor-signaling complex determine which TNF response is predominant. Hepatocytes are primarily resistant to TNF-α-induced cell death and even proliferate in response to TNF-α under certain circumstances such as partial hepatectomy.[3] However, marked TNF-α-mediated hepatocytotoxicity does occur in the presence of inhibitors of transcription,[4] impaired anti-oxidant defense,[5] or in the absence of transcription factor nuclear factor (NF)-κB activity.[2,6]

In hepatocytes, as in most cells, NF-κB activity is enhanced by TNF-α. The importance of NF-κB in hepatocyte homeostasis was demonstrated in the fetal death of NF-κB/RelA-null mice with extensive apoptosis of the fetal liver, suggesting that NF-κB activation may be needed to protect liver cells from TNF-induced cell death.[7] Partial rescue of NF-κB/RelA-deficient embryos by breeding with TNF-α- or TNFR1-null strains suggests that NF-κB

Supported by the National Institutes of Health (grants R01HL63039 to G.P., T32ES07026 and T32HL72061 to J.R., K08AI055888 to R.P., CA095127 to E.T., and P30ES01247 to G.P. and R.P.).

Accepted for publication February 7, 2005.

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inhibition increases liver sensitivity specifically to TNF-α-induced cell death.[8] Evidence suggests that the survival effect of NF-κB activation is related to transcriptional induction of a number of anti-apoptotic genes including the caspase inhibitors, cIAP 1 and cIAP 2, and the TNF receptor-associated factors, TRAF1 and TRAF2, that interact to inhibit caspase 8 activation in response to TNF-α.[9]

TNF receptor-associated factors (TRAFs) are members of a family of proteins characterized by an amino terminal RING finger, several zinc fingers, and a carboxyl-terminal TRAF domain, that mediate interactions of the TRAF proteins with receptors and secondary signal transduction proteins. The functions of the TNF-α receptors, TNFRI and TNFRII, are modified by their recruitment of TRAF1 and TRAF2. TRAF2, along with the receptor-interacting protein, RIP, has a crucial role in the TNF receptor-mediated activation of both NF-κB and AP-1 and in the initiation of an anti-apoptotic response.[10] TRAF2 mediates the interaction of TNFR1 with the NF-κB-inducing IKK complex[11] and with cIAP1.[12] Overexpression of TRAF2 activates NF-κB whereas expression of a dominant-negative TRAF2, lacking the RING finger, blocked TNF-induced NF-κB activity.[13] Cells from TRAF2−/− mice failed to induce AP-1 in response to TNF-α, had a marked reduction in IKK complex formation, and were highly susceptible to apoptosis.[14] Because TRAF2 is critical to AP-1 and NF-κB activation and to a prosurvival response to TNF-α, regulation of TRAF2 protein levels and subcellular localization would be expected to be highly regulated, yet TRAF2 is ubiquitously expressed and although TNFR2 activity increases degradation of TRAF2, on a whole tissue or cell basis, expression of TRAF2 is primarily constitutive.[15,16] These observations suggest that regulation of TRAF2 activity depends at least in part on its availability for recruitment to receptor signaling complexes. Recent studies suggest that TRAF1 regulates subcellular localization and turnover of TRAF2.[17]

TRAF1 is unique among the TRAF proteins in that it lacks the amino-terminal RING and zinc fingers, its basal expression is restricted to lung, testis, and spleen, and it is induced by active NF-κB.[18] The physiological function of TRAF1 is less well understood but a number of studies suggest that it is a negative regulator of TNF-α-induced cytotoxicity. Transgenic mice overexpressing TRAF1 in T lymphocytes demonstrated reduced antigen-induced apoptosis supporting an anti-apoptotic TRAF1 function.[19] In addition, TRAF1−/− mice demonstrated hypersensitivity to TNF-α-induced skin necrosis.[20] There are conflicting reports regarding the role of TRAF1 in NF-κB and AP-1 regulation. Lymphocytes of the TRAF1−/− mice, unlike wild-type (WT), demonstrated increased NF-κB and AP-1 activation in response to TNF-α.[20] Other studies suggest that the presence of TRAF1 inhibits TRAF2-mediated transcription factor activation.[21,22] The controversy regarding TRAF1 in NF-κB activation may be explained in part by the observation that full-length TRAF1 protein modulates transcription factor activation in a receptor-selective manner but a naturally occurring, caspase-mediated C-terminal cleavage product of TRAF1 functions as a general inhibitor of NF-κB.[23] TRAF1 appears to modulate NF-κB and AP-1 activation in a complex manner, both dependent and independent of TNF receptor family member and TRAF2.

TRAF1 is expressed by alveolar macrophages and is highly regulated in pulmonary epithelial cells by TNF-α.[24] In the current study of intratracheal TNF-α treatment of TRAF1−/− mice, we demonstrate a protective role of TRAF1 in preventing hepatotoxicity, limiting circulating TNF-α levels and TNF-α synthesis in pulmonary cells. In addition, the unique observation that TRAF1 is present and inducible in the resident macrophages of the liver, the Kupffer cells, suggests that the protein may also modulate the direct response of these cells to TNF-α.

## Materials and Methods

### Animals

The TRAF1-null (TRAF1−/−) strain was generated by homologous recombination as previously described.[20] BALB/c-TRAF1−/− mice were generated by backcrossing of 129/SV-TRAF1−/− mice onto BALB/c mice for eight generations. Control, WT BALB/cJ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Both control and transgenic strains were bred and maintained in microisolator cages in specific pathogen-free rooms at the University of Rochester Medical Center. All animal care and experimental protocols follow the guidelines of the Institutional Animal Care and Use Committee.

### Intratracheal TNF-α Exposure

Age- and sex-matched 8- to 10-week-old WT and TRAF1−/− mice were treated with control saline or 0.02, 1.0, or 5.0 μg/50 μl rmTNF-α per mouse (R&D Systems, Inc., Minneapolis, MN), delivered by blunt needle endotracheal intubation under light isoflurane anesthesia. Whole blood, collected by cardiac puncture, and liver tissue were isolated 20 hours after treatment. A subset of mice were treated by intraperitoneal injection with anti-TNFRI blocking antibody (100 μg, clone 55R-170; R&D Systems), isotype control IgG (Southern Biotechnology, Birmingham, AL) or diluent saline 24 hours before intratracheal TNF-α treatment.

