The Lysophosphatidic Acid Receptor LPA$_1$ Promotes Epithelial Cell Apoptosis after Lung Injury

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Increased epithelial cell apoptosis in response to lung injury has been implicated in the development of idiopathic pulmonary fibrosis (IPF), but the molecular pathways promoting epithelial cell apoptosis in this disease have yet to be fully identified. Lysophosphatidic acid (LPA), which we have previously demonstrated to mediate bleomycin lung injury–induced fibroblast recruitment and vascular leak in mice and fibroblast recruitment in patients with IPF, is an important regulator of survival and apoptosis in many cell types. We now show that LPA signaling through its receptor LPA$_1$ promotes epithelial cell apoptosis induced by bleomycin injury. The number of apoptotic cells present in the alveolar and bronchial epithelia of LPA$_1$–deficient mice was significantly reduced compared with wild-type mice at Day 3 after bleomycin challenge, as was lung caspase-3 activity. Consistent with these *in vivo* results, we found that LPA signaling through LPA$_1$ induced apoptosis in normal human bronchial epithelial cells in culture. LPA-LPA$_1$ signaling appeared to specifically mediate anoikis, the apoptosis of anchorage-dependent cells induced by their detachment. Similarly, LPA negatively regulated attachment of R3/1 rat alveolar epithelial cell line cells. In contrast, LPA signaling through LPA$_1$ promoted the resistance of lung fibroblasts to apoptosis, which has also been implicated in IPF. The ability of LPA-LPA$_1$ signaling to promote epithelial cell apoptosis and fibroblast resistance to apoptosis may therefore contribute to the capacity of this signaling pathway to regulate the development of pulmonary fibrosis after lung injury.

Keywords: pulmonary fibrosis; apoptosis; epithelial cells, lysophosphatidic acid; LPA$_1$

Repetitive lung injury and aberrant wound-healing responses are thought to contribute to the pathogenesis of idiopathic pulmonary fibrosis (IPF) (1). We have recently generated evidence that the bioactive lipid lysophosphatidic acid (LPA) may drive several of the wound-healing responses thought to contribute to the development of pulmonary fibrosis after lung injury, including fibroblast recruitment and vascular leak (2). LPA signals through specific G protein–coupled receptors, at least five of which have been definitively established and designated

CLINICAL COMMENTARY

Increased epithelial cell apoptosis, coupled with increased fibroblast resistance to apoptosis, has been implicated in idiopathic pulmonary fibrosis (IPF), but the molecular pathways responsible for these divergent susceptibilities of epithelial cells and fibroblasts to apoptosis have yet to be fully identified. Our findings provide evidence that that lysophosphatidic acid (LPA) signaling through the lysophosphatidic acid receptor LPA$_1$ may contribute to this "apoptosis paradox" in IPF. We demonstrate that LPA-LPA$_1$ signaling promotes epithelial cell apoptosis but fibroblast resistance to apoptosis, both of which may contribute to the dramatic ability of this signaling pathway to regulate the development of pulmonary fibrosis after lung injury, and highlight the therapeutic potential of targeting this pathway in IPF.

LPA$_1$–LPA$_5$ (3). We found that mice deficient in one of these receptors, LPA$_1$, are dramatically protected from fibrosis and mortality induced by bleomycin challenge (2). Fibroblast recruitment and vascular leak were significantly attenuated in LPA$_1$–deficient (LPA$_1$ knockout [KO]) mice in this model of pulmonary fibrosis. We also found that LPA levels were increased in bronchoalveolar lavage samples from patients with IPF, that LPA$_1$ was highly expressed by fibroblasts recovered from these samples, and that inhibition of LPA$_1$ markedly reduced fibroblast responses to the chemotactic activity of these samples, all suggesting that LPA signaling through LPA$_1$ contributes to the excessive accumulation of fibroblasts that occurs in the lungs of patients with IPF.

In addition to mediating fibroblast recruitment and vascular leak, LPA regulates an extensive array of developmental, physiological, and pathophysiological processes, including cell proliferation, cell differentiation, cytoskeletal rearrangement, and cell survival. Regarding cell survival, LPA has pervasive but varied effects, promoting the survival or apoptosis of many different cell types (4). Exaggerated lung epithelial cell apoptosis appears to play a central role in pulmonary fibrogenesis. Increased numbers of apoptotic cells have been observed in the alveolar and bronchial epithelia of patients with IPF (5, 6). Induction of pulmonary epithelial cell apoptosis in mice by pulmonary delivery of anti-Fas antibody (7, 8) or transgenic overexpression of transforming growth factor (TGF)-β (9) results in the development of fibrosis, as does targeted injury of alveolar epithelial cells (10). Epithelial cell apoptosis is also prominent in the bleomycin model of pulmonary fibrosis, in which intratracheal challenge leads to the rapid appearance of apoptosis in bronchial and alveolar epithelial cells. This early phase of apoptosis resolves but is followed by a second wave of apoptosis in the second week after challenge (11). To investigate

whether LPA contributes to increased epithelial cell apoptosis induced by fibrogenic lung injury, we compared the time course of apoptosis induced by intratracheal bleomycin challenge in wild-type (WT) and LPA_1 KO mice. We found that the early phase of apoptosis was significantly attenuated in the absence of LPA_1 expression, suggesting that LPA signaling through LPA_1 promotes epithelial cell apoptosis. Consistent with these *in vivo* results, we found that LPA signaling through LPA_1 induced apoptosis in normal bronchial airway epithelial (NHBE) cells in culture.

