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Potential Role of Apoptotic Macrophages in Pulmonary Inflammation and Fibrosis

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Induction of apoptosis has been associated with a variety of exposures which result in inflammatory and fibrotic lung disorders. Macrophages are key regulatory cells in the lung; however, the role of apoptotic macrophages in those pulmonary disorders is not well characterized. In the present investigation, apoptotic macrophages were instilled into the lungs of rats to study directly the pulmonary responses to apoptotic cells. The effects of apoptotic macrophages on lung inflammation and fibrosis, as well as associated protein expression of TNF-α, TGF-β, and matrix metalloproteinases (MMPs) were examined. Induction of macrophage apoptosis was carried out in vitro using a variety of known apoptosis inducers. Intratracheal administration of apoptotic macrophages (5×10^6 cells/rat) into the lung of rats caused an increase in pulmonary infiltration of macrophages and lung cell apoptosis 4 weeks after the treatment as indicated by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. In contrast, pulmonary instillation of saline or normal control macrophages had no effect. Histological analysis of lung sections showed collagen deposition and fibrotic lesions after apoptotic cell treatment but not in control groups. Immunohistochemical studies revealed increased expression of TNF-α, TGF-β, MMP2, and MMP9 in the treatment group 4 weeks after the treatment. These results suggest a role for macrophage apoptosis in the initiation of these lung disorders. This study provides direct evidence that apoptotic macrophages can induce lung inflammation and fibrosis and that this induction may be associated with increased expression of TNF-α, TGF-β, MMP2, and MMP9. J. Cell. Physiol. 194: 215–224, 2002. Published 2002 Wiley-Liss, Inc.†

Apoptosis has been described as a physiologic cell death program critical for homeostasis. It is characterized by cytoplasmic blebbing, cell shrinkage, nuclear fragmentation (~180 bp fragments), and formation of apoptotic bodies that are ingested by neighboring cells (Wyllie et al. 1980). Unlike necrosis, which results in cellular disintegration and subsequent release of toxic and injurious contents, apoptosis is thought to provide an injury-limiting mechanism because the plasma membrane remains intact during the apoptotic process. There is no release of proinflammatory stimuli and this has been considered to be important in the resolution of inflammation (Savill et al., 1993). However, uncleared apoptotic bodies could undergo secondary necrosis (Savill and Haslett, 1995). Apoptosis provides beneficial effects by maintaining and regulating tissue homeostasis, but detrimental effects may occur when the process of apoptosis and clearance is abnormally regulated. Dysregulation of apoptosis may be deleterious: minimal apoptosis has been shown to lead to cancer and autoimmune diseases, whereas excessive apoptosis may lead to Alzheimer's disease (Thompson, 1995).

Apoptosis may also play an important role in pulmonary diseases. The induction of apoptosis has been shown to be associated with various lung disorders including acute lung injury (Bardales et al., 1996), diffuse alveolar damage (Guinee et al., 1996), and idiopathic pulmonary fibrosis (Kuwano et al., 1996, 1999a). Agents that induce lung inflammation and

Abbreviations: Alveolar macrophages, AM; transforming growth factor-β, TGF-β; tumor necrosis factor-α, TNF-α; matrix metalloproteinase, MMP; terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, TUNEL; dimethyl sulfoxide, DMSO; ethylenediaminetetraacetic acid, EDTA; Tris base, boric acid and EDTA buffer, TBE.

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fibrosis including bleomycin (Kuwano et al., 1999b, 2000), silica (Iyer et al., 1996), immune complexes (Nomoto et al., 1997), and endotoxin (Cox et al., 1995) have also been shown to induce lung cell apoptosis directly. Repair after lung injury requires the elimination of inflammatory and unwanted cells from the tissue through phagocytosis (Polnovsky et al., 1993). Because apoptosis and phagocytosis proceed rapidly in tissues, the presence of a small percentage of in situ apoptotic cells indicate biologically significant changes (Wyllie et al., 1980; Marthyn et al., 1998). In spite of the association between lung disorder and apoptosis and the fact that a number of agents which induce lung disorder are known, the role of apoptotic cells themselves in pathogenesis of lung disorders has not been demonstrated.

The objective of this study was to test the hypothesis that an excessive numbers of apoptotic cells may induce pulmonary inflammation and fibrotic lung disease. Such conditions may occur when an agent directly induces lung cell apoptosis or in conditions where the clearance of apoptotic cells and products is defective. In vivo experimental models and clinical studies suggest that failure to remove apoptotic cells from inflammatory foci contributes to a persistent state of inflammation in various organs and tissues (Grigg et al., 1991; Haslett et al., 1994; Cox et al., 1995). However, there is no evidence to date that directly links apoptosis and lung disorders. Specifically, whether or not apoptosis can directly cause lung disorders in the absence of other stimulating agents remains to be established. This is an important question, since in certain pulmonary diseases such as idiopathic pulmonary fibrosis no identifiable stimulus can be detected, yet the pathologic condition persists. To address this question we instilled apoptotic alveolar macrophages (AM) directly into the lungs of normal rats and monitored the pulmonary responses. We also measured the corresponding changes in key inflammatory and fibrogenic mediators, for example, TNF-α, TGF-β, and MMP, known to be involved in lung pathogenesis. The specific questions to be addressed in this study are whether or not apoptosis by itself can cause lung inflammation and fibrosis in the absence of any stimulating agents, and if so, what is the underlying mechanism.

MATERIALS AND METHODS

In vitro induction of macrophage apoptosis

To induce apoptosis, one million AM were treated in vitro with several known apoptosis-inducing agents including salicylates (Life Technologies, Grand Island, NY), TGF-β (R&D Systems, Minneapolis, MN), ethanol (Pharmco Products, Brookfield, CT), crystalline silica (Min-U-Sil5, U.S. Silica, Berkeley Springs, WV), or dimethyl sulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ). Primary AM were isolated from different strains of rats including Brown Norway (BN), Sprague–Dawley (SD), and Fischer 344 (F344) by bronchoalveolar lavage. A normal rat AM cell line NR8383 (American Type Culture Collection, Manassas, VA) was also tested. The cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS), 4 mg/ml glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Induction of AM apoptosis was performed in RPMI-1640 medium containing 5% FBS using different concentrations of the test agents and varying exposure times as indicated in the Results. After specific treatments, the cells were scraped, washed, and resuspended in phosphate-buffered saline without calcium or magnesium (PBS), and then analyzed for apoptosis by DNA ladder and TUNEL assays as described below.

