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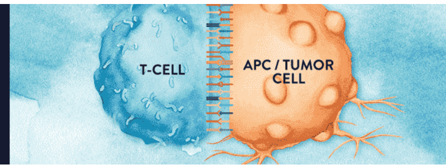

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Repression of Bleomycin-Induced Pneumopathy by TNF

Misuzu Kuroki,* Yuji Noguchi,[1†] Michihide Shimono,[†] Kazunori Tomono,* Takayoshi Tashiro,* Yuichi Obata,[‡] Eiichi Nakayama,[†] and Shigeru Kohno*

Idiopathic pulmonary fibrosis is a chronic inflammatory lung disease with interstitial fibrosis. As a potent proinflammatory cytokine, TNF has been suggested to play critical roles in the pathogenesis of the human disease and its animal model, bleomycin-induced pneumopathy. However, studies using TNF-deficient mice have demonstrated that TNF also has an anti-inflammatory function. To determine the role of TNF in pulmonary inflammation induced by bleomycin, we injected bleomycin intratracheally into TNF-deficient mice. In this study, we demonstrated persistent and intense inflammation in TNF-deficient mice due to reduced apoptosis of inflammatory cells. We also showed that in TNF-deficient mice, challenge via airways with murine, but not human rTNF, efficiently eliminated inflammatory cells from the bronchoalveolar space by apoptosis, and thus promoted tissue repair of damaged lungs. Contrary to previous reports that showed that TNF was a central mediator of pulmonary inflammation, we have demonstrated that TNF is essential for repressing pulmonary inflammation in bleomycin-induced pneumopathy. ***The Journal of Immunology,*** **2003, 170: 567–574.**

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Idiopathic pulmonary fibrosis (IPF)[2] (1) is a chronic lethal disorder characterized by persistent inflammation and fibrosis of the alveolar septa (1). Although its etiology is unknown, locally produced cytokines and growth factors such as TNF (2, 3), TGF-β (4, 5), and platelet-derived growth factor (6, 7) have been suggested to promote infiltration of inflammatory cells and proliferation of fibroblasts. Because of its pathophysiological similarity to IPF, bleomycin (BLM)-induced pneumopathy has been frequently used as an animal model of IPF. Because treatment with anti-mouse TNF polyclonal Abs (8) or a TNF antagonist (9) prevents pulmonary inflammation in BLM-injected mice, TNF has been considered to be the crucial mediator that promotes lung inflammation triggered by BLM. Using p55p75 TNFR$^{-/-}$ mice, Ortiz et al. (10) recently showed resistance of these mice to BLM-induced pneumopathy, confirming the detrimental role of TNF in this condition.

TNF was initially recognized as the factor that induced hemorrhagic necrosis of transplanted tumors in mice (11). Cloning of the TNF gene and the subsequent generation of recombinant TNF and TNF-specific mAbs facilitated further analyses of the biological properties of TNF, elucidating that TNF is one of the most potent proinflammatory cytokines (12, 13). However, studies using TNF$^{-/-}$ mice revealed a new biological facet of TNF. Following single injection of heat-inactivated *Corynebacterium parvum*, delayed and intense inflammatory responses associated with ascites and hepatosplenomegaly were observed in TNF$^{-/-}$ mice at a time when granuloma formation and inflammation were completely resolved in TNF$^{+/+}$ mice (14). Consistent with this observation was that TNF$^{-/-}$ mice immunized with myelin basic protein exhibited prolonged myelin-specific T cell reactivity with development of late-onset and chronic autoimmune encephalomyelitis (15). These two observations indicated the anti-inflammatory property of TNF. Because TNF$^{-/-}$ mice showed no inflammatory responses or disease signs in the early phase in both experimental systems, TNF may exert its anti-inflammatory function in the late phase of inflammation or the disease course.

In this study, we have examined the effects of TNF deficiency on pulmonary inflammation using TNF$^{-/-}$ mice. In this work, we demonstrate persistent infiltration and failure of elimination of inflammatory cells from the bronchoalveolar space in TNF$^{-/-}$ mice. Challenge via airways with murine, but not human rTNF induced apoptosis of inflammatory cells, indicating that TNF eliminates inflammatory cells by apoptosis through interaction with p75 TNFR. We also evaluated the long-term effects of TNF inhalation on pulmonary inflammation and tissue repair of damaged lungs.

Materials and Methods

Mice

TNF$^{-/-}$ mice were generated and maintained at the Ludwig Institute for Cancer Research at Memorial Sloan-Kettering Cancer Center (New York, NY). TNF$^{-/-}$ mice were backcrossed to C57BL/6 mice for at least five generations. C57BL/6 mice were purchased from Charles River (Yokohama, Japan). All mice were maintained under specific pathogen-free conditions in the Laboratory Animal Center for Biomedical Science at Nagasaki University. Eight- to 12-wk-old female mice were used for experiments. Experiments were performed in accordance with the protocol approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University.

Abs and rTNF

The following Abs were used: goat polyclonal Abs specific to murine CD120a (p55 TNFR) or CD120b (p75 TNFR) from R&D Systems (Minneapolis, MN); rabbit anti-goat IgG Abs conjugated with FITC from Jackson ImmunoResearch Laboratories (West Grove, PA); anti-CD3 mAb (17A2) labeled with FITC from BD PharMingen (San Diego, CA); and anti-CD120a (HM104) and anti-CD120b (HM102) mAbs labeled with PE from Caltag Laboratories (Burlingame, CA). rTNF protein was obtained from Peprotech (London, U.K.).

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[2] Abbreviations used in this paper: IPF, idiopathic pulmonary fibrosis; BAL, bronchoalveolar lavage; BALF, BAL fluid; BLM, bleomycin; FasL, Fas ligand.