In contrast, LPA signaling through the same receptor, LPA_1, promoted resistance of primary mouse lung fibroblasts (PMLFs) to apoptosis. Fibroblasts and myofibroblasts appear to be abnormally resistant to apoptosis in IPF (12). In addition to the abilities of LPA and LPA_1 to promote fibroblast recruitment and vascular leak, LPA-LPA_1 signaling may also contribute to the development of pulmonary fibrosis after lung injury by promoting epithelial cell apoptosis but fibroblast resistance to apoptosis. Loss of these effects of LPA-LPA_1 signaling on epithelial cell and fibroblast apoptosis may therefore represent two additional mechanisms through which LPA_1 KO mice are dramatically protected from bleomycin-induced fibrosis, as we have previously observed (2). Some of the data presented in this paper have previously been reported in abstract form (13).

MATERIALS AND METHODS

Animals and Bleomycin Administration

Experiments comparing LPA_1 KO and WT mice used sex- and weight-matched offspring of mice heterozygous for the LPA_1 mutant allele, which were hybrids of the C57Bl/6 and 129Sv/J genetic backgrounds (14). These mice on average had similar assortments of genes from the C57Bl/6 and 129Sv/J backgrounds. Experiments measuring bronchoalveolar lavage (BAL) LPA concentrations used WT C57Bl/6 mice purchased from the NCI-Frederick Mouse Repository. All bleomycin-challenged mice received 3 U/kg of bleomycin (Gensia Sicor Pharmaceuticals, Irvine, CA) in a total volume of 50 μl sterile saline by intratracheal injection. All experiments used mice that were 6 to 10 weeks of age, and all mice were maintained in a specific pathogen–free environment certified by the American Association for Accreditation of Laboratory Animal Care. All experiments were performed in accordance with National Institute of Health guidelines and protocols approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Lung Immunohistochemical and Immunofluorescence Staining

Lungs excised for immunostaining were inflated to 25 cm H_2O and fixed with 10% buffered formalin. Terminal deoxynucleotidyl transferase dUTP nick end labeling $(TUNEL)^+$, $p53^+$ and $p21^+$ cells, and $TUNEL^+/T1\alpha^+$ and $TUNEL^+$/prosurfactant protein C^+ cells were identified in multiple paraffin-embedded 5-μm sections of the entire mouse lung by immunohistochemical and immunofluorescence staining as described in the online supplement.

Lung Caspase-3 Activity

Caspase-3 activity was determined in homogenates of whole sets of mouse lungs using a Caspase-3 Fluorometric Assay Kit (Biovision, Mountain View, CA) according to the manufacturer's instructions.

Mouse BAL and LPA Analysis

BAL samples for analysis of LPA levels were obtained as previously described (2). LPA concentrations were determined by electrospray ionization mass spectrometry by an investigator blinded to the identity of the samples as previously described (15). Concentrations of 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 LPA were measured and added to determine total BAL LPA concentrations.

NHBE, R3/1, and PMLF Cell Culture and Apoptosis, Detachment, and Attachment Assays

NHBE cells (Lonza, Basel, Switzerland) were initially cultured in tissue culture–treated polystyrene flasks (Corning, Lowell, MA) in bronchial epithelial cell growth medium (Lonza) according to the manufacturer's instructions and used when 60 to 70% confluent at passages 5 to 8. R3/1 cells, a rat alveolar epithelial cell line with several characteristic features of type I cells (16, 17), were the kind gift of Dr. Roland Koslowski (University of Technology, Dresden, Germany) and were cultured in DMEM medium with 10% FBS. PMLFs were isolated from WT and LPA_1 KO mice and cultured in tissue culture–treated polystyrene flasks in DMEM with 15% FBS according to the standard methods of our laboratory (18). All cells were cultured at 37°C and 5% CO_2 in a humidified incubator. NHBE cells and PMLF apoptosis assays and NHBE cells and R3/1 cell detachment and attachment assays were performed as described in the online supplement.

NHBE Cell Cytofluorescence Staining

NHBE cells for fluorescence staining were transferred to Lab-Tek 4 well Permanox chamber slides (Nunc-Nalgene; Thermo Scientific, Rochester, NY) at 50,000 cells per well and cultured directly in bronchial epithelial cell growth medium with 0.1% fatty acid–free BSA (Sigma Aldrich, St. Louis, MO) with or without 18:1 LPA (Avanti Polar Lipids, Alabaster, AL) for 3 hours. Cells were fixed with paraformaldehyde, and labeling of vinculin or F-actin was performed with fluorescein isothiocyanate–conjugated mouse antihuman vinculin antibody (Sigma Aldrich) or fluorescein phalloidin (Molecular Probes; Invitrogen), respectively. Slides were then mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Statistics

Differences between groups in experiments with two groups were analyzed for statistical significance with two-tailed Student's *t* tests using Microsoft Excel software. Differences between groups in experiments with more than two groups were analyzed for statistical significance by one-way ANOVA. When ANOVA rejected the hypothesis of equality of the group means, pairwise comparisons were made between groups with Bonferroni post-test corrections. *P* values of < 0.05 were considered statistically significant for all comparisons, and all errors represent SEM.