### Ribonuclease Protection Assay

Total RNA was extracted by Phase Lock Gel II columns (Eppendorf-5 Prime, Inc., Westbury, NY) from lung tissue homogenized and lysed in 4 mol/L guanidinium isothiocyanate (Kodak Chemical Co., Rochester, NY), 0.5% *N*-lauryl sarcosine and 25 mmol/L sodium citrate (Sigma Chemical Co., St. Louis, MO) as previously described.[25] TNF-α, MIP-2, MCP-1, TNFRI, TNFRII, interleukin (IL)-1RI, and IL1RII mRNA levels, normalized to rpL32 mRNA content, were quantified by ribonuclease protection assays performed with commercial reagents and protocols (Riboquant; Pharmingen, Inc., San Diego, CA). Radiolabeled, single-strand RNA probes from templates mCK5

and mCR4 were synthesized at room temperature using ($\alpha^{32}$P)-UTP (3000 Ci/mmol, EasyTides; New England Nuclear, Boston, MA) and T7 polymerase. RNA samples (5 $\mu$g by absorbance at 260 nm), including murine RNA and yeast tRNA (2 $\mu$g) as positive and negative controls, were dried, resuspended in 8 $\mu$l of hybridization buffer plus 2 $\mu$l of radiolabeled probe ($3 \times 10^5$ cpm/$\mu$l), denatured at 90°C and incubated overnight at 56°C. After incubation, single-stranded RNA was digested in an RNase A/T1 cocktail, followed by proteinase K. The protected radiolabeled RNA fragments were resolved on a 6% acrylamide/urea gel (Life Technologies Inc., Grand Island, NY), using radiolabeled probe (1000 to 2000 cpm) as size markers. The intensity of protected probe bands in dried gels was quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and normalized to rpL32 mRNA content of each sample.

### *Histology*

The left lung was inflation-fixed at 10 cm $H_20$ pressure with 10% buffered formalin for 24 hours, dehydrated, and paraffin-embedded. The left liver lobe was similarly prepared after immersion in 10% buffered formalin. Tissue sections (4 $\mu$m) were analyzed by light microscopy using hematoxylin and eosin (H&E) stain, and assayed for DNA fragmentation by fluorescent microscopy using a terminal dUTP nick-end labeling system (TUNEL) (Roche Diagnostics, Indianapolis, IN). Kupffer cells were identified by fluorescence immunohistochemistry with anti-F4/80 antibody with 4,6-diamidino-2-phenylindole nuclear counterstain, as previously described.[26]

### *Kupffer Cell Isolation*

Mice were anesthetized with isoflurane, the liver exposed and the portal vein perfused with warm Hanks' balanced salt solution (HBSS) + 0.5 mmol/L ethylenediamine tetraacetic acid for 2 minutes and then with warm collagenase buffer (HBSS, 5% fetal bovine serum, 5 mmol/L $CaCl_2$, 0.32 mg/ml collagenase, 0.16 $\mu$g DNase) for 3.5 minutes, as previously described.[27] The liver was excised, placed in collagenase buffer for 5 minutes at 37°C, and then placed on ice. The liver capsule was removed and the liver mashed and shaken to release cells. Cells were filtered through a 70-$\mu$m strainer and centrifuged ($50 \times g$, 2.5 minutes, 4°C). The supernatant was collected and centrifuged (1400 rpm, 10 minutes, 4°C). The pellet was resuspended in 2.6 ml and mixed with 7 ml of 0.276 g/ml Nycodenz (Sigma Chemical Co.) solution. HBSS (4 ml) was layered on top of the Nycodenz. The cell gradient was centrifuged (1500 rpm, 25 minutes). Cells at the interface were removed, washed three times with media (RPMI 1640, 10% fetal bovine serum, 100 U/ml Pen/Strep) and either placed in culture or homogenized in anti-protease buffer. Purity of cultured cells (>98%) was verified by morphology and latex bead endocytosis assay.[27]

### *Western Blot Analysis*

Frozen lung, thymus, spleen, and liver tissue samples, ~100 $\mu$g, were homogenized in protein lysis buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L sodium chloride, 2 mmol/L ethylenediamine tetraacetic acid, 25 mmol/L sodium fluoride, $\beta$-glycerolphosphate, 0.1 mmol/L sodium vanadate, 1 mmol/L phenylmethyl sulfonyl fluoride, 0.2% Triton X-100, 0.3% Igepal CA-630, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin A, 10 $\mu$g/ml aprotinin; Sigma Chemical Co.). Total protein content of the lysates was determined by bicinchoninic acid assay using commercial reagents and protocol (Pierce, Inc., Rockford, IL). Proteins (10 $\mu$g) in Laemmli buffer were size-separated on polyacrylamide-sodium dodecyl sulfate gels and transferred to polyvinylidene difluoride membrane. Membranes were blocked overnight in phosphate-buffered saline (PBS) with 5% nonfat dry milk at 4°C before a 1-hour incubation in PBS, 5% nonfat dry milk, 0.05% Tween-20 with a rabbit, anti-TRAF1 polyclonal IgG antibody (0.1 $\mu$g/ml, sc-874; Santa Cruz Biotechnology, Santa Cruz, CA) or subsequently, a rabbit, anti-actin polyclonal IgG antibody (1:5000, A-2066; Sigma). Membranes were then washed in PBS with 0.05% Tween-20 and incubated in peroxidase-conjugated anti-rabbit secondary IgG (1:5000, Santa Cruz Biotechnology). Immunodetection was performed using enhanced chemiluminescence protocols and reagents (ECL Plus; Amersham, Arlington Heights, IL).