Cell necrosis determination

Necrosis of AM treated with DMSO was determined by lactate dehydrogenase (LDH) activity assay. LDH is a cytoplasmic enzyme that is released when the cell membrane is damaged or lyzed. The assay was performed using the supernatants from AM treated with or without DMSO in vitro. LDH activity was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm using an LDH assay kit (Roche Diagnostic Systems, Montclair, NJ).

Apoptosis determination

Apoptosis of AM was determined by DNA ladder and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays (Kerr et al., 1972; Wyllie et al., 1980). In the DNA ladder assay, DNA from treated and control cell samples was isolated and analyzed for DNA fragmentation using agarose gel electrophoresis. Three micrograms of the DNA were subjected to gel electrophoresis at 20 V for 18 h, through a 2% TBE agarose gel containing 1 μg/ml ethidium bromide. The separated DNA bands were visualized under a UV transilluminator and then photographed. For the TUNEL assays, the cell samples, or in some cases lung tissue sections, were formalin-fixed, paraffin-embedded, and analyzed for apoptosis using a TUNEL assay kit (Promega, Madison, WI). This system measures fragmented DNA by catalytically incorporating fluorescein-12-dUTP at the 3′-OH DNA polymeric tail, as described previously (Gravrieli et al., 1992). The apoptotic cell nuclei were visualized as a yellow-green fluorescent signal using fluorescence microscopy. Normal cell nuclei were counterstained with propidium iodide, which gives red fluorescence. TUNEL positive but LDH negative cells in our experiments indicated that this method is specific to apoptotic cells.

In vivo exposure studies

Lung response to apoptotic AM was studied in pathogen-free, inbred male BN rats (200–250 g) by a single intratracheal administration of 1 ml of cell suspension in sterile PBS. The BN rats were chosen in this study since they are inbred and more susceptible to apoptosis induction than the inbred F344 rats. SD rats are not inbred and thus their use may induce adverse immunologic reactions. The BN rats were anesthetized by an intraperitoneal injection of 0.6 ml of 1% sodium methohexital (Brevital; Eli Lilly, Indianapolis, IN) and instillations were performed with a ball-tipped 18-gauge animal feeding needle. One milliliter of sterile PBS containing 5×10^6 normal AM or DMSO-induced apoptotic AM ($\sim$20% apoptotic cells) from BN rats were instilled per rat. Prior to instillation, all apoptotic cell samples were washed thoroughly with sterile PBS to remove residual DMSO. The animals were sacrificed

4 weeks after the instillations. The right lungs were lavaged by flushing with 4 ml of sterile PBS through a tracheal cannula. The left lungs were fixed via airway instillation with 10% buffered formalin for histological evaluation.

Bronchoalveolar lavage and differential cell count

Rats were euthanized by exsanguinations under anesthesia with an intraperitoneal injection of 0.2 ml of 260 mg/ml sodium pentobarbital (Pentosol; Med-Pharmex, Inc., Pomona, CA) after specific treatments. A tube was inserted surgically into the trachea. The left main bronchus was clamped and the right lung was lavaged with 4 ml of ice-cold PBS. The lung was lavaged three times to collect cellular contents. The first and subsequent lavages were combined, centrifuged, and the cell pellets were resuspended in 1 ml PBS. Cell counts and differentials were determined using a Coulter Multisizer II (Coulter Electronics, Hialeah, FL).

Histological studies

The left lung was fixed by intratracheal instillation of formalin at 20 cm H_2O. After measurements of fixed lung volume, the lungs were embedded in paraffin, and sectioned at 5 μm. The sections were mounted on glass slides, deparaffinized, rehydrated, and then stained with hematoxylin and Sirius Red (Junqueira et al., 1979) to assess lung inflammation and fibrosis. The total numbers of normal and apoptotic AM as well as the volume fraction of Sirius Red-stained collagen tissues were determined using standard morphometric methods by two individuals blinded to the experimental protocols.

Immunohistochemistry

Immunohistochemical analysis of lung tissue samples was performed according to the manufacturer's protocols (Thermo Shandon, Pittsburgh, PA) using DAKO peroxidase kit (DAKO Corp., Carpinteria, CA) and liquid DAB substrate kit (ZYMED Laboratories, Inc., So. San Francisco, CA). Briefly, tissue sections were deparaffinized in xylene, rehydrated, and microwaved in citrate buffer, pH 6.0, for antigen retrieval. After the sections were treated with 3% H_2O_2:methanol (1:1) to block endogenous peroxidases, they were incubated at 4°C overnight with primary antibodies at the following concentrations: 20 μg/ml for rabbit (polyclonal) anti-rat TNF-α (Biosource, Camrillo, CA); 20 μg/ml for mouse (monoclonal) anti-human TGF-β1 (R&D systems); 8 μg/ml for mouse (monoclonal) anti-rat MMP2, MMP9, and MMP10 (Lab Vision Corp., Fremont, CA). Control antibody treatment with either primary or biotinylated secondary antibody alone gave negative background results. MMP2 and MMP10 primary antibodies are biotin-conjugated monoclonal antibodies which did not need the secondary antibody.

Statistical analysis

Analysis of variance and Duncan's multiple comparison test (Duncan, 1955) were used to evaluate the significance between measurements. All tests were 2-sided tests, and $P < 0.05$ was considered to be significant. Data are given as mean ± SE.