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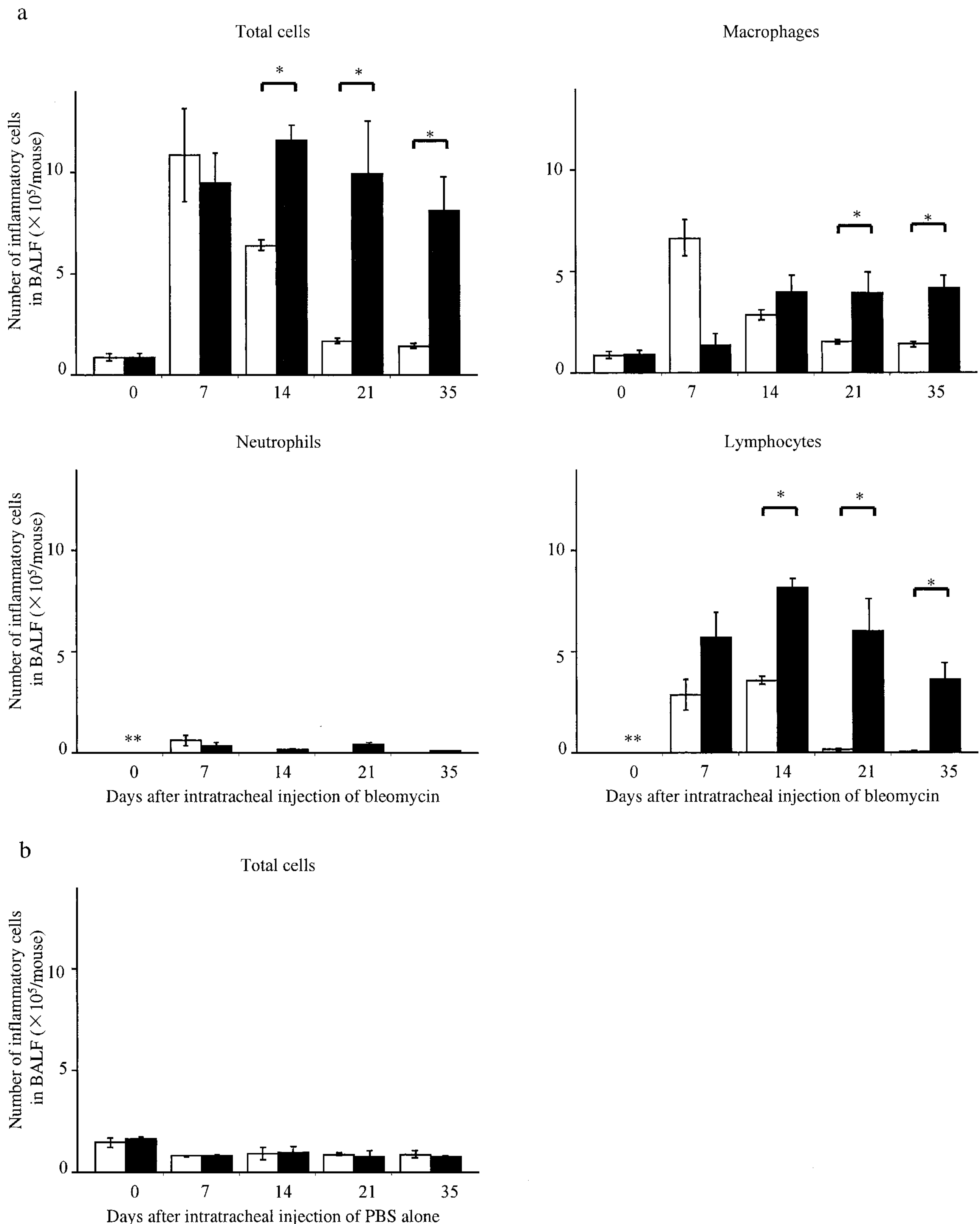

FIGURE 1. Cellular composition in BALF. Number of cells in BALF harvested from $TNF^{+/+}$ (open bars) and $TNF^{-/-}$ (filled bars) mice after BLM instillation (*a*) or sterile PBS treatment (*b*). BALF on day 0 was harvested from nontreated mice. Data represent mean ± SEM ($n = 5$ for each group). *, $p < 0.01$. **, Few of neutrophils and lymphocytes were observed in nontreated mice.

Intratracheal injection of BLM, bronchoalveolar lavage (BAL), and preparation of cells from lung tissue

After anesthesia with sodium pentobarbital, 5 U/kg mouse weight of bleomycin hydrochloride (Nippon Kayaku, Tokyo, Japan) was administered intratracheally through i.v. catheters. For control, 50 μl sterile PBS was comparably administered. For BAL, a tracheal cannula was inserted into the tracheal lumen after sacrificing mice by cervical dislocation. Lungs were lavaged five times with 1-ml aliquots of sterile PBS. After washing

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twice with PBS, cells were suspended in 0.5 ml PBS. Lung cells were isolated, as described previously (16). Total cell counts were performed using trypan blue and a hemocytometer. Differential cell counts were performed in May-Giemsa-stained cytocentrifuge preparations of recovered cells. At least 200 cells were counted using a microscope.

Histological examination

Excised lungs were fixed with 10% formaldehyde neutral buffer solution for 24 h and embedded in paraffin. Sections (5 μm) were placed on glass slides and deparaffinized, followed by staining with H&E.

Hydroxyproline assay

Lungs were homogenized in PBS and hydrolyzed in concentrated HCL at 100°C for 20 h. The hydroxyproline content of each sample was determined as previously described (17).