RESULTS

Bleomycin-induced Apoptosis of the Alveolar Epithelium Is Dependent on LPA_1

Bleomycin delivered as a single intratracheal challenge has been demonstrated to lead to the rapid appearance of apoptosis in bronchial and alveolar epithelial cells. This early phase of apoptosis resolves but is followed by another wave of apoptosis in the second week after challenge (11). To determine if LPA-LPA_1 signaling influenced epithelial cell apoptosis during the development of pulmonary fibrosis, we first compared the time course of alveolar epithelial apoptosis induced by bleomycin in the lung parenchyma of WT and LPA_1 KO mice. TUNEL staining of lung sections from mice killed at multiple time points after bleomycin challenge demonstrated that the early phase of apoptosis was significantly attenuated in the absence of LPA_1 expression (Figures 1A–1C). Minimal numbers of $TUNEL^+$ cells were present in the lungs of WT and LPA_1 KO mice at baseline on Day 0 before bleomycin challenge (*see* Figures E1A and E1B in the online supplement). The number of $TUNEL^+$ cells increased in the alveolar epithelium of WT mice at Day 1 after bleomycin challenge, remained elevated at Day 3, and declined at Day 5 (Figures 1A and 1C). The number of $TUNEL^+$ cells in the alveolar epithelium of LPA_1 KO mice increased to levels similar to those of WT mice at Day 1 after bleomycin challenge
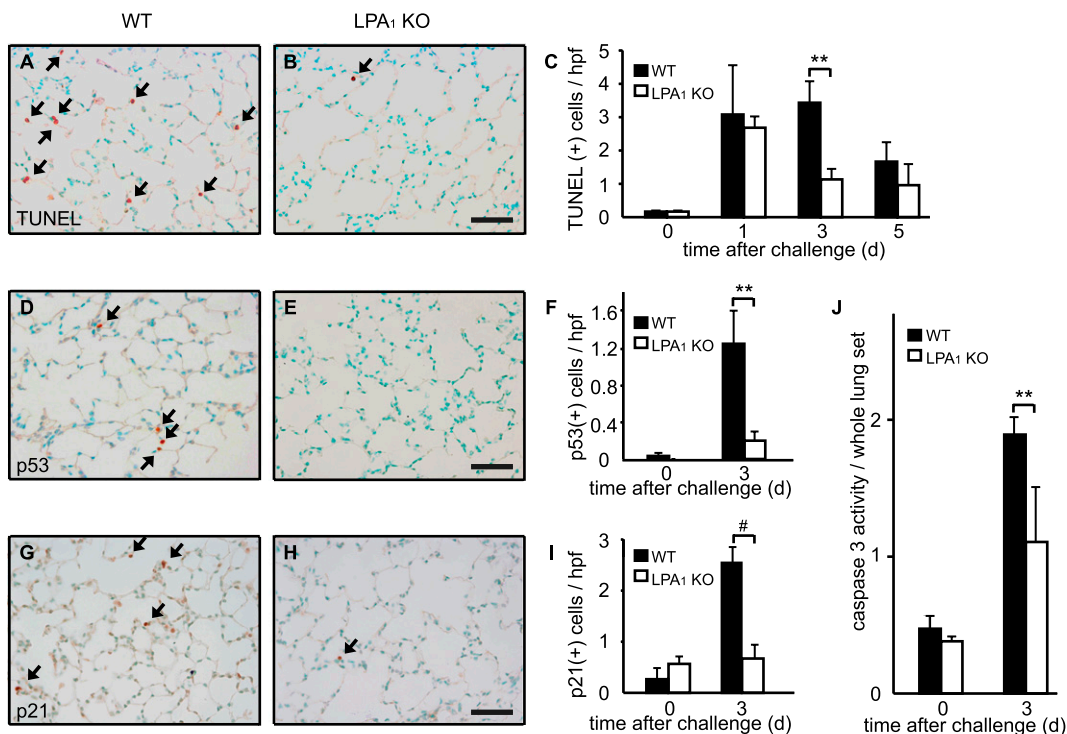
Figure 1. Bleomycin-induced alveolar epithelial apoptosis was attenuated in the lysophosphatidic acid receptor (LPA₁) knock-out (KO) mice. (*A–C*) The increase in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)$^+$ cells induced by bleomycin in the alveolar epithelium was attenuated in LPA₁ KO mice. Representative TUNEL/peroxidase-stained sections of (*A*) wild-type (WT) and (*B*) LPA₁ KO mouse lungs 3 days after bleomycin challenge. *Scale bars* = 50 μm for all images. (*C*) Mean numbers of TUNEL$^+$ cells per high-power field ± SEM in lung sections of WT and LPA₁ KO mice at Days 0, 1, 3, and 5 after bleomycin challenge. Data were pooled from two independent experiments; the combined numbers of samples were $n = 3$ (Day 0) or 6 (Days 1, 3, and 5) for WT and LPA₁ KO mice. **$P < 0.01$, WT versus LPA₁ KO mice at Day 3 after bleomycin challenge; significance for this comparison, and for all subsequent comparisons performed for experiments with only two experimental groups, was determined by two-tailed Student's *t* test. (*D–F*) The increase in p53$^+$ cells induced by bleomycin in the alveolar epithelium was attenuated in LPA₁ KO mice. Representative p53/peroxidase-stained sections of (*D*) WT and (*E*) LPA₁ KO mouse lungs 3 days after bleomycin challenge. (*F*) Mean numbers of p53$^+$ cells per high-power field ± SEM in WT and LPA₁ KO lung sections at Day 0 and Day 3 after bleomycin challenge. $n = 3$ (Day 0) or 7 (Day 3) for each genotype. **$P = 0.01$, WT versus LPA₁ KO mice at Day 3. (*G–I*) The increase in p21$^+$ cells induced by bleomycin in the alveolar epithelium was attenuated in LPA₁ KO mice. Representative images of p21/peroxidase-stained sections of (*G*) WT and (*H*) LPA₁ KO mouse lungs 3 days after bleomycin challenge. (*I*) Mean numbers of p21$^+$ cells per high-power field ± SEM in WT and LPA₁ KO lung sections at Day 0 and Day3 after bleomycin challenge. $n = 3$ (Day 0) or 7 (Day 3) for each genotype. $^\#P < 0.001$, WT versus LPA₁ KO mice at Day 3. (*J*) Mean caspase 3 activity/whole lung set ± SEM measured in WT and LPA₁ KO lung homogenates at Day 0 and Day 3 after bleomycin challenge. $n = 7$ to 10 for each genotype at each time point. **$P = 0.01$, WT versus LPA₁ KO mice at Day 3.