RESULTS

Induction of apoptosis of AM from different strains of rats

In order to investigate the role of apoptotic AM in pulmonary disorders, first apoptotic AM were prepared in vitro and their apoptotic activities characterized using TUNEL and DNA ladder assays. Primary AM from SD, BN, and F344 rats, as well as the normal rat AM cell line (NR8383) were assessed for their apoptotic activities. This study was carried out to determine the susceptibility of AM from different strains of rats to apoptosis induction and to evaluate their suitability as apoptotic cell models for subsequent in vivo studies. The cells were treated with various known apoptosis inducers including ethanol (Singhal et al., 1998), TGF-β (Fukuda et al., 1993), sodium salicylates (Klampfer

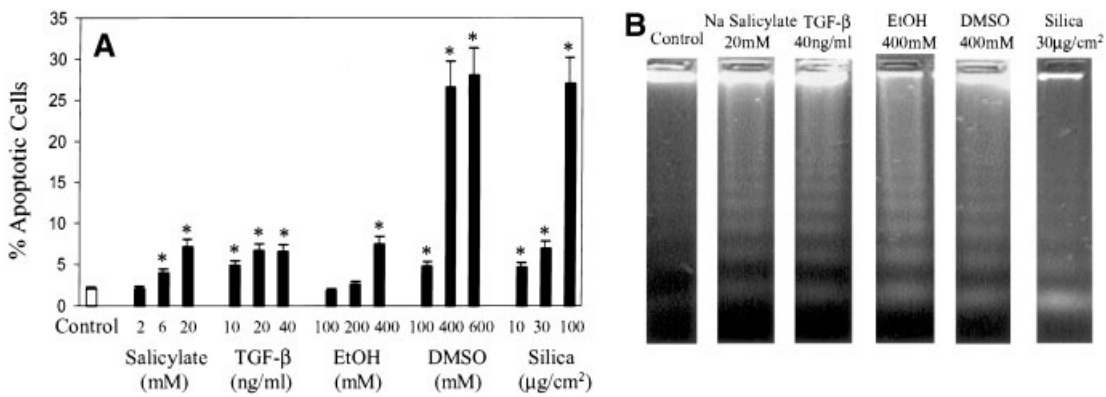


Fig. 1. Induction of AM apoptosis in vitro by different agents. **A**: TUNEL assay. AM from SD rats were incubated in 12-well culture plates in RPMI-1640 medium containing 5% FBS in the presence or absence of test agents at indicated concentrations for 9 h at 37°C. After incubation, the cells were labeled for TUNEL assay and the percentage of apoptotic cells was determined by fluorescence microscopy. A minimum of 1,000 cells were counted in each sample. Values are means ± SE, n = 3. *$P < 0.05$ versus non-treated control. **B**: DNA ladder assay. The figure depicts corresponding gel electrophoresis patterns of DNA isolated from AM of SD rats. AM were treated as described above. Note that all treated samples show classic DNA ladder patterns as compared to the control.

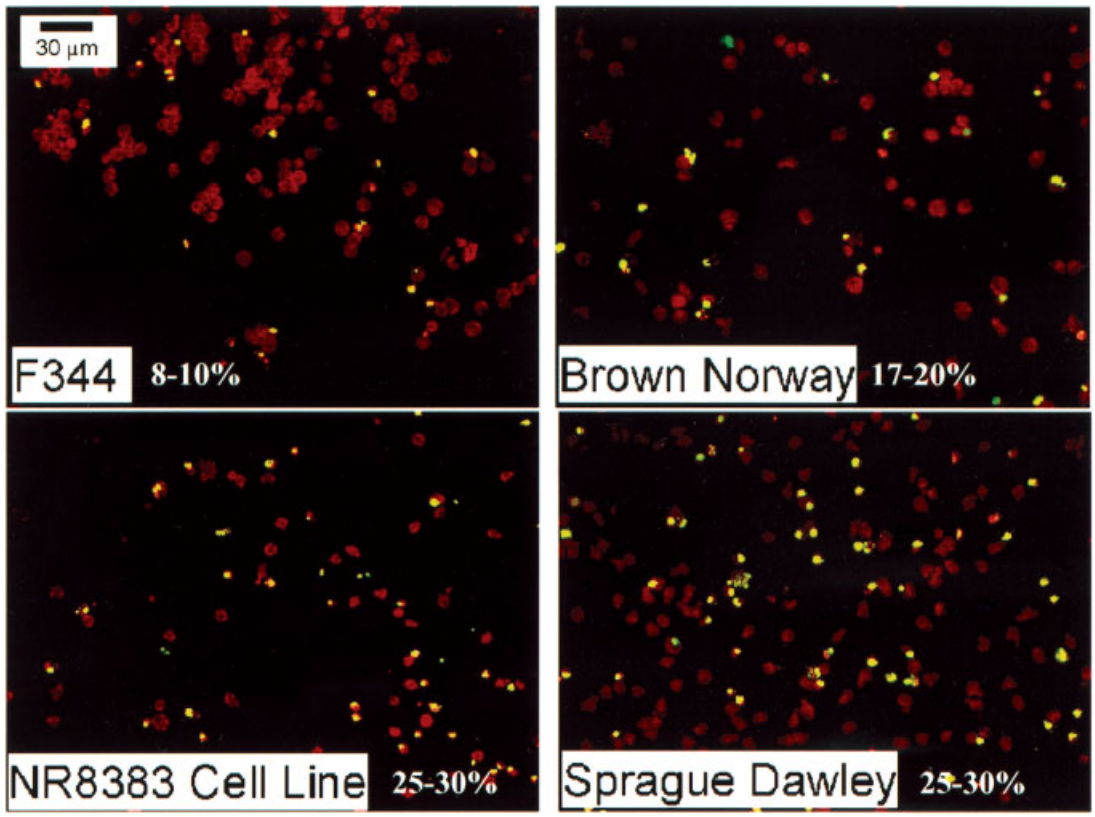


Fig. 2. Induction of AM apoptosis by DMSO. AM from different strains of rats were treated in vitro with 400 mM DMSO for 9 h. Apoptotic cells were stained with TUNEL reagent and exhibited yellow-green fluorescence, while normal cells were counter-stained with propidium iodide and fluoresced red. Values indicate percentage apoptosis from three separate experiments. Panels present representative data from different treatment groups (n = 3 rats/group).