Flow cytometry

Cells (1×10^5–2×10^5) infiltrating into the bronchoalveolar space were stained with each Ab for 30 min on ice in PBS with 1% FCS. The annexin V-FITC apoptosis detection kit (BD PharMingen) was used to detect apoptotic cells in bronchoalveolar space. A total of 1×10^4 cells were analyzed for flow cytometry with a FACSCalibur using CellQuest software (BD Biosciences, San Jose, CA).

ELISA for TNF

To determine concentrations of TNF, lungs from mice were homogenized in 0.9% NaCl and centrifuged at 15,000 × g for 10 min at 4°C. Supernatants (100 μl) were added onto TNF ELISA plates purchased from TFB (Tokyo, Japan). Assays were performed using reagents supplied by the manufacturer.

Airway challenge with rTNF

TNF$^{-/-}$ mice were put in a closed chamber and challenged via airways with various concentrations of rTNF solution by ultrasonic nebulization.

Statistical analysis

All results were expressed as mean ± SEM. The unpaired two-tailed Mann-Whitney (nonparametric) test was used to analyze the data. Values of p less than 0.05 denoted a significant difference.

Results

Persistent pulmonary inflammation induced by BLM in TNF$^{-/-}$ mice

We investigated pulmonary inflammation induced by intratracheal injection of BLM. As shown in Fig. 1*a*, the number of inflammatory cells in BAL fluid (BALF) reached a peak level on day 7 and decreased thereafter in TNF$^{+/+}$ mice. In contrast, persistent infiltration of inflammatory cells was observed in BALF from TNF$^{-/-}$ mice. Cells in BALF harvested from TNF$^{+/+}$ mice after day 14 were predominantly macrophages, whereas significant number of lymphocytes was observed in BALF from TNF$^{-/-}$ mice even on day 35 after BLM (Fig. 1*a*). Intratracheal injection of sterile PBS alone caused no infiltration of inflammatory cells in the bronchoalveolar space (Fig. 1*b*). For further analysis, cells from lung tissue were isolated on day 21 after BLM instillation. Total cell and differential cell counts were compared in TNF$^{+/+}$ and TNF$^{-/-}$

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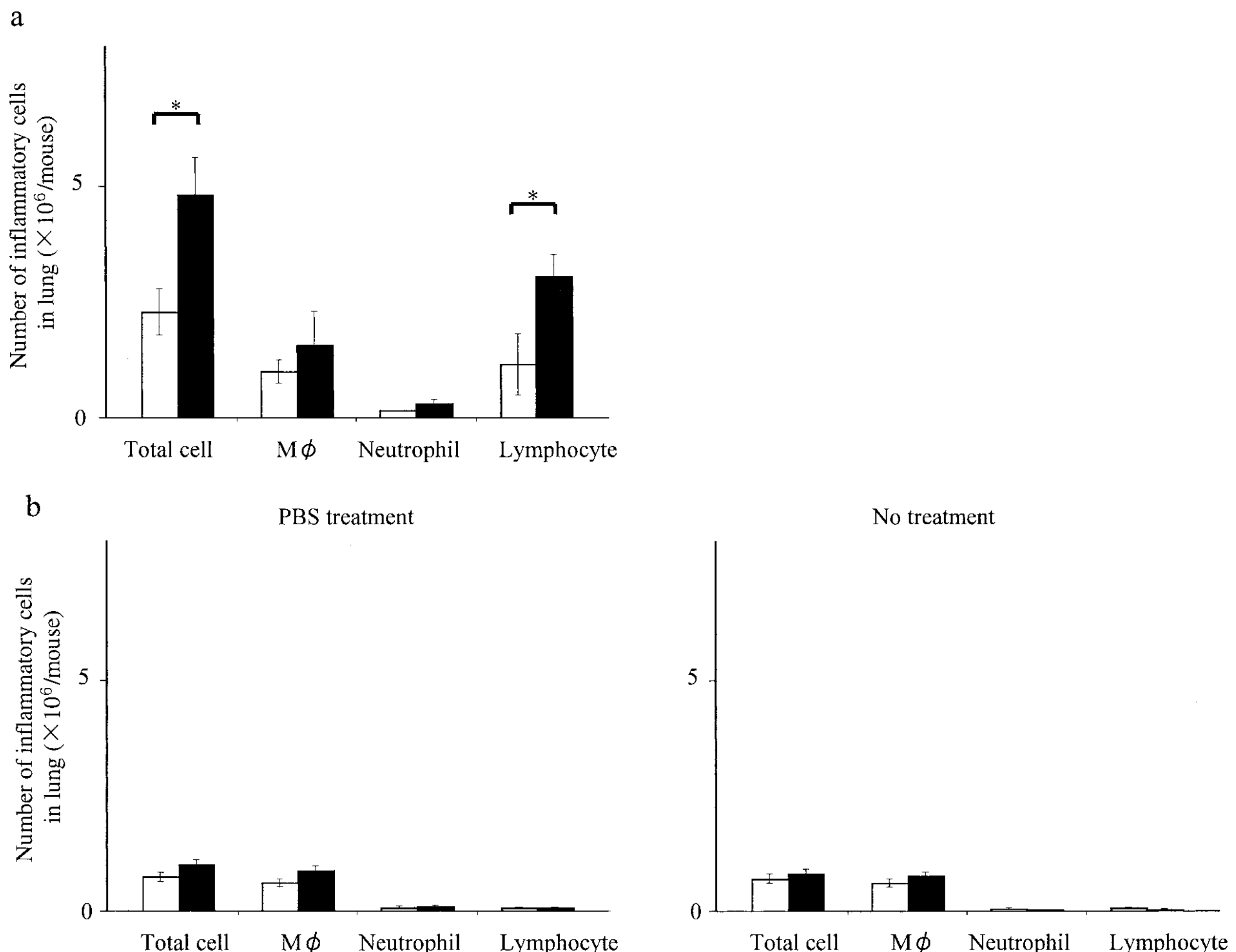

FIGURE 2. Cellular composition of lung cells. Number of cells in lungs isolated from TNF$^{+/+}$ (open bars) and TNF$^{-/-}$ (filled bars) mice on 21 days after BLM instillation (*a*) or sterile PBS treatment and none (*b*). Data represent mean ± SEM (n = 5 for each group). *, $p < 0.01$.