but declined earlier. Consequently, at Day 3 after bleomycin challenge, TUNEL$^+$ cells were reduced by 67% in LPA₁ KO compared with WT mice (Figures 1B and 1C).

To confirm that the early phase of bleomycin-induced apoptosis was attenuated in the lung in the absence of LPA₁ expression, we compared the expression of p53 and p21 and the activity of caspase 3 in WT and LPA₁ KO mice at Day 3 after bleomycin challenge. Up-regulation of the tumor suppressor/apoptosis regulatory proteins p53 and p21 has been demonstrated in apoptotic cells in the alveolar and bronchial epithelia of patients with IPF (5, 6) and in mouse alveolar epithelial cells during the early phase of bleomycin-induced apoptosis (19). Minimal numbers of p53$^+$ and p21$^+$ cells were present in the lungs of WT and LPA₁ KO mice at Day 0 (Figures E1C–E1F). The increase in the number of p53$^+$ cells observed at Day 3 after bleomycin challenge was significantly attenuated in the lungs of LPA₁ KO compared with WT mice (Figures 1D–1F); at this time point, p53$^+$ cells were reduced by 84% in LPA₁ KO compared with WT mice. The increase in the number of p21$^+$ cells at Day 3 after bleomycin challenge was similarly attenuated in LPA₁ KO mice (Figures 1G–1I), with 74% fewer p21$^+$ cells being present in LPA₁ KO compared with WT mice. Up-regulation of caspase 3, one of the effector caspases of apoptosis, has also been associated with apoptosis of bronchiolar and alveolar epithelial cells in patients with IPF (6) and in bleomycin-challenged mice (20). The increase in lung caspase 3 activity induced by bleomycin at Day 3 after bleomycin challenge was significantly mitigated in LPA₁ KO mice

(Figure 1J); at this time point, lungs from LPA₁ KO mice contained only 55% of the caspase 3 activity present in WT lungs. At Day 1 after bleomycin challenge, lung caspase 3 activity was increased to a similar extent in LPA₁ KO and WT mice, although the increases were not significant in either genotype (data not shown).

To determine whether apoptotic cells in the alveolar epithelium of WT and LPA₁ KO mice at early time points after bleomycin challenge were type I or type II alveolar epithelial cells (AECs), we performed TUNEL staining in conjunction with immunofluorescence staining of T1α, a marker of type I AECs (21) (Figure E2), or pro-surfactant protein C (pro-SP-C), a marker of type II AECs (Figure E3), on lung sections from mice killed at Day 0 before bleomycin challenge or at Day 3 after bleomycin challenge. As seen previously, minimal numbers of TUNEL$^+$ cells were present in the lungs of WT and LPA₁ KO mice at baseline on Day 0 (Figures E2A and E2B; E3A and E3B). At Day 3 after bleomycin challenge, most TUNEL$^+$ cells in WT and LPA₁ KO mice demonstrated costaining for T1α (Figures E2C and E2D). The specificity of T1α staining for type I AECs is demonstrated in Figure E2E, in which T1α expression is absent from the pulmonary endothelium, the bronchial epithelium, and cells in the alveolar epithelium that appear to be type II AECs. In contrast, few TUNEL$^+$ cells in both genotypes of mice demonstrated costaining for the type II AEC marker pro-SP-C at Day 3 after bleomycin challenge (Figures E3C and E3D). However, whereas pro-SP-C staining appeared uniform throughout the lung parenchyma at

Day 0 before bleomycin challenge, staining for this type II cell marker appeared to decrease in areas of lung injury at Day 3 after bleomycin challenge. There was a paucity of pro-SP-C staining in areas with substantial numbers of TUNEL$^+$ cells, consistent with a prior report of type II cell expression of SP-C declining after bleomycin challenge (Figure E3E) (22). Therefore, although our staining suggests that substantial apoptosis of type I AECs occurs at Day 3 after bleomycin challenge, we cannot exclude the possibility that substantial type II cell apoptosis is concurrently occurring.