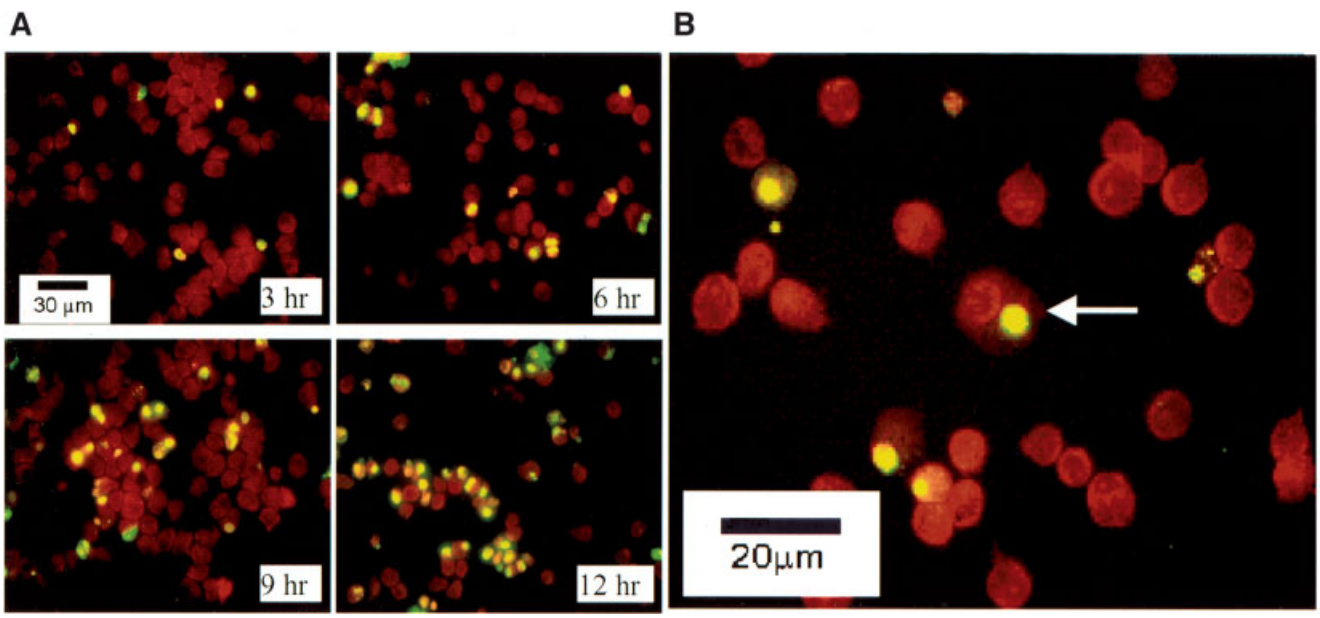


Fig. 3. Time course of apoptosis and phagocytosis of apoptotic bodies. **A**: DMSO-induced apoptosis in AM obtained from SD rats. Cells were treated in vitro with 400 mM DMSO at 37°C for 3, 6, 9, and 12 h as indicated. Apoptotic cells were stained with TUNEL and counter-stained with propidium iodide. **B**: AM ingesting apoptotic bodies at 12 h treatment with DMSO. The arrow shows TUNEL-positive cell (apoptotic body) in the cytoplasm of AM.

et al., 1999), DMSO (Marthyn et al., 1998), and crystalline silica (Nomoto et al., 1997) in an attempt to find the most efficient apoptosis inducer for AM. Figure 1A shows the results from TUNEL assay using AM from SD rats. These results indicate that DMSO was the most effective agent in inducing AM apoptosis. To further confirm the TUNEL results, DNA ladder assays were performed in cells treated with apoptosis inducers as described above. The DNA ladder assay measures DNA fragmentation, a biochemical hallmark of apoptosis. All treated cell samples showed classic DNA ladders whereas the non-treated control sample showed minimal DNA fragmentation (Fig. 1B). Kinetic patterns of apoptosis in response to DMSO were similar in all strains (data not shown). These results confirm the reliability of the TUNEL assay in detecting cell apoptosis. This assay was subsequently used in all apoptosis studies.

Figure 2 illustrates the differential effect of DMSO on apoptosis of AM from different strains of rats following a 12-h treatment. Maximum apoptotic response was observed in AM from SD rats and in NR8383 macrophages (25–30%), while AM from BN and F344 rats showed 12–17% and 8–10% apoptosis, respectively. These results demonstrate a wide range in the efficiency with which apoptotic cells are generated in different strains of rats.

Time course of AM apoptosis

Time-dependent induction of apoptosis of AM by DMSO can be seen in Figure 3A. Increasing levels of AM apoptosis was observed for up to 12 h. Higher magnification of the cell samples reveals inclusions of apoptotic bodies in the cytoplasm of normal AM (Fig. 3B), indicating the preservation of phagocytic function of the treated cells.

Assay for cell necrosis in apoptotic cell population

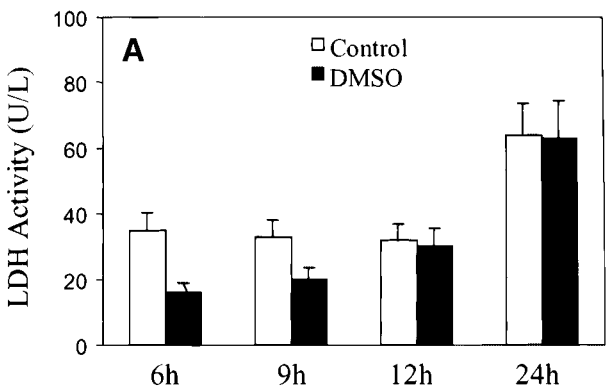
In order to study the effect of apoptotic cells on lung pathology, the apoptotic cell samples must be free of necrotic cells. Necrotic cells may be induced during the process of apoptosis induction and their presence could lead to tissue inflammation and injury due to the release of toxic and inflammatory contents. To determine whether the apoptotic cell samples might contain necrotic cells we assessed cell lysis using LDH assay. Our results showed that DMSO treatment did not cause an increase in LDH release over non-treated control over a 24-h period (Fig. 4A). These results suggest that the apoptotic cell samples used in the instillation studies (9-h treatment) are relatively free of necrotic cells. To study the possible delayed necrotic effect of DMSO, cells were treated with DMSO for 12 h and after being washed they were kept in 5% FBS culture medium for up to 4 days. LDH analysis of these cell samples showed no significant difference in LDH release between treated and non-treated cells at all times (Fig. 4B).