FIGURE 3. Histological analysis of lung damage induced by BLM. Lungs were collected on days 14 (*a*, *b*) and 75 (*c*, *d*) after BLM injection. Lungs from $TNF^{+/+}$ (*a*, *c*) and $TNF^{-/-}$ (*b*, *d*) mice. The paraffin sections were H&E stained and photographed at ×40 magnification. Results are representative of four mice in each group.

mice. An increase in total cell and lymphocyte numbers was observed in mice injected with BLM. More total lung cells and lymphocytes were seen in $TNF^{-/-}$ mice than $TNF^{+/+}$ mice (Fig. 2*a*). No significant increase in total cells and lymphocytes was observed in mice injected with sterile PBS alone, compared with nontreated mice (Fig. 2*b*). Histological examination revealed typical inflammatory changes induced by BLM, such as infiltration of lymphocytes and neutrophils, thickening of alveolar septa, and proliferation of fibroblasts in lung specimens from both $TNF^{+/+}$ (Fig. 3*a*) and $TNF^{-/-}$ mice (Fig. 3*b*) on day 14 after BLM. In $TNF^{+/+}$ mice, inflammatory responses gradually subsided with restoration of normal alveolar structures (Fig. 3*c*), whereas massive infiltration of lymphocytes and a honeycomb structure was observed in $TNF^{-/-}$ mice on day 75 after BLM instillation (Fig. 3*d*). Only unilateral lungs were damaged macroscopically and histologically in $TNF^{-/-}$ mice that survived >35 days after BLM instillation. All the data shown were from mice in which bilateral lungs were affected macroscopically or histologically, except $TNF^{-/-}$ mice with long survival. The lung hydroxyproline content was measured in $TNF^{+/+}$ and $TNF^{-/-}$ mice on day 21 after BLM. No significant difference in the amount of hydroxyproline was observed (Fig. 4).

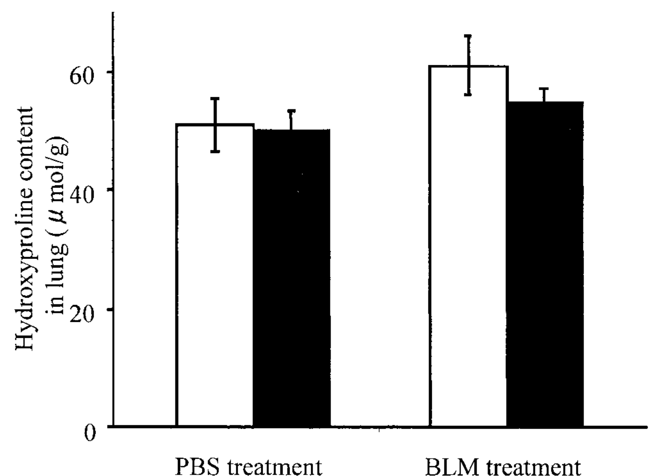

FIGURE 4. Hydroxyproline content in lungs after BLM instillation. Lungs were collected on day 21 after BLM or sterile PBS injection. Hyroxyproline content was measured in $TNF^{+/+}$ (open bars) and $TNF^{-/-}$ (filled bars) mice. Data represent mean ± SEM ($n = 5$ for each group).

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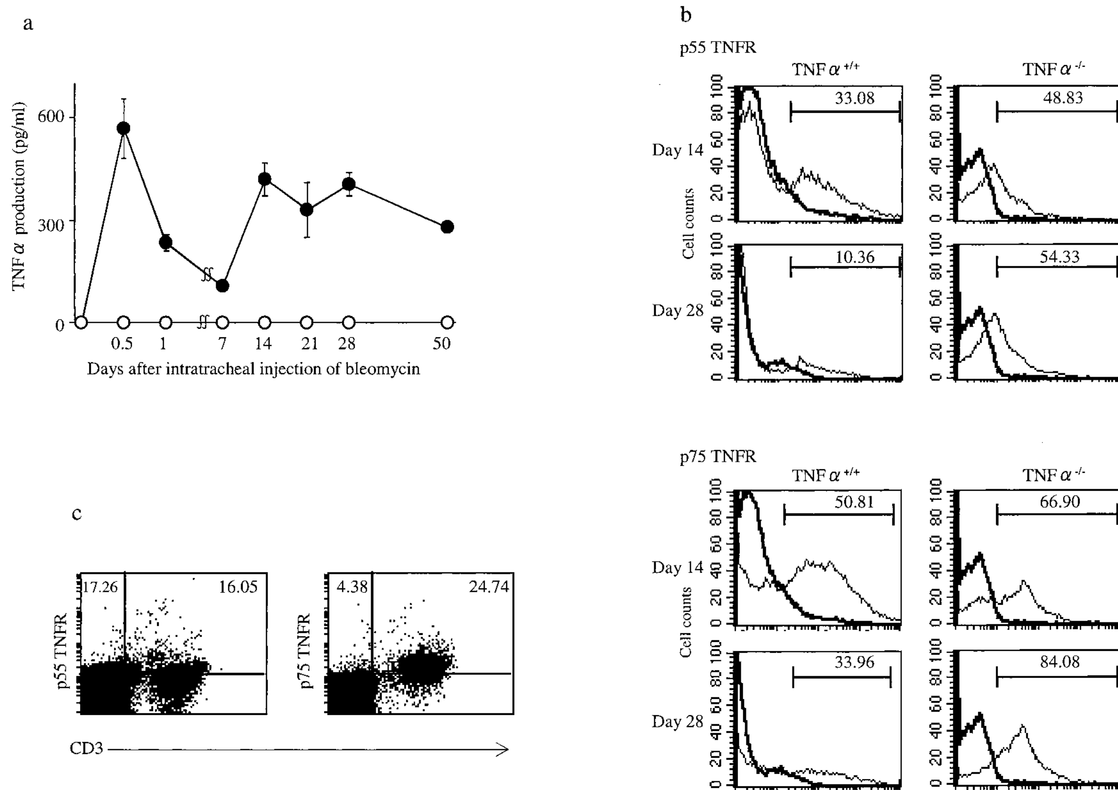