Bleomycin-Induced Apoptosis of the Bronchial Epithelium Is Dependent on LPA$_1$

Increased numbers of apoptotic cells have been observed in the bronchial epithelium as well as in the alveolar epithelium in the bleomycin model of pulmonary fibrosis (11) and in the lungs of patients with IPF (5, 6). We therefore compared the time course of bronchial epithelial apoptosis induced by bleomycin in the airways of WT and LPA$_1$ KO mice. TUNEL staining of lung sections from mice killed at multiple time points after bleomycin challenge demonstrated that the early phase of apoptosis was also significantly attenuated in the bronchial epithelium in the absence of LPA$_1$ expression (Figures 2A–2G). No TUNEL$^+$ cells were observed in the airways of WT or LPA$_1$ KO mice at baseline on Day 0 before bleomycin challenge (Figures 2A, 2B, and 2G). As observed in the alveolar epithelium, the number of TUNEL$^+$ cells increased in the bronchial epithelium of WT and LPA$_1$ KO mice to a similar extent at Day 1 after bleomycin challenge (Figures 2C, 2D, and 2G). However, as also observed in the alveoli, the number of TUNEL$^+$ cells in the airways of LPA$_1$ KO mice declined earlier than in WT mice, so that at Day 3 after bleomycin challenge, TUNEL$^+$ cells were reduced by 76% in LPA$_1$ KO compared with WT mice (Figures 2E, 2F, and 2G). Taken together, our results indicate that apoptosis induced early after bleomycin injury is significantly reduced in the airways and in the alveoli in the absence of LPA-LPA$_1$ signaling in LPA$_1$ KO mice.

LPA Accumulates in the Airspaces Early after Bleomycin Lung Injury

We have previously reported that LPA is present in increased concentrations in BAL recovered from mice at later time points after bleomycin challenge, from Day 5 to Day 14, indicating that it accumulates in the airspaces at these times (2). The dependence of bronchial and alveolar epithelial apoptosis on LPA$_1$

expression earlier after bleomycin suggests that increased amounts of LPA are also present in the lungs before these time points. To confirm this, we measured the concentrations of six major LPA species (16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 LPA) in BAL recovered from unchallenged mice (Day 0) and mice 1 and 3 days after bleomycin challenge by electrospray ionization mass spectrometry. Of these six LPA species, 16:0 and 22:6 are the most abundant in the plasma of unchallenged mice (23). The BAL concentrations of each of these species except 22:6 LPA increased at these early time points (Figure 3A). 18:0 LPA demonstrated the greatest increase, rising 3.5-fold between Days 0 and 1, and then returning to its baseline level at Day 3. Total BAL LPA concentrations, determined as the sum of the six major species of LPA measured, were elevated at 1 and 3 days after bleomycin challenge (Figure 3B). Increased levels of LPA were therefore present in the airspaces early post-bleomycin challenge, when bronchial and alveolar epithelial apoptosis was found to depend on LPA$_1$ expression. Although we have not formally evaluated BAL levels of LPA in bleomycin-challenged LPA$_1$ KO mice, we have previously found evidence to suggest that the generation of LPA is preserved in these mice deficient for one of LPA's receptors (2).