Lung cell apoptosis after instillation with apoptotic AM

Numerous TUNEL-positive lung cells are seen in tissue sections obtained 4 weeks after the pulmonary instillation of apoptotic AM (Fig. 5A,B). Most apoptotic lung cells are macrophages and few are epithelial cells. Minimal apoptotic cells were observed in the lungs of rats treated with non-apoptotic AM or PBS. The lack of inflammatory effect from instillation of untreated AM is comparable to the failure of inflammation induction by bone marrow derived macrophages instilled into lungs (Lambrech et al., 2000).

Pulmonary responses to apoptotic cell instillation

BN rats were used in this study since they are inbred animals and their AM are more susceptible to in vitro apoptosis induction than the inbred F344 rats. Apoptotic AM used in the instillation studies were treated with DMSO for 12 h and then thoroughly washed to remove any residual DMSO. Instillation of the apoptotic AM (5×10^6 cells/rat, ~20% apoptotic cells) into the rat lungs caused a significant increase in macrophage cell number



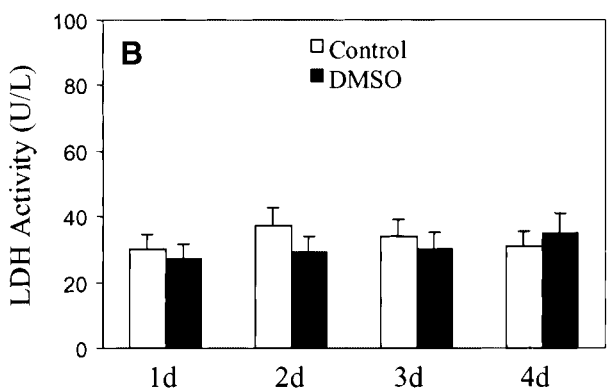
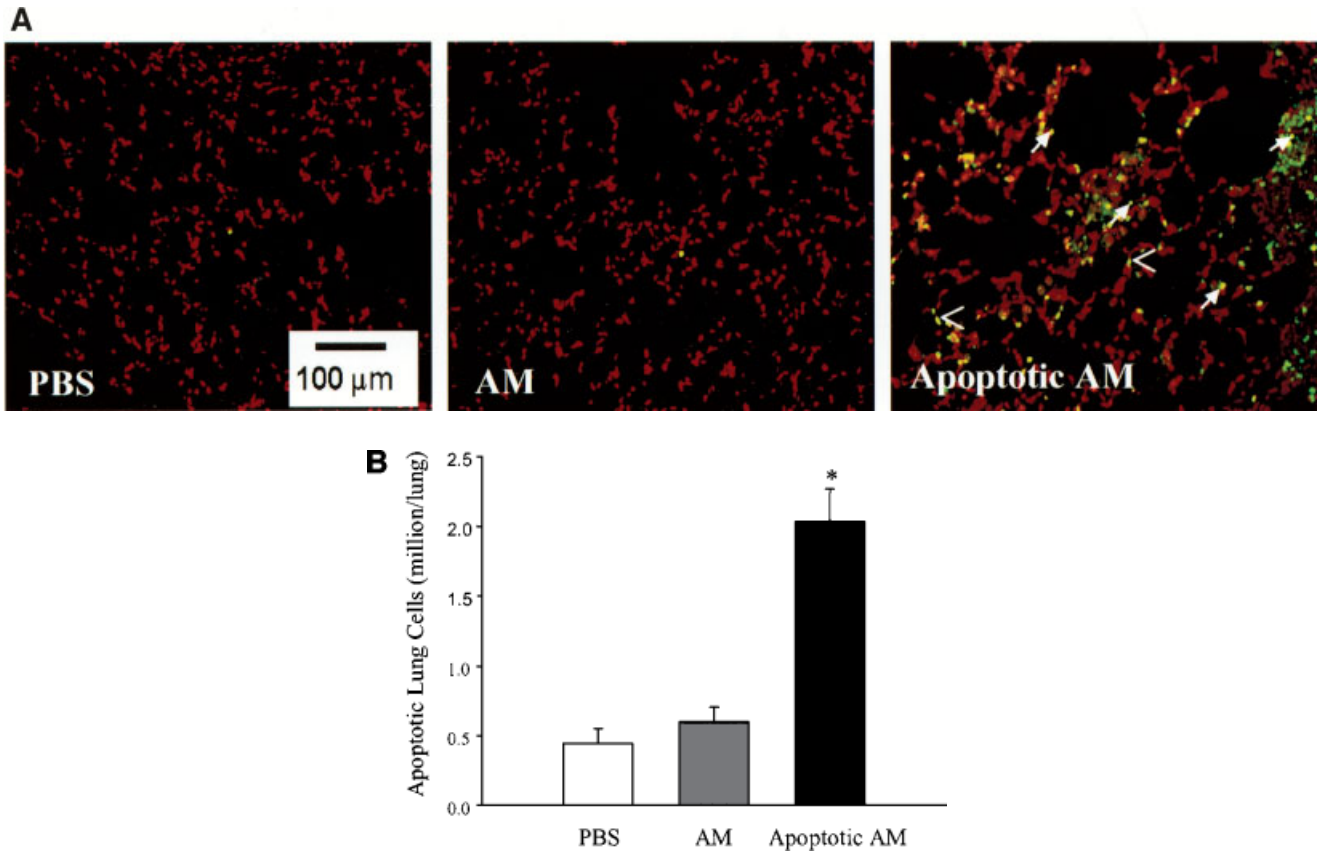


Fig. 4. Effect of DMSO on cellular LDH release. **A**: AM were treated in vitro with 400 mM DMSO for 6, 9, 12, and 24 h at 37°C, after which LDH levels in cell supernatants were determined. The figure shows the effect of DMSO on AM obtained from BN rats. Similar results were also obtained when AM from different strains of rats were used (data not shown). **B**: AM from BN rats were treated with DMSO for 9 h, and after being washed they were kept in 5% FBS culture medium for 1, 2, 3, and 4 days. Values were means ± SE, n = 4. No significant difference was observed between DMSO-treated and control cells ($P < 0.5$).