### *Bronchoalveolar Lavage (BAL) and Cell Culture*

The trachea was intubated and the lungs isolated before exhaustive lavage with warmed normal saline (37°C, 1 ml × 10). Samples (50 to 100 $\mu$l) of the combined first 2 ml of BAL were frozen at −80°C until assayed for total protein and TNF-$\alpha$ by bicinchoninic acid assay and enzyme-linked immunosorbent assay (ELISA), respectively, using commercial reagents and protocols (Pierce, Inc.) and (DuoSet, R&D Systems). The remaining lavage fluid from all 10 aliquots was pooled and centrifuged at $250 \times g$ for 10 minutes at 4°C. The pelleted cells were resuspended in 1 ml of 0.15 mol/L $NH_4Cl$, 0.01 mol/L $NaHCO_3$ solution for 10 minutes to lyse red blood cells, then washed twice with Hanks' balanced salt solution and resuspended in tissue culture plates in RPMI media (Life Technologies Inc.), 10% fetal bovine serum, 1% antibiotic/antimycotic, and 10 $\mu$g/ml brefeldin A, the latter added to inhibit TNF-$\alpha$ secretion. The cells were cultured for 4 hours (37°C, 5% $CO_2$) then harvested by centrifugation (1000 rpm, 4°C, 5 minutes). Cell pellets were lysed in a protein lysis buffer (see Western blot assay) and stored at −80°C until assayed for total protein and TNF-$\alpha$. Serum TNF-$\alpha$ was also measured by ELISA.

### *Statistical Analysis*

Quantitative data were analyzed by single-factor analysis of variance and Fischer's protected least significant difference statistic using Stat View 4.0 statistical analysis software (SAS Institute, Cary, NC).

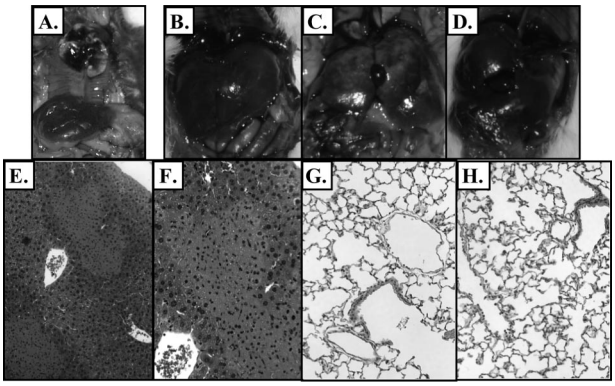


**Figure 1.** Acute hepatocytotoxicity in TRAF1−/− liver 20 hours after intratracheal treatment with rmTNF-α (5 μg/mouse). **A:** Intratracheal instillation of methylene blue and immediate necropsy confirmed treatment of pulmonary compartment. **B** and **C:** Gross liver pathology of TRAF1−/− mouse treated with diluent saline (**B**) or intratracheal rmTNF-α (**C**) (5 μg/mouse) as compared to BALB/c (WT) mouse treated with intratracheal rmTNF-α (**D**). **E** and **F:** Photomicrographs of H&E-stained TRAF1−/− liver 20 hours after intratracheal rmTNF-α treatment. **G** and **H:** Photomicrographs of H&E-stained WT (**G**) and TRAF1−/− (**H**) lung 20 hours after intratracheal rmTNF-α treatment. Original magnifications: ×200 (**E**, **G**, **H**); ×400 (**F**).

## *Results*

To determine the significance of TRAF1 expression in TNF-α-induced lung disease, TRAF1-null (−/−) mice were analyzed after intratracheal delivery of recombinant murine (rm) TNF-α. Before experiments with TNF-α, intratracheal instillation of 10% methylene blue (50 μl/mouse) was used to test the specificity and distribution of intratracheal treatment (Figure 1A). Mice were sacrificed immediately after intratracheal instillation and the distribution of dye was noted. The majority of instilled dye was deposited in the lungs in a perihilar distribution. Little to no dye was detected in the esophagus or stomach.

### *Intratracheal TNF-α Induced Ocular Exudates and Hepatocytotoxicity in TRAF1−/− Mice*

Because previous experiments demonstrated significant increase in lung chemokine expression within 20 hours of intratracheal rmTNF-α (5 μg/mouse) treatment,[25] this model was used to test the phenotype of TRAF1-null mice in TNF-α-induced injury. BALB/c (WT) mice treated with TNF-α were healthy in appearance at the time of harvest. In contrast, the TNF-α TRAF1−/−-treated mice demonstrated decreased spontaneous movement, huddling, and ruffled fur. These mice frequently had exudates around the eyes and were light sensitive as compared to the WT controls (data not shown). Necropsy was striking for gross liver abnormalities described as sharply demarcated dusky areas in the intratracheal TNF-α-treated TRAF1−/− mice but not in the saline-treated knockout mice, nor in the saline or TNF-α-treated WT (Figure 1; B to D). There was no other evidence of gross organ damage. Light microscopy of H&E-stained lung sections demonstrated mild injury with subtle increase in cells, consistent with macrophages, in the air space (Figure 1, G and H). Results of total protein and LDH assay suggested a trend toward increased injury in the TRAF1−/− TNF-treated lungs but without statistical significance (Table 1). Total cells per ml lavaged from the TNF-treated lungs tended to be reduced, but not significantly, in comparison to saline-treated controls, independent of TRAF1 expression. Reduction in BAL cells is proposed to be due to activation and adhesion to the airway surface. Light microscopy of H&E-stained liver from intratracheal TNF-α-treated TRAF1−/− mice showed patches of periportal and central lobular damage with condensation of nuclei and loss of cellular definition (Figure 1, E and F). The affected areas were heterogeneous in size and the overall severity variable within the treatment group. Such abnormalities were rarely detected in TNF-α-treated WT mice and were not seen in saline-treated WT or TRAF1−/−.