LPA Induces Epithelial Cell Apoptosis through its Receptor LPA$_1$

To investigate whether LPA is able to induce lung epithelial cell apoptosis by acting directly on these cells, we determined the effects of LPA on normal human bronchial epithelial (NHBE) cell apoptosis in culture. Apoptotic cells were identified in these experiments by flow cytometry after staining with annexin V and propidium iodide (24) as annexin V-positive, PI-negative cells (Figure E4). The effects of mediators on lung epithelial cell apoptosis have been demonstrated to depend on the extracellular matrix proteins with which the cells are in contact. For example, TGF-β has been demonstrated to induce apoptosis of lung epithelial cells grown on matrigel, which is representative of the basement membranes normally present in the lung but not of lung epithelial cells grown on fibronectin, which is representative of the extracellular matrix present in the injured or fibrotic lung (25). To investigate the effects of LPA on epithelial cell apoptosis early after lung injury before significant matrix remodeling, we exposed subconfluent NHBE cells grown on matrigel to 1 μM LPA. This concentration of LPA significantly induced NHBE cell apoptosis (Figure 4A). We have previously found that the process of collecting BAL dilutes the mouse epithelial lining fluid approximately 65-fold (18).
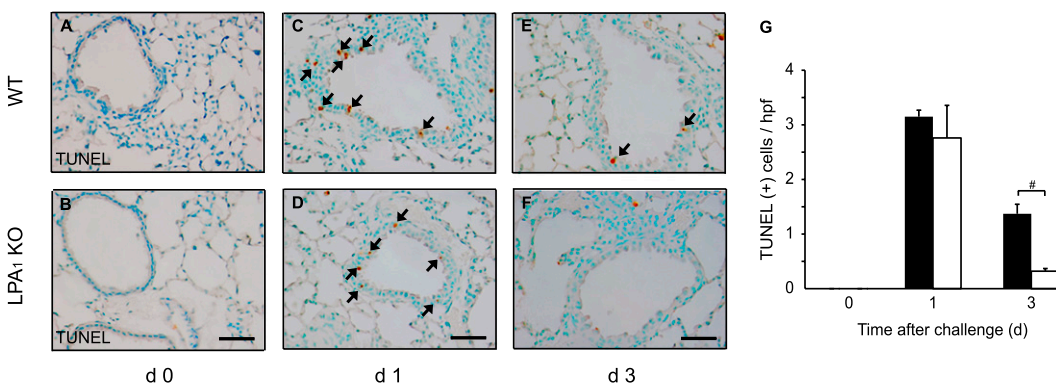
Figure 2. Bleomycin-induced bronchial epithelial apoptosis was attenuated in LPA$_1$ KO mice. The increase in TUNEL$^+$ cells induced by bleomycin in the bronchial epithelium was attenuated in LPA$_1$ KO mice. Representative TUNEL/peroxidase-stained sections of WT and LPA$_1$ KO mouse lungs at Day 0 (*A, B*), Day 1 (*C, D*), and Day 3 (*E, F*) after bleomycin challenge. *Scale bars =* 50 μm for all images. (*G*) Mean numbers of TUNEL$^+$ cells per high-power field ± SEM in lung sections of WT and LPA$_1$ KO mice at Day 0, 1, and 3 after bleomycin challenge. Data were pooled from two independent experiments; the combined numbers of samples were $n = 3$ (Day 0) or 6 (Days 1 and 3) for WT and LPA$_1$ KO mice. $^\#P < 0.001$, WT versus LPA$_1$ KO mice at Day 3 after bleomycin challenge.
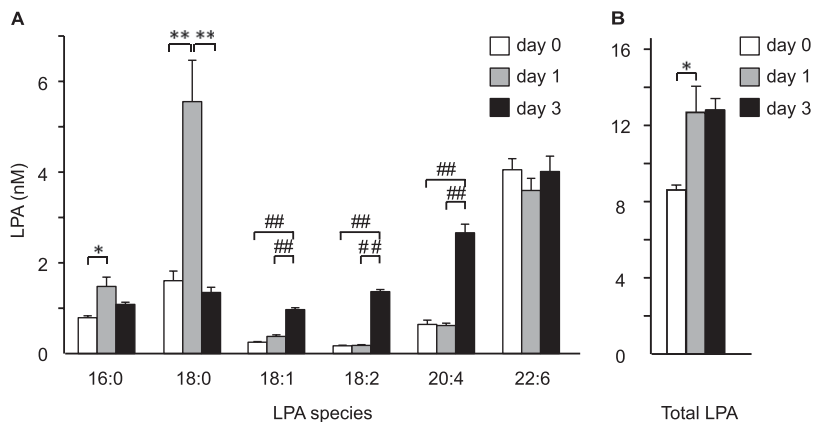
Figure 3. Bleomycin increased LPA levels early after injury. (*A*) Mean concentrations of six LPA species (16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 LPA) and (*B*) mean total LPA concentrations in BAL of C57Bl/6 mice at Day 0, 1, and 3 after bleomycin challenge. Three independent experiments were performed. Data represent the means of the values produced in the individual experiments ± SEM; the values produced in the individual experiments themselves were the means of $n = 5$ mice per time point. Significant differences are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ##$P < 0.0001$. Significance for these comparisons, and for all subsequent comparisons performed for experiments with more than two experimental groups, was determined by pairwise comparisons made with Bonferroni post-test corrections after one-way ANOVA rejected the hypothesis of equality of the group means.

Accounting for this dilution, our BAL measurements indicate that LPA concentrations in this range were present in the lungs at Days 1 and 3 post-bleomycin challenge. LPA concentrations are unlikely to be uniform throughout the epithelial lining fluid after bleomycin challenge. Rather, at focal sites of lung injury and repair, LPA epithelial lining fluid concentrations would be expected to be considerably higher than determined by our BAL measurements. These data suggest that direct effects of LPA on lung epithelial cells contribute to the epithelial apoptosis observed early after bleomycin injury.