A
PBS
100 μm
AM
Apoptotic AM
B
Apoptotic Lung Cells (million/lung)
2.5
2.0
1.5
1.0
0.5
0.0
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PBS
AM
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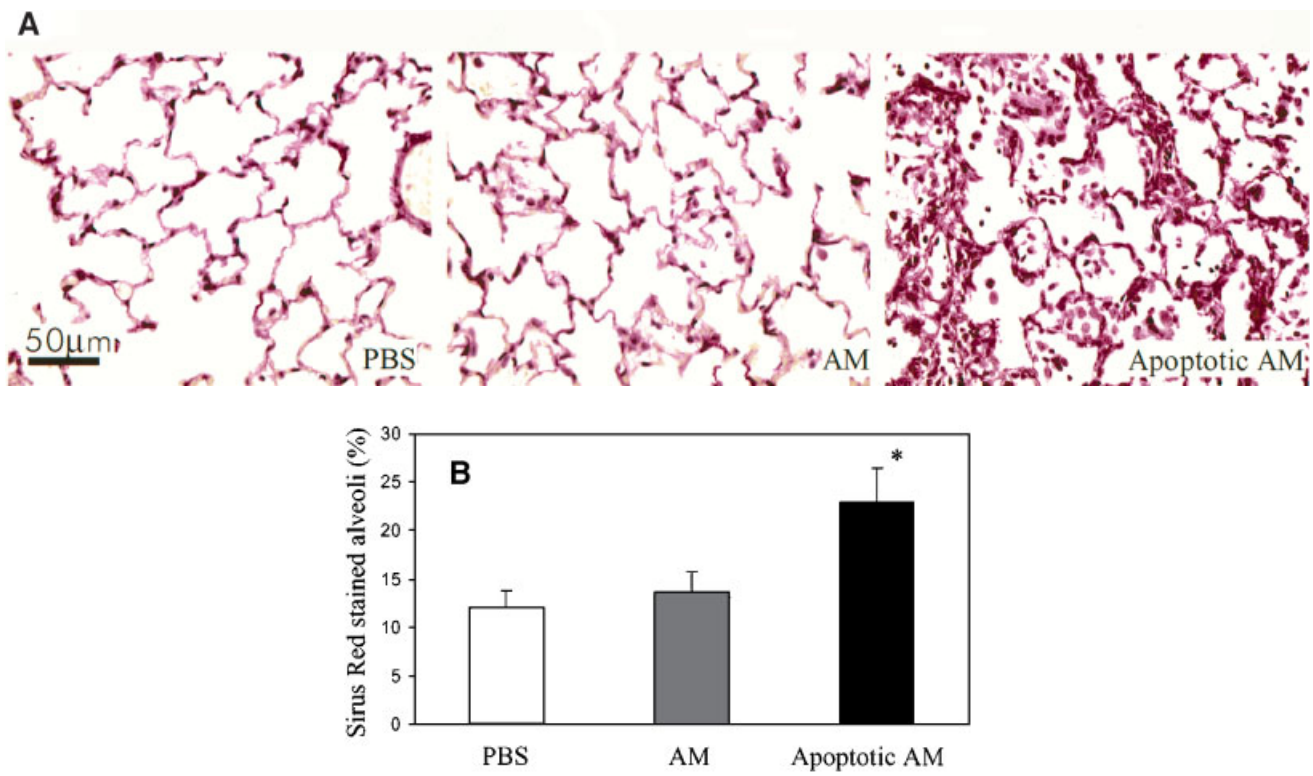
Fig. 5


A
50μm
PBS
AM
Apoptotic AM
B
Sirus Red stained alveoli (%)
30
25
20
15
10
5
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PBS
AM
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Fig. 7

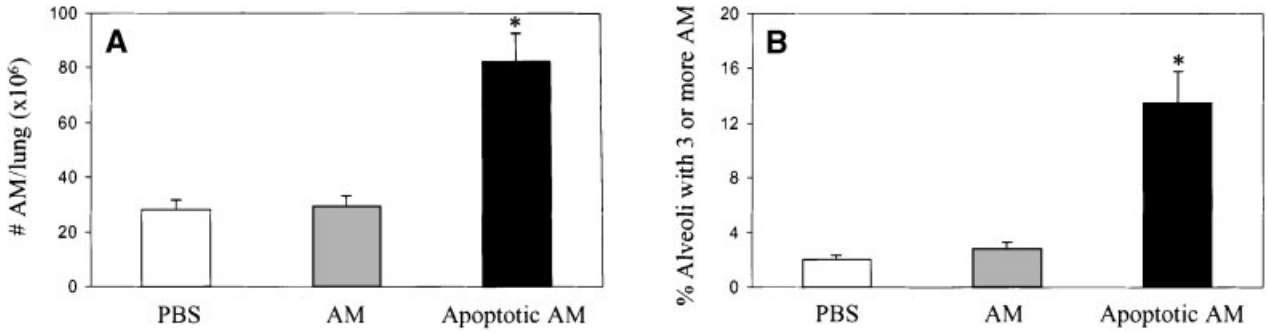

Fig. 6. Effect of apoptotic cell treatment on macrophage cell number. **A**: The number of macrophages 4 weeks after treatment with or without apoptotic cells. Experiments were conducted using BN rats and apoptotic BN macrophages. **B**: Effect of apoptotic cell treatment on the percentage of alveoli containing 3 or more AM. An alveolus with three or more AM is considered to be an inflamed site since normal rat lung contains an average of 1 AM/alveolus. Values are means ± SE, n = 6 rats/group. $^*P < 0.05$ versus PBS control group.

at 4 weeks post-treatment in both morphometric analysis (Fig. 6A) and lavaged cell count (data not shown). No significant change in the number of lung macrophages was observed after the treatment period when a low dose of apoptotic AM (1×10^6 cells/rat) was used (result not shown). Likewise, treatment of the rats with normal control AM (5×10^6 cells/rat) or PBS had no effect. Morphometric analysis of the number of inflamed alveoli, i.e., those containing three or more AM, also indicates a substantial increase in lung inflammation following the high-dose apoptotic AM instillation (Fig. 6B). These results suggest that apoptotic AM were able to induce pulmonary infiltration of macrophages and cause lung inflammation in a dose dependent manner.