Fluorescent TUNEL was positive and nuclear in patches corresponding to sites of liver injury seen by light microscopy, suggesting the presence of DNA strand breaks and potential apoptotic cell death (Figure 2; A to E). Dual localization of TUNEL-positive cells with Kupffer cell anti-F4/80 immunohistochemistry, representing resident liver macrophages, demonstrated that nuclear damage involved primarily hepatocytes. Occasional Kupffer cells were also TUNEL-positive both within the islands of severe liver injury as well as in more peripheral regions (Figure 2F). The dual labeling suggests either primary Kupffer cell damage or phagocytosis of damaged nuclei. Neither DNA laddering nor caspase 3 activity, the latter assessed by Western blot analysis and by *in vitro* activity assay using fluorogenic DEVD-AMC substrate, were detected (data not shown), however the injury may have advanced beyond the apoptotic stage by the time of analysis 20 hours after treatment.

**Table 1.** Bronchoalveolar Lavage of WT and TRAF1−/− Mice 20 Hours after Intratracheal Saline or rmTNF-α

| BAL analysis | Saline | | TNF-α | |
|---|---|---|---|---|
| | WT | TRAF1−/− | WT | TRAF1−/− |
| Cell number × $10^6$/ml | 5.1 ± 0.6 | 5.1 ± 1.0 | 3.5 ± 0.3 | 4.8 ± 1.3 |
| Macrophages (%) | 93.5 ± 6.1 | 96.9 ± 2.3 | 93.1 ± 3.2 | 98.1 ± 0.4 |
| Neutrophils (%) | 6.2 ± 6.1 | 2.7 ± 2.2 | 6.6 ± 3.1 | 1.2 ± .3 |
| Lymphocytes (%) | 0.2 ± 0.1 | 0.4 ± 0.1 | 0.2 ± 0.2 | 0.7 ± 0.4 |
| LDH | 47.0 ± 4.5 | 57.5 ± 4.3 | 70.5 ± 17.3 | 121.5 ± 25.8 |
| Total protein | 255.6 ± 17.0 | 292.0 ± 30.3 | 352.5 ± 58.9 | 450.0 ± 108.2 |

Mean +/− SEM; n = 7–8; $P > 0.1$ between strains in all cases.
LDH, lactate dehydrogenase.

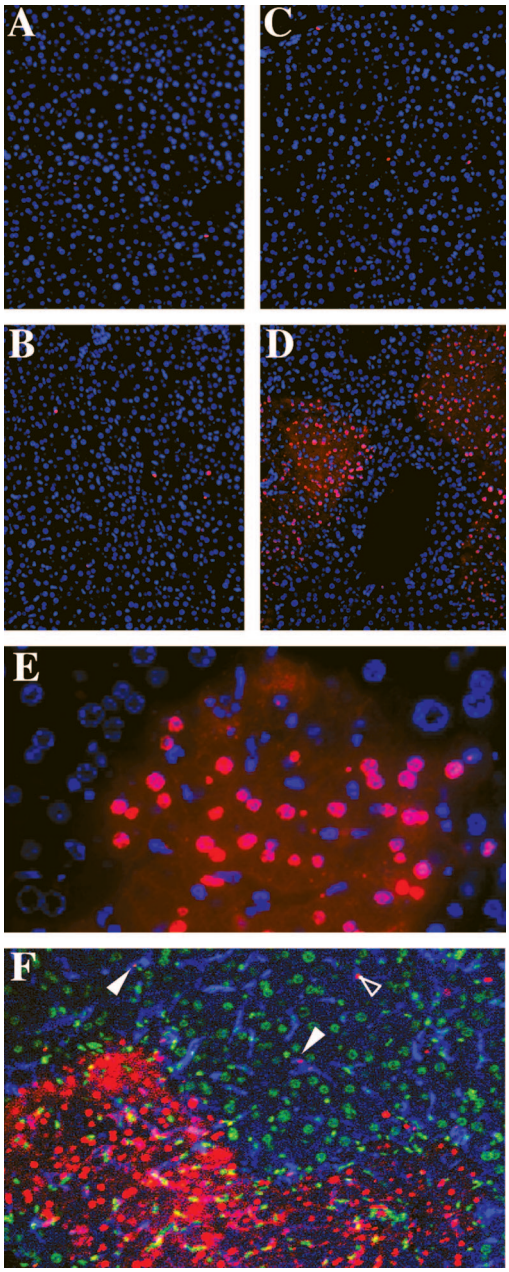


**Figure 2.** Positive TUNEL assay in TRAF1−/− mice treated intratracheally with rmTNF-α. **A** to **E:** Liver sections harvested 20 hours after treatment of WT (**A**, **B**) and TRAF1−/− (**C–E**) mice with intratracheal saline (**A**, **C**) or rmTNF-α (**B**, **D**, **E**), evaluated by TUNEL assay (red nuclei) and 4,6-diamidino-2-phenylindole counterstain (blue nuclei). **F:** TRAF1−/− liver 20 hours after intratracheal TNF-α treatment assayed by TUNEL (red), anti-F4/80 antibody immunohistochemistry marking Kupffer cells (blue), and Sytox green nucleic acid counterstain. **Arrows** indicate TUNEL-positive Kupffer cells (4-μm sections). Original magnifications: ×200 (**A–D**, **F**); ×400 (**E**).

## *TRAF1 Protein Is Induced in Kupffer Cells by Intratracheal TNF-α*

TRAF1 was originally reported to be expressed in lung, thymus, spleen, and testis and has since been shown to be highly inducible in pulmonary epithelial cells as well as T lymphocytes. To determine expression of the protein in normal mouse liver, Western blot analysis for TRAF1 and, as control, β-actin, was performed on whole tissue homogenates prepared from lung, thymus, spleen, and liver of BALB/c and TRAF1−/− mice 20 hours after intratracheal treatment with saline or TNF-α. Using antibody specific for the amino-terminus (N19), TRAF1 protein was detected in lung, thymus, and spleen of WT animals (Figure 3A). As expected, no bands consistent with TRAF1 protein were detected in TRAF1−/− mouse tissues. TRAF1 protein was not detected in up to 60 μg of whole liver homogenate of either WT or knockout animals, despite TNF-α treatment, suggesting little or no expression of the protein in the hepatocytes that compose the bulk of liver tissue.