FIGURE 5. Production of TNF in lungs and expression of TNFRs after BLM instillation. *a*, TNF production in lungs was determined by ELISA. $TNF^{+/+}$ (●) and $TNF^{-/-}$ mice (○). Data represent mean ± SEM ($n = 5$ for each group). *b* and *c*, Flow cytometric analysis of expression of TNFRs on inflammatory cells in BALF. Numbers represent percentage of TNFR-positive cells. Results are representative of five mice.

Production of TNF in lungs and expression of TNFRs on inflammatory cells in BALF

TNF production in the lungs of $TNF^{+/+}$ mice after BLM instillation showed a biphasic response; TNF production reached a peak level 12 h after BLM injection, followed by a transient decline, and then resumed increasing after day 7 (Fig. 5*a*). TNF was persistently produced until day 50. No TNF was detectable in $TNF^{-/-}$ mice. Flow cytometric analysis revealed that expression of p55 and p75 TNFRs was up-regulated on inflammatory cells in BALF from both $TNF^{+/+}$ and $TNF^{-/-}$ mice on day 14 (Fig. 5*b*). Inflammatory cells expressing TNFRs were still detected in the bronchoalveolar space of $TNF^{-/-}$ mice on day 28. Transient expression of TNFRs was observed 12 h after BLM instillation. However, no expression was detected during the period from day 1 to day 12. Most p75 TNFR-positive cells were $CD3^+$ T cells, whereas p55 TNFR was expressed in both T and non-T cells (Fig. 5*c*).

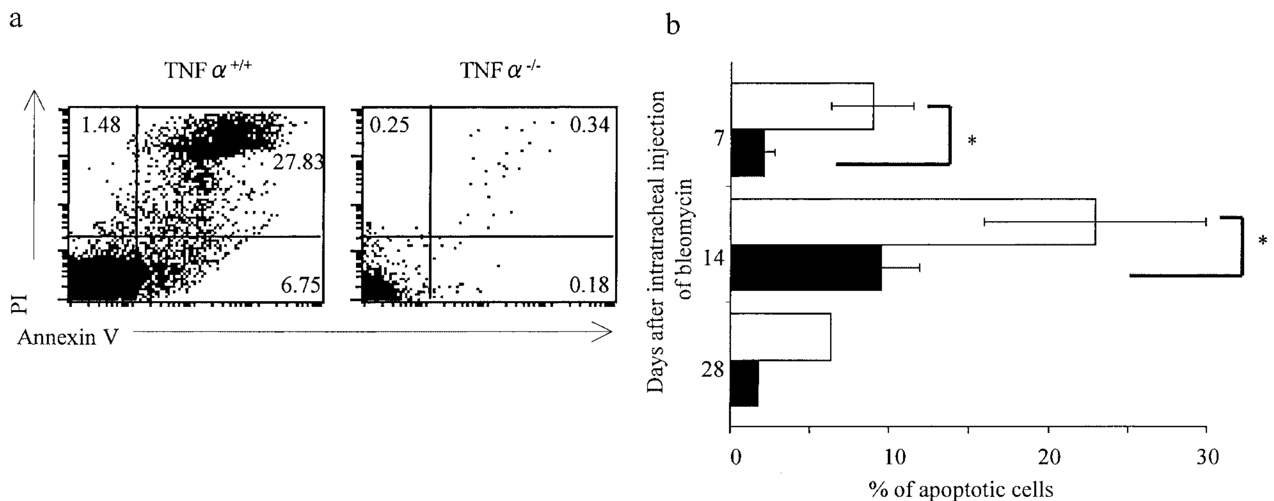

FIGURE 6. Apoptotic cells in the bronchoalveolar space after BLM instillation. *a*, Detection of apoptotic cells by flow cytometric analysis. BAL was performed on day 14 after BLM. Numbers represent percentage of annexin V- and/or propidium iodide-positive cells. Results are representative of five mice. *b*, Annexin V-positive cells were counted as apoptotic cells. $TNF^{+/+}$ mice (open bars) and $TNF^{-/-}$ (filled bars). Data represent mean ± SEM ($n = 5$ for each group), except for those obtained on day 28, which represent pooled inflammatory cells harvested from five mice. *, $p < 0.05$.