Pulmonary instillation of apoptotic AM also causes pulmonary fibrosis as indicated by the increased Sirius Red staining of lung collagen (Fig. 7A) and calculated collagen volume (Fig. 7B). The co-localization of accumulated macrophages and collagen in the tissue sections also suggest a relationship between these two processes in lung pathology.

Induction of inflammatory and fibrotic cytokines by apoptotic AM

To investigate the potential mechanisms of apoptotic AM-induced lung disorders, we used immunohistochemistry to localize key mediators that are known to be involved in the inflammatory and fibrotic processes, for example, TNF-α, TGF-β, and MMPs. The expression of these proteins is visualized as the brown color in the macrophage cytoplasm (Fig. 8). We found that apoptotic AM were able to induce increased expression of TNF-α, TGF-β, and MMP2, whereas the control normal AM or PBS had no or minimal effects. A similar result was obtained from MMP9 but not from MMP10 immunohistochemical staining (data not shown).

DISCUSSION

We have demonstrated the differential susceptibility to apoptosis induction of AM from different strains of rats. Maximum inducibility of AM apoptosis was found to be 30% in AM from SD rats and NR8383 macrophages, 17% in AM from BN rats and 10% in AM from F344 rats following an in vitro treatment with DMSO. The limited level of AM apoptosis in these studies is believed to be due to efficient engulfment of apoptotic cells by phagocytic macrophages. Supporting this notion is the evidence showing that macrophages ingest apoptotic bodies during the process of apoptosis induction in vitro (Fig. 3B).

Instillation of apoptotic AM into the rat lungs was shown to result in an increase in the number of apoptotic lung cells 4 weeks after the treatment. Since apoptotic cells are known to be rapidly phagocytosed in vivo (Wyllie et al., 1980; Bursch et al., 1990) and in vitro (this paper) in a matter of hours, it seems unlikely that the apoptotic cells observed 4-weeks after the treatment are the same cells initially instilled. Instead, an in vivo induction of apoptosis appears to be subsequently induced after the apoptotic AM treatment. This notion of secondary apoptosis is supported by previous in vitro studies showing that macrophages ingest apoptotic cells within 15 min and degrade them within 30 min (Savill et al., 1993; Savill, 1994). Subsequent studies also show that macrophages ingesting apoptotic bodies release soluble factors that trigger apoptosis of "bystander" cells (Brown and Savill, 1999). Several findings from our study also support this concept: 1) our in vitro data show

Fig. 5. Detection of apoptosis in vivo by TUNEL using lung sections. **A**: BN rats were instilled with 1 ml of sterile PBS, normal AM, or DMSO-induced apoptotic AM from BN rats (5×10^6 cells/rat). After 4 weeks, the animals were sacrificed and lung sections were prepared for TUNEL as described in the Materials and Methods. Note that most apoptotic cells in these lung sections are macrophages (closed arrow) and a few are epithelia cells (open arrow). **B**: Quantitation of apoptotic lung cells of Figure 5A. Numbers are means ± SE, n = 6 rats/group. $^*P < 0.05$ versus PBS control group.

Fig. 7. Detection of lung fibrosis. **A**: Collagen staining of BN rat lung sections by Sirius Red 4 weeks after instillation of PBS, normal AM, or apoptotic AM. **B**: Calculated collagen volume is shown. Values are means ± SE, n = 6 rats/group. $^*P < 0.05$ versus PBS control group.