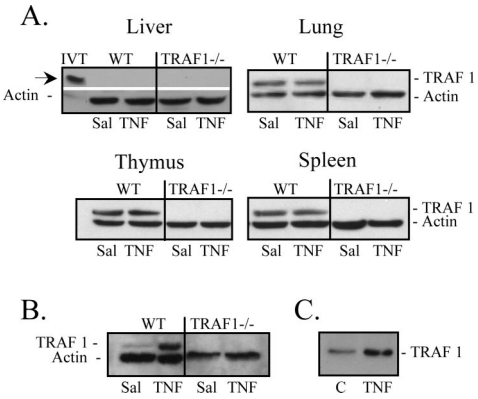


**Figure 3.** TRAF1 protein detected in lung, thymus, and spleen and induced by TNF-α in Kupffer cells. **A** and **B:** Western blot analysis of protein samples (25 μg) using anti-amino terminal TRAF1 (N19) antibody followed by anti-actin antibody for standardization. **A:** Whole liver, lung, thymus, and spleen homogenates isolated from WT and TRAF1−/− mice 20 hours after intratracheal treatment with saline (Sal) or rmTNF-α (TNF). TRAF1 was detected in lung, spleen, and thymus of WT but not TRAF1−/− mice or in whole liver homogenates (**arrow**). IVT: *In vitro*-transcribed TRAF1 protein as positive control. **B:** TRAF1 was detected in Kupffer cells isolated from liver of WT mice 20 hours after intratracheal saline and to a greater level after intratracheal TNF-α. **C:** Anti-TRAF1 Western analysis of lysates of Kupffer cells isolated from untreated WT mice treated *in vitro* with control media (C) or rmTNF-α (TNF, 25 ng/ml) for 6 hours before protein harvest.

To determine whether the resident macrophages of the liver express TRAF1 protein, as has been demonstrated in alveolar macrophages,[24] Kupffer cells were isolated from liver of BALB/c mice 20 hours after intratracheal treatment with saline or rmTNF-α. Protein consistent with TRAF1 was detected in Kupffer cells of saline-treated animals and was markedly induced in Kupffer cells isolated from WT mice after TNF-α treatment (Figure 3B). TRAF1 protein was not detected in Kupffer cells of TRAF1−/− mice. Kupffer cells were also isolated from untreated, WT livers and treated for 6 hours *in vitro* with control media or with TNF-α (25 ng/ml) before isolation for Western analysis. TRAF1 protein was present at low levels in media-treated Kupffer cells and was induced *in vitro* by TNF-α (Figure 3C).

## *In TRAF1−/− Mice the Degree of Hepatocytotoxicity Was Dose-Dependent and Associated with Elevated Serum Liver Enzymes and Serum TNF-α*

Supporting the histopathological evidence of liver injury, serum ALT and AST aminotransferase levels were markedly elevated in the intratracheal TNF-α-treated TRAF1−/− animals, as compared to the WT controls, and correlated with

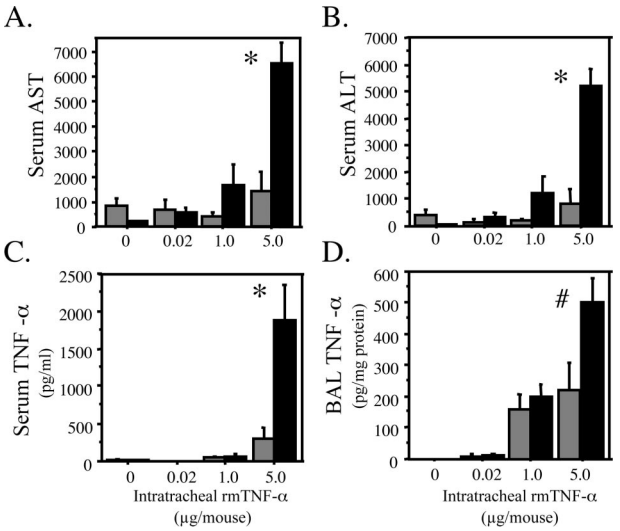


**Figure 4.** TNF-α dose and TRAF1-dependent increase in serum aminotransferase and TNF-α levels. AST (**A**) and ALT (**B**) were measured in serum isolated by intracardiac puncture from WT (**gray bars**) or TRAF1−/− (**black bars**) mice 20 hours after intratracheal treatment with increasing doses of rmTNF-α. *, $P < 0.001$ TRAF1−/− as compared to WT at same dose. For dose versus AST or ALT, $R^2 = 0.71$ and 0.69, respectively, in TRAF1−/− and $R^2 < 0.3$ in WT. Serum (**C**) and BAL (**D**) were assayed by mTNF-α ELISA, 20 hours after intratracheal treatment of WT (**gray bars**) and TRAF1−/− (**black bars**) mice with increasing doses of rmTNF-α. *, $P < 0.01$; #, $P < 0.03$ TRAF1−/− compared to WT at same TNF dose. Mean ± SEM, $n = 7$ to 11.

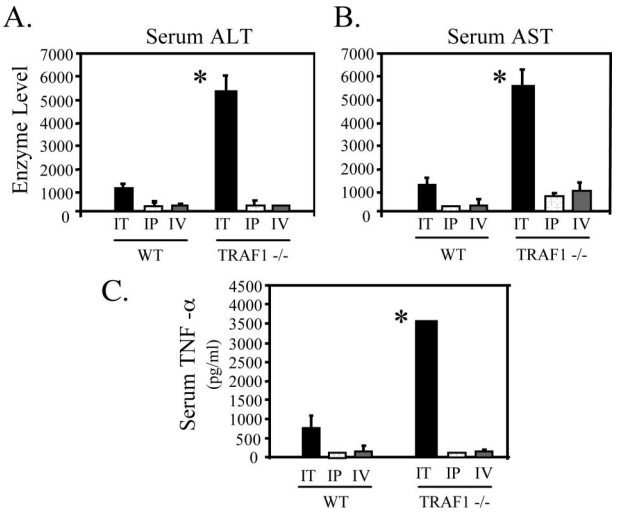


**Figure 5.** Intratracheal but not intraperitoneal or intravenous rmTNF-α induced hepatocytotoxicity and elevated serum TNF-α in TRAF1−/− mice. Serum harvested from WT and TRAF1−/− mice 20 hours after treatment with rmTNF-α (5 μg/mouse) either intratracheally (IT), intravenously (IV, tail vein), or intraperitoneally (IP) were assayed for ALT (**A**), AST (**B**), or murine TNF-α (**C**), as described in Materials and Methods. Mean ± SEM, $n = 3$ to 5. *, $P < 0.01$ intratracheal TNF-α treatment of TRAF1−/− as compared to all other values.

the severity of gross liver injury. The degree of hepatocellular injury in the TRAF1−/− mice, as measured by serum ALT and AST, was dependent on intratracheal TNF-α dose (Figure 4, A and B). No dose-dependent increase in serum ALT or AST was detected in WT mice.