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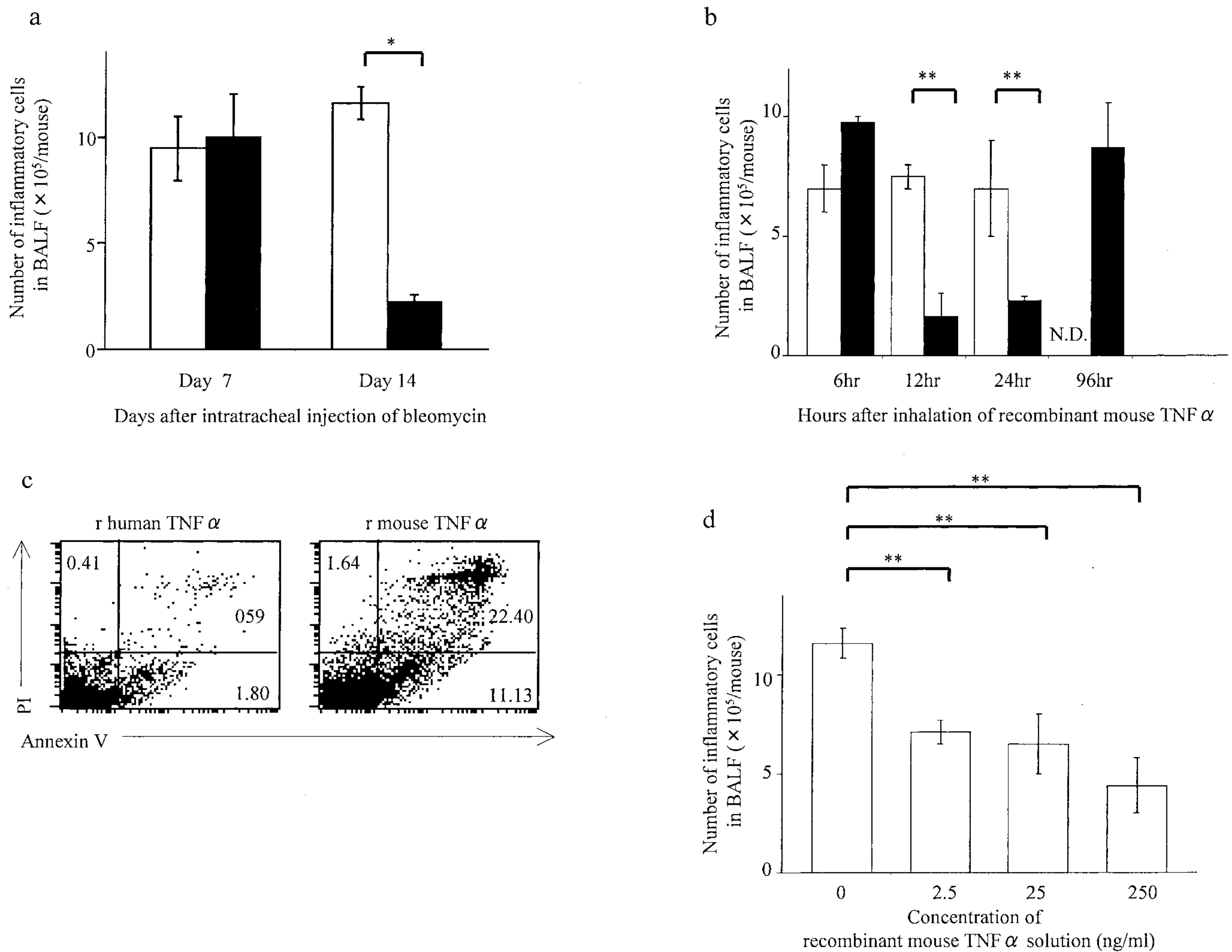

FIGURE 7. Effects of airway challenge with rTNF on induction of apoptosis of inflammatory cells. *a*, Number of inflammatory cells in the BALF harvested from $TNF^{-/-}$ mice challenged via airways with murine rTNF (filled bars) or PBS alone (open bars) after BLM. The concentration of murine rTNF was 250 ng/ml in PBS. BAL was performed 48 h after airway challenge with TNF. Data represent mean ± SEM ($n = 5$ for each group). *, $p < 0.01$. *b*, Number of inflammatory cells in BALF harvested from $TNF^{-/-}$ mice challenged with murine (filled bars) or human rTNF (open bars) on day 14 after BLM instillation. TNF concentration was 250 ng/ml in PBS. BAL was performed sequentially after airway challenge with TNF. Data represent mean ± SEM ($n = 5$ for each group). **, $p < 0.05$. ND, not done. *c*, Detection of apoptotic cells by flow cytometric analysis. Cells were harvested from $TNF^{-/-}$ mice challenged with murine or human rTNF on day 14 after BLM. BAL was performed 6 h after airway challenge with TNF. Numbers represent percentage of annexin V- and/or propidium iodide-positive cells. Results are representative of five mice. *d*, Number of inflammatory cells in BALF harvested from $TNF^{-/-}$ mice challenged with various concentrations of murine rTNF on day 14 after BLM instillation. BAL was performed 48 h after airway challenge with murine TNF. Data represent mean ± SEM ($n = 5$ for each group). **, $p < 0.05$.

Apoptotic effects of murine rTNF on inflammatory cells in the bronchoalveolar space

We then examined apoptosis of inflammatory cells in the BALF after BLM injection. Flow cytometric analysis revealed that significant numbers of inflammatory cells in BALF from $TNF^{+/+}$ mice were apoptotic, whereas fewer apoptotic cells were observed in $TNF^{-/-}$ mice (Fig. 6, *a* and *b*). To confirm the direct effects of TNF on induction of inflammatory cell apoptosis in BALF, $TNF^{-/-}$ mice were challenged via airways with rTNF protein. Inflammatory cells in BALF from $TNF^{-/-}$ mice were counted 48 h after airway challenge with murine rTNF protein solution at a concentration of 250 ng/ml. Fewer inflammatory cells were observed in $TNF^{-/-}$ mice challenged with TNF than in those that inhaled PBS alone on day 14 after BLM instillation (Fig. 7*a*). However, no significant difference was observed in the number of inflammatory cells in the BALF between TNF-treated and untreated $TNF^{-/-}$ mice on day 7 (Fig. 7*a*). The effect of murine TNF was transient, with inflammatory cells appearing again in the bronchoalveolar space 96 h after TNF challenge (Fig. 7*b*). Airway challenge with human rTNF did not eliminate inflammatory cells (Fig. 7*b*), indicating that signals through p75 TNFR are critical in inducing apoptosis (Fig. 7*c*). The apoptotic effect of murine TNF on inflammatory cells was observed at a concentration as low as 2.5 ng/ml (Fig. 7*d*).