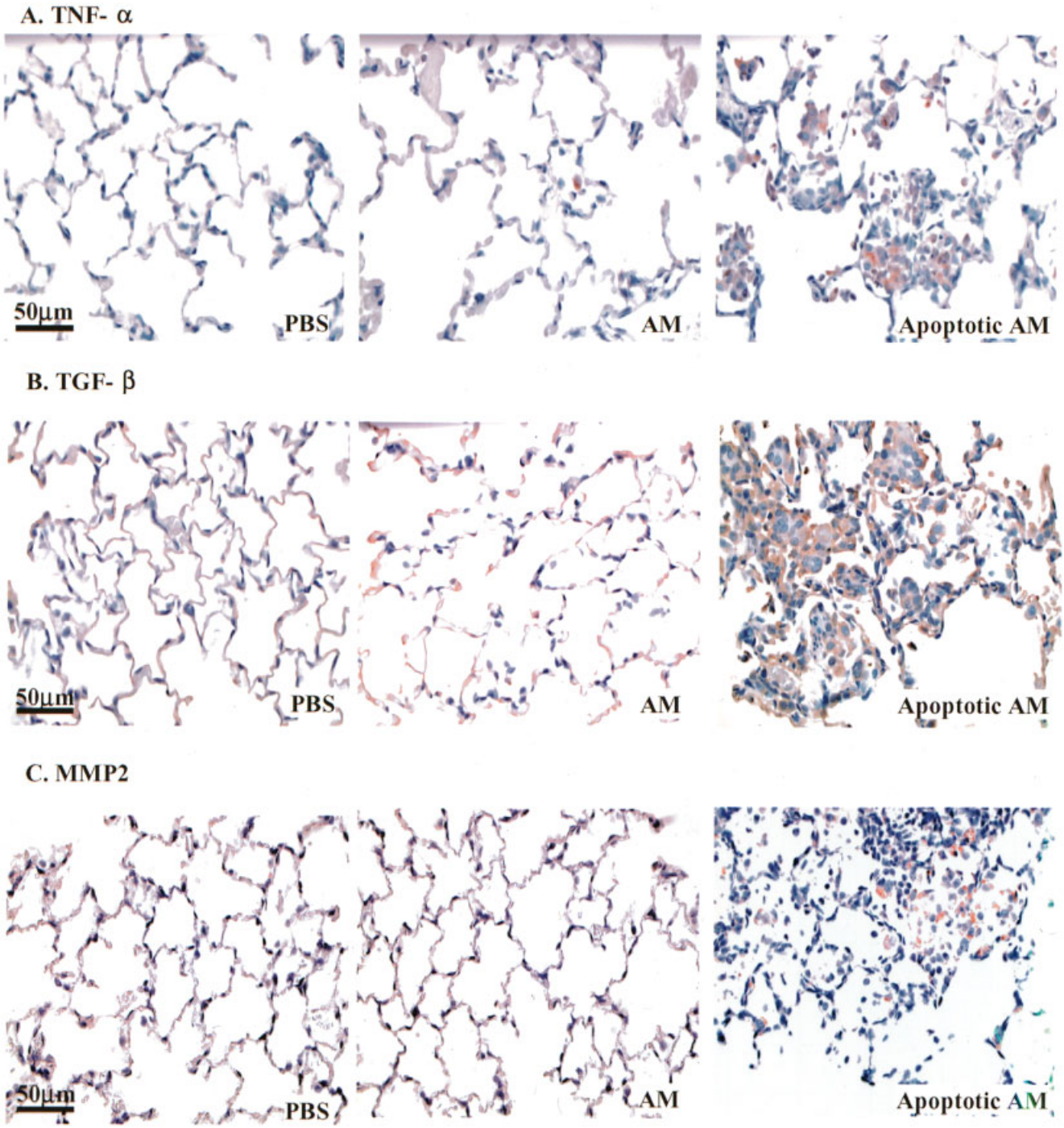

Fig. 8. Immunohistochemical detection of cytokines. Representative lung sections from BN rats at 4 weeks after instillation of PBS, normal AM, or apoptotic AM (n = 6 rats/group). **A**: TNF-α, (**B**) TGF-β, (**C**) MMP2. These proteins, stained as a brown color, are shown in the macrophage cytoplasm. Similar result was obtained from MMP9 but not from MMP10 immunohistochemical staining (data not shown).

that phagocytosis of apoptotic cells occurs at or before 12 h; 2) increased macrophage infiltration in response to in vivo exposure to apoptotic cells occurs concurrently with the observed increase in apoptosis of resident macrophages in exposed lungs; 3) lung cell apoptosis is not limited to macrophages but also includes epithelial cells; and 4) increased expression of TNF-α and TGF-β, two major cytokines known to induce apoptosis, was also observed at the time of apoptosis detection. These findings, along with the observed absence of effects of normal non-apoptotic AM on lung cell apoptosis, macrophage infiltration, and expression of apoptosis-inducing

cytokines, strongly indicate the development of secondary apoptosis of resident lung cells after exposure to apoptotic AM.

Studies on the pathogenesis of pulmonary disorders have thus far been focused on the cellular events that initiate and amplify pulmonary responses to pathologic stimuli. However, certain pathologic conditions persist in the absence of any identifiable stimuli, raising the possibility that such conditions result not from ongoing stimulation but from failure of the normal resolution processes. Because apoptosis plays an important role in the normal resolution process, its alteration may result in pathologic conditions. In this study we have shown that pulmonary administration of apoptotic AM causes pulmonary inflammation and fibrosis. This is the first demonstration that apoptosis by itself can cause lung disorders in the absence of inciting agents. The ability of apoptotic AM to induce lung cell apoptosis suggests that the phagocytic clearance system of the lung may be impaired or overwhelmed by massive waves of apoptosis. Such impairment could lead to development of secondary necrosis of apoptotic cells, which results in release of their inflammatory and cytotoxic contents.

We have also shown that pulmonary instillation of apoptotic AM results in an upregulation of inflammatory and fibrogenic mediators including TNF-α, TGF-β, and MMP2. These mediators have also been shown to be overexpressed following pulmonary exposure to a variety of pneumotoxic agents. TNF-α is a major cytokine, which participates in the recruitment of inflammatory cells and in the development of pulmonary inflammation (Fujita et al., 2001). TNF-α has been proposed to be directly linked to the fibrotic process, since TNF-α antibodies inhibit bleomycin and silica induced fibrosis (Pignet et al., 1989, 1990). Furthermore, MMP and TGF-β are known fibrogenic mediators (Swiderski et al., 1998; Martin et al., 2000; Vu and Werb, 2000). Recent studies have shown that macrophage ingestion of apoptotic cells causes an increased release of TGF-β (Fadok et al., 1998; McDonald et al., 1999). Overproduction of this cytokine by excessive apoptotic and phagocytic stimuli may contribute to the observed fibrotic response.

Our study suggests that at sufficiently high levels of apoptosis, lung clearance mechanisms may be overwhelmed by the need to remove apoptotic cells. Thus a reduction in the clearance of apoptotic products would also reduce the clearance of inhaled bacteria and airborne pollutants within the alveolar region of the lungs. Under circumstances where exposure to bacteria and/or airborne particles occurs in concert with a high level of apoptosis in the lungs, an augmented pathological cycle may arise in which the accumulated bacteria, toxic pollutants, and/or apoptotic cells induce additional apoptosis, further reducing the lung clearance and leading to additional accumulation of toxic apoptotic bodies and inhaled pollutants.

In conclusion, pulmonary administration of apoptotic AM results in an increased level of apoptotic cell death, which appears to overwhelm the phagocytic clearance mechanism necessary to maintain homeostasis. This condition promotes pulmonary infiltration of macrophages which contributes to lung inflammation and fibrosis. The mechanisms by which AM apoptosis induce pulmonary disorders may involve increased expression of inflammatory and fibrogenic mediators such as TNF-α, TGF-β, MMP2, and MMP9, which are demonstrated in this study.

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