Elevations of serum TNF-α detected in patients or in experimental models after an inflammatory stimulus are typically short-lived as the cytokine is rapidly cleared from the circulation. However, the severity of illness in TNF-related disease is, in many cases, correlated with serum or BAL TNF-α levels.[28–31] To characterize the systemic effects of intratracheal TNF-α, serum cytokine levels were measured after treatment of WT and TRAF1−/− mice. A dose-dependent increase in TNF-α levels was measured by ELISA in both serum and BAL 20 hours after intratracheal treatment (Figure 4, C and D). Serum and BAL TNF-α levels were significantly higher at 20 hours in the TRAF1−/− mice than in the WT after treatment with 5 μg rmTNF-α (Figure 4C). Of note is that at the rmTNF 1 μg/mouse dose, serum and BAL levels of measured TNF-α in WT animals were not significantly different from that in the TRAF1−/− mice, yet there was a trend toward increased serum liver enzyme levels in TRAF1−/− but not WT at this dose, potentially dissociating the severity of liver injury from the serum TNF-α level.

## *Intraperitoneal and Intravenous Administration of rmTNF-α Did Not Induce Hepatocytotoxicity in WT or TRAF1−/− Mice*

To determine whether the liver toxicity demonstrated after intratracheal delivery of TNF was a unique effect of the site of delivery, mice were treated either by intraperitoneal or intravenous (tail vein) injection with an equivalent dose of rmTNF-α (5 μg/mouse) as was given intratracheally. Intraperitoneal and intravenous administration of TNF-α induced significantly less hepatocytotoxicity in TRAF1−/− mice compared to intratracheal administration as reflected in much reduced levels of serum transaminases (Figure 5, A and B). Gross liver injury was noted in only one TRAF1−/− mouse treated intraperitoneally and the lesions were small and infrequent. Serum TNF-α levels were elevated at 20 hours after treatment only in intratracheally treated animals, where they remained significantly higher in the TRAF1−/− compared to WT mice (Figure 5C).

## *Alveolar Macrophages Isolated from Intratracheal TNF-α-Treated TRAF1−/− Mice Produce More TNF-α Than Do Cells from WT Animals*

Because intratracheal treatment with TNF-α was more injurious than intravenous or intraperitoneal treatment, it appeared likely that the TRAF1−/− lung was hyperresponsive to exogenous TNF-α and potentially released increased quantities of hepatotoxic inflammatory mediators. Figure 6A demonstrates that lung mRNA content for the TNF receptors and IL-1 receptors was not different between saline-treated WT and TRAF1−/− mice. Each receptor mRNA level, except for TNFRI (p55), was induced after TNF-α treatment, notably to a significantly greater extent in the TRAF1−/− than in the WT lungs. Figure 6B also indicates that MIP-2 expression, presented as a representative chemokine previously demonstrated to be induced by exogenous TNF-α in the lung, was also induced to a greater extent at both the RNA and protein level in the TRAF1-null lungs. In contrast, MCP-1 was markedly elevated after TNF-α treatment but did not appear differentially regulated between WT and

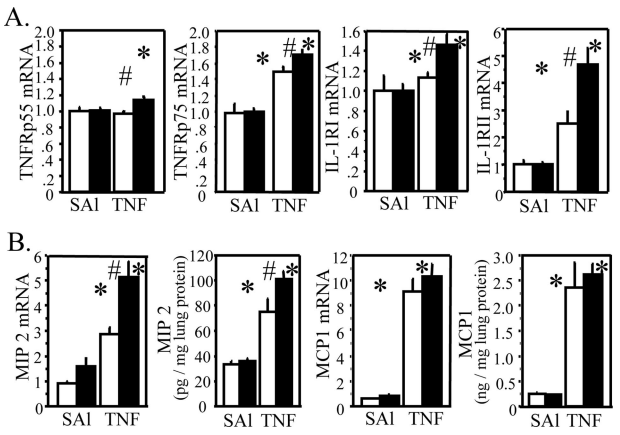


**Figure 6.** Increased inflammatory response in TRAF1−/− lung after intratracheal TNF-α. **A** and **B:** mRNA encoding TNFRI, TNFRII, IL-RI, and IL-RII (**A**) and MIP-2 and MCP-1 (**B**) were measured by ribonuclease protection assays in RNA isolated from lung homogenates of WT (**white bars**) or TRAF1−/− (**black bars**) mice, 20 hours after intratracheal treatment. **B:** MIP-2 and MCP-1 protein were also measured in lung homogenates by ELISA. *, $P < 0.001$ TNF versus saline-treated strain-specific control; #, $P < 0.05$ TRAF1−/− versus WT both TNF-α treated. Mean ± SEM, $n = 5$ to 6.

TRAF1−/− mice. These findings, along with a trend toward increased LDH and protein leak into BAL fluid (Table 1), do support greater TNF-induced injury in the TRAF1−/− lungs.