We next evaluated the long-term effects of murine TNF challenge on BLM-induced pulmonary inflammation. Because the effect of TNF was transient, challenge via airways with 250 ng/ml murine TNF solution was started on day 14 after BLM and repeated twice weekly for 4 wk. Murine TNF treatment effectively subdued pulmonary inflammation in $TNF^{-/-}$ mice. Fewer inflammatory cells and partial restoration of normal alveolar structure were observed in $TNF^{-/-}$ mice challenged with murine TNF (Fig. 8*a*), whereas massive pulmonary inflammation was noted in TNF-untreated $TNF^{-/-}$ mice in which only

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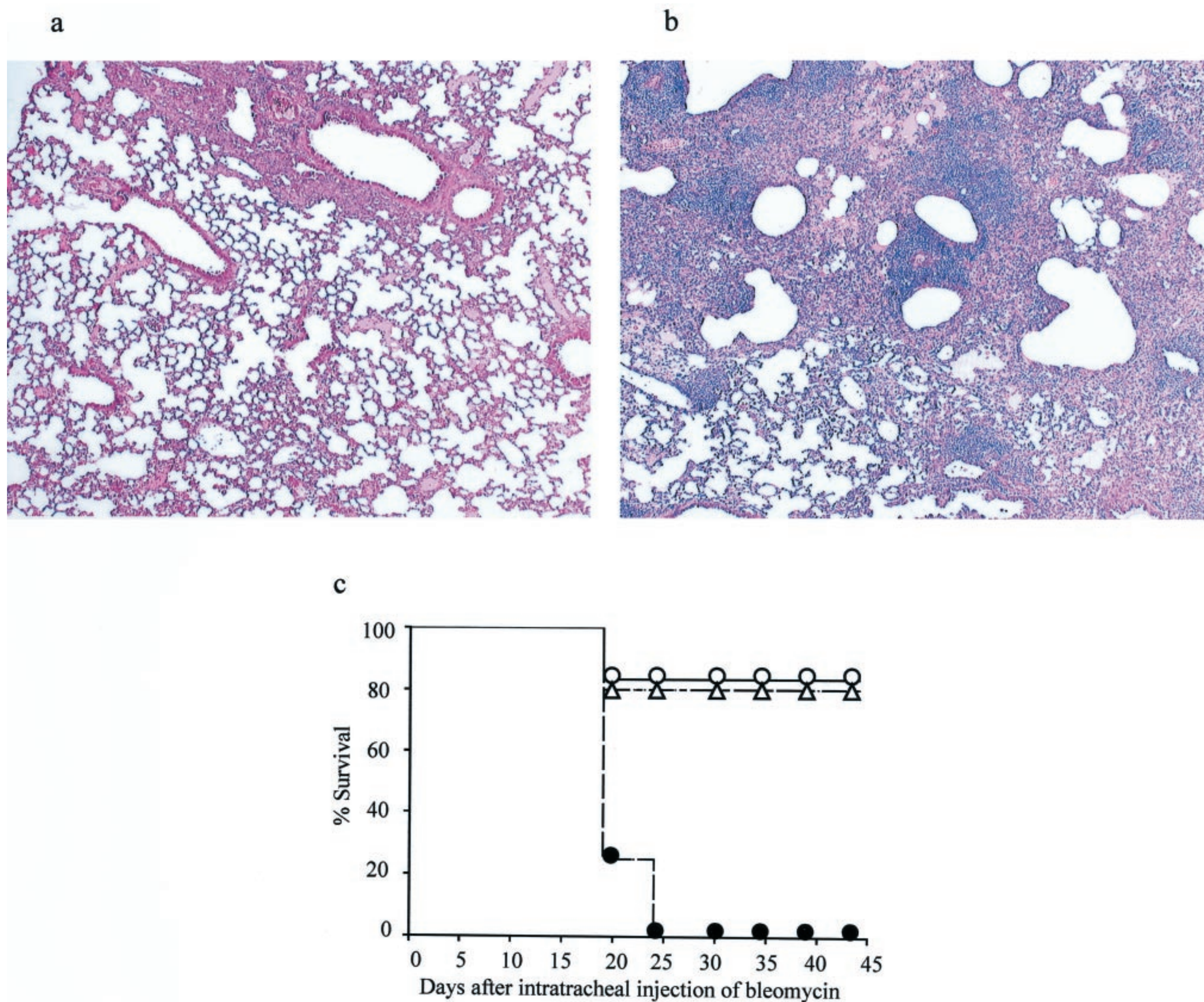