In our experiments with NHBE cells, we found that exposure to LPA made these cells noticeably easier to detach with trypsin-EDTA. To begin to examine whether promotion of NHBE cell detachment might contribute to LPA's ability to induce apoptosis in these cells, we compared the effect of LPA on the apoptosis of subconfluent NHBE cells grown in low- versus high-attachment conditions. Standard tissue culture polystyrene is treated to make its surface negatively charged and hydrophilic when medium is added, promoting cell attachment and spreading (26). The surface of polystyrene that is not tissue culture–treated remains neutrally charged and hydrophobic, reducing cell attachment. In contrast to cells grown on matrigel, which we used to model physiologic basement membrane composition, LPA did not significantly induce apoptosis of NHBE cells grown on high-attachment tissue culture–treated polystyrene (data not shown). LPA did significantly and dose dependently induce apoptosis of NHBE cells grown on low-attachment (not tissue culture–treated) polystyrene (Figure 4B), leading us to hypothesize that LPA's

ability to promote epithelial cell apoptosis is dependent on its ability to promote epithelial cell detachment, a hypothesis we subsequently explored further.

In the experiments presented in Figures 4A and 4B, we demonstrated that LPA promotes NHBE apoptosis using 18:1 LPA, the most commonly used laboratory reagent for activation of LPA receptors (3). However, 18:0 was the LPA species largely responsible for the increase in BAL LPA concentration observed at Day 1 after bleomycin challenge (Figure 3A). We therefore investigated the effects of 18:0 LPA on NHBE apoptosis and found that it also significantly induced NHBE apoptosis at a concentration of 1 μM (Figure 4C). Given this similarity in the effects of 18:0 and 18:1 LPA on NHBE apoptosis, the rest of our experiments were performed with 18:1 LPA only.

To investigate which of LPA's receptors mediate its ability to induce lung epithelial cell apoptosis, we first assessed NHBE cell mRNA expression of the five established, high-affinity cognate receptors LPA$_1$ through LPA$_5$, as well as P2Y5, a relatively low-affinity receptor that is likely to join the LPA receptor family as LPA$_6$ (3). Consistent with previously reported results (27), we confirmed that these cells predominantly expressed LPA$_1$, LPA$_2$, and LPA$_3$ (Figure E5). To distinguish between these three receptors, we determined whether 18:0 LPA–induced NHBE cell apoptosis was inhibited by AM095, a new LPA receptor antagonist that we have recently demonstrated to be highly specific for LPA$_1$ (28). This antagonist has been demonstrated to inhibit LPA activation of LPA$_1$ in a dose-
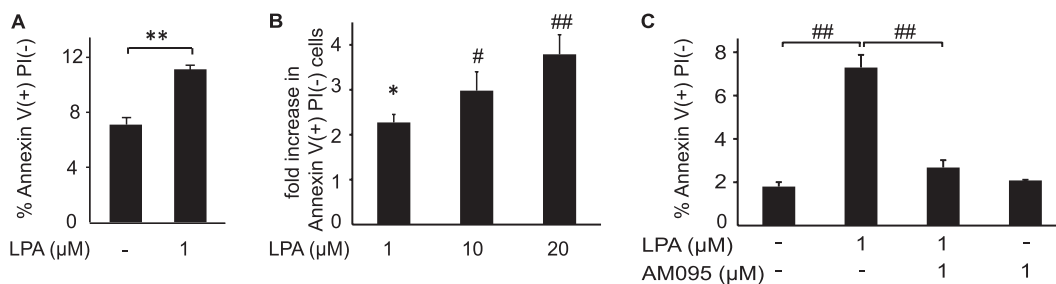
Figure 4. LPA signaling through LPA$_1$ promoted lung epithelial cell apoptosis. (*A*) LPA (18:1) induced the apoptosis of normal human bronchial epithelial (NHBE) cells grown on matrigel. Apoptotic cells were identified by flow cytometry after staining with annexin V and propidium iodide (24) as annexin V-positive, PI-negative cells. Data from one of three independent experiments are presented as mean percentages of annexin V(+) PI(−) cells ± SEM. $n = 3$ cultures per treatment condition in each experiment. **$P < 0.01$, LPA-treated versus untreated NHBE cells. (*B*) LPA (18:1) dose-dependently induced the apoptosis of NHBE cells grown on low-attachment polystyrene. Four independent experiments were performed. Data are presented as mean fold increases in percentages of apoptotic [annexin V(+), PI(−)] cells in LPA-treated NHBE cells compared with untreated cells from the four individual experiments ± SEM; the values produced in the individual experiments themselves were the means of $n = 3$ cultures per treatment condition. *$P < 0.05$, #$P < 0.001$, and ##$P < 0.0001$, NHBE cells treated with 1, 10, or 20 μM LPA, respectively, versus untreated cells. (*C*) LPA (18:0) also induced NHBE cell apoptosis, and this effect was abrogated by AM095, a selective LPA$_1$ receptor antagonist. Data are from a representative experiment with $n = 3$ cultures per treatment condition. ##$P < 0.0001$, LPA-treated versus untreated NHBE cells and AM095-treated LPA-treated NHBE cells versus LPA-treated NHBE cells.

dependent manner (29). AM095 abrogated the ability of 18:0 LPA to induce NHBE cell apoptosis (Figure 4C), suggesting that the pro-apoptotic effects of LPA on NHBE cells were mediated by LPA_1.