Because TNF-α protein levels were greater in BAL and serum of TRAF1-null mice, we sought to determine whether TRAF1−/− pulmonary macrophages produced more TNF-α in response to stimulation than did WT. Cells were harvested by BAL of WT and TRAF1−/− mice 20 hours after intratracheal TNF-α (5 μg/mouse) or saline treatment. By differential cell count, 93 to 98% and 7 to 2% of the BAL cells were, respectively, macrophages and neutrophils in WT and TRAF1−/− TNF-α-treated mice (Table 1). The lavaged cells were washed twice and placed in tissue culture medium. TNF-α was measured by ELISA in the cell pellet and samples of conditioned media collected after a 4-hour culture in medium with brefeldin A. The values were normalized to the total protein content of the cell pellet. Despite the brefeldin A, small amounts of TNF-α were secreted into the media of cells from TNF-α-treated animals but not saline controls perhaps representing shedding of transmembrane TNF (Figure 7A). The conditioned media of *in vivo* TNF-α-treated, TRAF1−/− cells contained threefold to fourfold

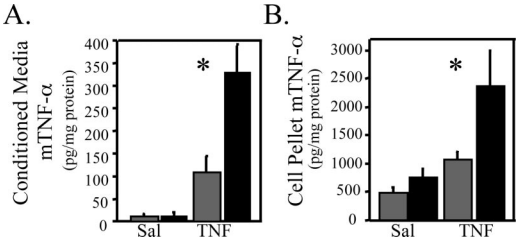


**Figure 7.** Cells lavaged from TRAF1−/− mice produced more TNF-α than did WT cells. Cells obtained from BAL of WT (**gray bars**) or TRAF1−/− (**black bars**) mice 20 hours after intratracheal treatment with saline or rmTNF-α were cultured in brefeldin A and RPMI media for 4 hours before harvest in anti-protease buffer. Murine TNF-α was assayed by ELISA in conditioned media (**A**) and cell pellet homogenates (**B**). The data are presented relative to the total protein concentration of the cell pellets. Mean ± SEM, $n = 6$ to 7 mice. *, $P < 0.05$ TRAF1−/− versus WT.

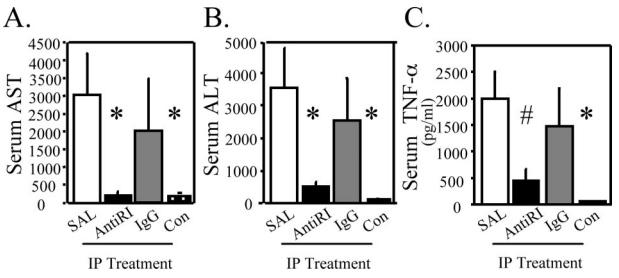


**Figure 8.** Neutralizing, anti-TNFRI antibody prevented elevation in serum levels of liver enzymes. TRAF1−/− mice were pretreated intraperitoneally with saline, TNFRI-blocking antibody, or an IgG isotype control, then treated intratracheal with rmTNF-α and harvested at 20 hours for serum AST (**A**), ALT (**B**), and TNF-α (**C**). Con represents levels in mice treated only intratracheally with saline. Mean ± SEM, $n = 10$, except $n = 3$ for IgG control. *, $P < 0.02$; #, $P < 0.05$ as compared to intratracheal TNF, intraperitoneal saline-treated mice.

more TNF-α than media conditioned by WT cells. Likewise, the cellular TNF-α content was approximately twofold greater in TRAF1-null than in WT cells (Figure 7B).

In most cases, TNF-α-induced pulmonary and hepatic injury has been shown to be mediated by the TNFRI receptor, yet TRAF1 also interacts with and likely modulates the activity of TNFRII. Pretreatment with TNFRI blocking antibody demonstrated that the increase in serum TNF-α and the increased sensitivity of TRAF1-null mice to liver injury was dependent on TNF receptor I activity (Figure 8).

## *Discussion*

Elevated serum and BAL levels of TNF-α correlate with severity of illness in cases of acute respiratory distress syndrome, sepsis, pneumonia, and viral pneumonitis.[28–30,32] In addition to local pulmonary effects, circulating TNF-α can cause fever and multiorgan dysfunction, including liver failure. Cellular responses to TNF-α are determined by the recruitment of a number of intracellular signal transduction proteins to interact with ligand-activated transmembrane TNF receptors. One of these proteins, TRAF1, a potential negative regulator of TNF signaling, is highly regulated in cells of the lung.[24] To determine the impact of reduced TRAF1 expression in lung, recombinant murine (rm) TNF-α was administered by intratracheal instillation to TRAF1−/− and to control BALB/c mice. Unexpectedly marked liver injury was noted in the TRAF1-deficient mice 20 hours after treatment as reflected in histopathology and TUNEL assay. A fivefold increase in serum liver enzyme levels, ALT and AST, was also observed in knockout mice versus WT controls. Occasional Kupffer cells, identified by F4/80 antigen immunostaining, as well as many hepatocytes, were TUNEL-positive. Western analysis demonstrated induction of TRAF1 protein in WT Kupffer cells, treated either *in vivo* or *in vitro* with TNF-α suggesting that the genetic removal of TRAF1 may directly affect the Kupffer cell ability to respond to TNF-α. The severity of liver injury correlated with elevated serum and BAL TNF-α levels that were greater and more sustained in TRAF1−/− animals than in the WT controls, suggesting that the knockout animals either cleared TNF-α more slowly or produced

more TNF-$\alpha$ in a positive feedback manner. Liver toxicity was prevented by pretreatment with a neutralizing, anti-TNFRI antibody, demonstrating that the injury was TNF receptor I mediated. It remains unclear if TNFRI activity in cells of the lung, the liver, or both was required. Serum TNF-$\alpha$ levels were also reduced by anti-TNFRI pretreatment, consistent with endogenous autoinduction of the cytokine. These findings are consistent with the hypothesis that TRAF1 functions as a negative regulator of TNF-$\alpha$ synthesis and cytotoxicity, serving to limit injury occurring in response to inflammatory stimuli.