FIGURE 8. Effects of long-term challenge with murine rTNF on pulmonary inflammation. Challenge via airways with 250 ng/ml murine TNF solution was started on day 14 after BLM and repeated twice weekly for 4 wk. *a* and *b*, Histological analysis of lungs harvested from murine TNF-treated (*a*) or untreated (*b*) $TNF^{-/-}$ mice. The paraffin sections were H&E stained and photographed at ×40 magnification. Lungs were harvested on day 42 after BLM. Only unilateral lungs were macroscopically and histologically damaged in murine TNF-untreated $TNF^{-/-}$ mice. Results are representative of three mice in each group. *c*, Percentage of survival of mice. $TNF^{-/-}$ mice challenged with mouse TNF (○; $n = 8$) or sterile PBS (●; $n = 8$). $TNF^{+/+}$ mice (Δ; $n = 8$) for control. It was confirmed that bilateral lungs were histologically damaged in all mice at the time of death or sacrifice on day 42 after BLM instillation. Data from two experiments were combined.

unilateral lungs were affected histologically (Fig. 8*b*). TNF treatment did not influence pulmonary inflammation in $TNF^{+/+}$ mice. To see the effects of inhalation of murine TNF on survival of $TNF^{-/-}$ mice injected with BLM, $TNF^{-/-}$ mice were intratracheally injected with BLM and then challenged via airways with 250 ng/ml murine TNF solution twice weekly for 4 wk. As shown in Fig. 8*c*, $TNF^{-/-}$ mice challenged with murine TNF exhibited prolonged survival.

Discussion

In this study, we demonstrated intense and persistent pulmonary inflammation induced by BLM in $TNF^{-/-}$ mice. Injection of TNF-specific Abs or soluble rTNFR proteins prevented pulmonary inflammation in mice intratracheally injected with BLM (8, 9). In contrast to those reports, our findings suggested that TNF is not necessary for promoting pulmonary inflammation triggered by BLM. The difference between the previous reports and our findings in effects of TNF on pulmonary inflammation may derive from the fact that $TNF^{-/-}$ mice were used in our experiments. In fact, $TNF^{-/-}$ mice injected with high doses of LPS showed the same symptoms as $TNF^{+/+}$ mice comparably treated with LPS (14), whereas mice pretreated with soluble rTNFR proteins were prevented from toxic effects of LPS (18). In addition, the delayed recovery from encephalomyelitis was observed in $TNF^{-/-}$ mice immunized with myelin oligodendrocyte glycoprotein (19), while treatment with anti-TNF mAb reduced severity of the disease in mice injected with myelin basic protein-specific T cells (20). No pulmonary inflammation or subsequent lung tissue damage was observed in p55p75 $TNFR^{-/-}$ mice injected intratracheally with BLM (10). Because lymphotoxin α and TNF share two TNFRs as the ligands (21), lymphotoxin α, rather than TNF, may be the crucial factor involved in boosting inflammation.

We showed that endogenous TNF was required for resolution of inflammation and subsequent remission of BLM-induced pneumopathy. In the absence of TNF, inflammatory cells accumulated in the bronchoalveolar space, and thus, the repair process of lung

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injury was impaired, which was often lethal to mice. In contrast to $TNF^{-/-}$ mice, clearance of inflammatory cells and restoration of normal alveolar structure were observed in $TNF^{+/+}$ mice, in which persistent production of TNF in lungs was observed until day 50 after BLM instillation. More apoptotic cells were observed in BALF harvested from $TNF^{+/+}$ than from $TNF^{-/-}$ mice, suggesting that TNF is an important mediator for inducing apoptosis of inflammatory cells.

Little is known about the relative contribution of Fas-Fas ligand (FasL) and TNF in mediating apoptosis associated with elimination of inflammatory cells. The involvement of Fas-FasL in BLM-induced pneumopathy has been controversial (22, 23). Fas expression was not detected on inflammatory cells in the BALF in either $TNF^{-/-}$ or $TNF^{+/+}$ mice, and challenge via airways of $TNF^{-/-}$ mice with murine rTNF effectively eliminated inflammatory cells from the bronchoalveolar space by apoptosis. We therefore conclude that TNF, but not Fas-FasL, plays a critical role in inducing apoptosis of inflammatory cells in BLM-induced pneumopathy. Because of the lack of expression of TNFRs on inflammatory cells in BALF, TNF inhalation was not effective on day 7 after BLM instillation. Long-term airway challenge of $TNF^{-/-}$ mice with murine TNF starting on day 14 was effective in reducing lung inflammation and prolonging survival of those mice, without causing weight loss and ruffled hair in $TNF^{-/-}$ mice. In this regard, local production of endogenous TNF and up-regulation of expression of TNFRs after the acute phase of inflammation may be an important host response for resolution of inflammation. It will be important to clarify the triggers of TNF production in the lungs and of up-regulation of TNFR expression on inflammatory cells. TNF is not required for inducing expression of TNFRs.

Our results showed that murine, but not human rTNF was effective in elimination of inflammatory cells from the bronchoalveolar space by apoptosis. Because human TNF binds only mouse p55 TNFR, but not mouse p75 TNFR (24), our findings in this study revealed the critical role of TNF-p75 TNFR in inducing apoptosis in vivo. In vitro analysis showed that the interaction between TNF and p75 TNFR induced apoptosis of activated $CD8^+$ T cells (25, 26). Receptor interacting protein, a Ser/Thr protein kinase, was suggested to be up-regulated in activated T cells by IL-2 and to mediate death signals through the interaction of TNF and p75 TNFR (27). Receptor interacting protein may be up-regulated in inflammatory cells and may mediate apoptosis of those cells in vivo.

In conclusion, we have demonstrated in the present study that endogenous and persistent production of TNF and up-regulation of p75 TNFR expression were indispensable for clearance of inflammatory cells from the bronchoalveolar space by apoptosis and tissue repair of damaged lungs in BLM-induced pneumopathy. Long-term airway challenge of $TNF^{-/-}$ mice with murine TNF was effective and safe, suggesting that TNF may have a clinical application in IPF.

Acknowledgments

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