In this study, sustained serum TNF-$\alpha$ levels and hepatotoxicity were seen only when TNF was delivered into the trachea suggesting that the lung response to TNF provided the stimulus that induced liver injury. The hepatotoxin produced by the TRAF1-null lung may be TNF-$\alpha$ itself. Enhanced TNF-$\alpha$ production by pulmonary inflammatory cells in the absence of TRAF1 expression may explain elevated BAL and serum TNF-$\alpha$ in TRAF1$-/-$ mice. In TRAF1$-/-$ mice, intraperitoneal or intravenous delivery of rmTNF-$\alpha$ resulted in nearly undetectable serum levels of TNF-$\alpha$ 20 hours after treatment, in marked contrast to the elevated serum TNF-$\alpha$ measured after intratracheal delivery of the cytokine suggesting that in these animals the lung was a source or reservoir of TNF-$\alpha$. Indeed, cells lavaged from the lung of TNF-$\alpha$-treated mice synthesized more TNF-$\alpha$ when cultured *in vitro* than did cells isolated from saline-treated mice. In addition, cells lavaged from TNF-$\alpha$-treated TRAF1$-/-$ animals produced significantly more TNF-$\alpha$ *in vitro* than did WT. These findings support the role of intrapulmonary production of TNF-$\alpha$ in a systemic response to primary lung disease, consistent with previous studies[33] and the further speculation that the impact of pulmonary TNF production may be heightened if systems that limit the autoinduction of TNF-$\alpha$ are impaired.

Although liver injury in TRAF1$-/-$ is associated with elevated serum TNF-$\alpha$, hepatocytes are relatively resistant to direct TNF-$\alpha$-induced cell death.[2] In addition, TRAF1 was not demonstrable in whole liver homogenates of WT mice, even after TNF-$\alpha$ exposure, suggesting that the majority of liver cells, the hepatocytes, do not express TRAF1 protein which in turn suggests that the hepatotoxicity induced by TNF in TRAF1$-/-$ animals was a bystander effect and not directly due to enhanced hepatocyte susceptibility to TNF-$\alpha$. In this case, we speculate that hepatocyte cell death is due to non-TNF, hepatotoxic mediators originating in the injured TRAF1-null lung or in the local environment of the hepatocytes such as due to Kupffer cell activation or dysfunction.

Increased lung injury in the absence of TRAF1 could result in enhanced liver injury due to release of hepatotoxic products of inflammation other than TNF. Increased TNFRII, IL-1R, and MIP-2 expression in TRAF1$-/-$ lung, along with a trend toward increased BAL LDH and protein, suggest more severe lung injury in these mice. The differences, however, were not striking and may be explained by the demonstrated increase in pulmonary TNF-$\alpha$. Recent evidence supports a role of JNK/AP-1 activity in hepatocyte cell death that can be activated by a number of products of inflammation including IL-1.[34] Hepatocytes do undergo TNF-related cell death under conditions of ischemia/reperfusion, overwhelming inflammation, and macrophage activation with attendant oxidant stress and products of inflammation such as Fas ligand and IL-l. It is possible that the lung releases enough of these factors to induce liver injury. It may also be that the hepatocyte microenvironment in the TRAF1-null mice is abnormal enough to sensitize the cells to TNF-induced death, perhaps due to ischemia or release of free radicals. A further potential explanation is that in the absence of TRAF1, dysregulation of CD-40, or other TNF superfamily receptor with which TRAF1 interacts, could occur as a secondary effect of TNF-$\alpha$ treatment and could contribute to further lung and liver injury.[35–37] Additional studies will determine whether TRAF1-dependent susceptibility to liver injury is a direct result of increased local or circulating TNF-$\alpha$ or is an indirect effect of products of enhanced lung injury.

Kupffer cells isolated from saline-treated WT mice did contain readily detectable TRAF1 protein. In addition, intratracheal TNF-$\alpha$ treatment significantly increased the level of TRAF1 protein detected in subsequently isolated Kupffer cells. *In vitro* treatment of isolated WT Kupffer cells with TNF-$\alpha$ also induced TRAF1 protein. That TRAF1 is not detected in the majority of liver cells but is induced in Kupffer cells presents the possibility that the increased sensitivity of TRAF1$-/-$ mice to pulmonary TNF-$\alpha$ is due, at least in part, to dysregulation of Kupffer cell TNF-$\alpha$ response in the absence of TRAF1 protein. TUNEL-positive Kupffer cells in intratracheal TNF-treated TRAF1$-/-$ mice is supportive of direct cell injury or the engulfment of damaged nuclei by active macrophages. Kupffer cells are reported to have a protective function in some types of liver injury. Increased acetaminophen-induced hepatoxicity was demonstrated in association with reduced production of hepatoprotective cytokines IL-10 and IL-6 in mice depleted of Kupffer cells by infusion of liposome/clodronate.[38] Studies with IL-10$-/-$ and IL-6$-/-$ mice support the suggestion that these cytokines, and other anti-inflammatory mediators produced by activated Kupffer cells, protect surrounding hepatocytes from injury.[38,39] If TRAF1$-/-$ Kupffer cells are more susceptible to TNF-induced cell death, their loss may sensitize the remaining hepatocytes to injury. Phagocytosis of apoptotic nuclei has also been shown to strongly activate Kupffer cells. TRAF1-null Kupffer cells may be more susceptible to activation resulting in the local release of heptotoxins, such as reactive oxidant species or IL-1. Future studies will test the hypothesis that TRAF1-deficient Kupffer cells are hypersensitive to TNF-$\alpha$-induced activation and cell death, and are less able to protect surrounding hepatocytes from TNF toxicity.

Pulmonary diseases are often complicated by systemic organ dysfunction, the latter an important cause of morbidity and mortality. In the case of acute respiratory distress syndrome, mortality is often attributed to multiorgan failure, highlighting the clinical significance of systemic effects of pulmonary injury. The current study reports an intratracheal instillation model of pulmonary TNF-$\alpha$ exposure in which acute liver injury was observed in the absence of TRAF1 protein. Conditions that reduce pulmonary expression of TRAF1, such as exposure to high levels of oxygen (data not

shown), may contribute to excessive TNF-$\alpha$ production with both local and systemic toxicity. Under these conditions, enhanced TNF-$\alpha$ production and tissue hypersensitivity may in turn contribute to the development of multiorgan failure in the setting of severe lung disease.

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