LPA Induces Epithelial Cell Apoptosis through Detachment

The detachment of anchorage-dependent cells, such as epithelial cells, induces apoptosis, a phenomenon termed "anoikis" (Greek, meaning the state of being without a home) (30). To investigate whether LPA induces lung epithelial cell apoptosis through the process of anoikis, we first confirmed the ability of LPA to induce epithelial cell detachment. We performed detachment assays in which NHBE cells were transferred to low-attachment tissue culture plates and then incubated with or without LPA for 2 hours. The tissue culture plates were then centrifuged in an inverted position, and the cells remaining in the wells were quantified. The adherence index of NHBE cells exposed to 1 μM LPA was 0.54 (i.e., LPA's ability to promote epithelial cell detachment reduced the cells remaining attached in these assays by 46%) (Figure 5A). We also investigated the ability of LPA to inhibit epithelial cell attachment in spreading assays in which the flattening of adherent cells is used to indicate their adhesion. Without exposure to LPA, 97% of NHBE cells had attached and flattened by 3 hours after transfer to high-attachment tissue culture–treated polystyrene plates. In contrast, only 40% of NHBE cells incubated with 1 μM LPA had attached and flattened in the same time period (Figure 5B). A broad range of LPA concentrations was able to inhibit the adhesion of unattached NHBE cells in these spreading assays, with statistically significant reductions in NHBE spreading produced by LPA concentrations as low as 0.01 μM LPA (Figure E6). LPA therefore promotes the detachment of adherent NHBE cells and inhibits the adhesion of unattached NHBE cells, suggesting that LPA may induce epithelial cell apoptosis after bleomycin lung injury by inducing anoikis.

In the process of anoikis, cell detachment from the extracellular matrix (ECM) induces apoptosis by disrupting survival signals generated by cell integrin–ECM interactions (31). These survival signals are generated through cytoskeletal rearrangements induced by integrin–ECM interactions, including the formation of focal adhesions (FAs) and actin filament stress fibers (32, 33). We therefore investigated the effects of LPA on FA and actin stress fiber formation in lung epithelial cells. We compared the development of FAs, identified by staining of the FA structural protein vinculin, in NHBE cells 3 hours after transfer to high-attachment tissue culture–treated permanox slides in the presence or absence of 1 μM LPA. NHBE cells seeded in the absence of LPA developed FAs, whereas LPA present at the time cells were transferred essentially completely inhibited initial FA formation (Figures 5C and 5D). Similarly, actin stress fibers were observed in NHBE cells 3 hours after transfer to tissue culture–treated permanox slides in the absence of LPA, whereas 1 μM LPA present at the time cells were transferred essentially completely inhibited actin stress fiber formation as well (Figures 5E and 5F).

To determine whether LPA also promotes the apoptosis of lung epithelial cells that have already become detached, we investigated the effects of LPA on NHBE cells cultured on ultra–low attachment plastic. This plastic is treated to be neutrally charged and hydrophilic to maintain cells in a suspended, unattached state. The presence or absence of 1 μM LPA did not significantly affect the percentage of NHBE cells that were apoptotic 24 hours after transfer to ultra–low attachment plastic (Figure 5G), indicating that LPA did not further increase the apoptosis of NHBE cells that were already unattached. These data further support our hypothesis that LPA's pro-apoptotic effects on lung epithelial cells result from its ability to induce the detachment of these cells.

Increased numbers of apoptotic cells have been observed in the alveolar and bronchial epithelia of patients with IPF (5, 6), and we found that bleomycin-induced apoptosis was reduced in the alveolar and bronchial epithelia of LPA_1 KO mice. To investigate whether LPA negatively regulates the attachment of alveolar epithelial cells in addition to NHBE cells, we investigated the effect of LPA on the spreading of R3/1 rat alveolar epithelial cell line cells. The R3/1 cell line, derived from fetal rat lung (16), expresses cytokeratins of the simple epithelial type numbers 7, 8, 18, and 19, which are characteristic for rat alveolar epithelial cells, as well as a variety of proteins typical, though not exclusive, for the type I cell phenotype, such as T1α, ICAM-1, Cav-1, and Cav-2 (17). Two of the three lectins known to bind to the surface of type I AECs, *Bauhinia purpurea* agglutinin and soybean agglutinin, bind to R3/1 cells. R3/1 cells also express moderate levels at the type II cell proteins for SP-A and SP-B, though not SP-C or SP-D (17). These cells consequently have been suggested to be a suitable tool for the *in vitro* study of alveolar epithelial cell biology (17), although they resemble type I cells more than type II. We first confirmed that R3/1 cells express the LPA1 receptor (Figure E7A). Without exposure to LPA, 97% of R3/1 cells had attached and flattened by 3 hours after transfer to high-attachment tissue culture–treated polystyrene plates, whereas only 64% and 29% of R3/1 cells incubated with 1 or 20 μM LPA, respectively, had attached and flattened in the same time period (Figures 5H, E7B, and E7C). LPA therefore appears to negatively regulate the attachment of alveolar and bronchial epithelial cells, although we have not investigated primary alveolar epithelial cells.

LPA Prevents Fibroblast Apoptosis through LPA_1

We have previously reported that LPA signaling through LPA_1 contributes to fibroblast accumulation in the lung after bleomycin injury by promoting the migration of these cells (2). In contrast to our findings with lung epithelial cells, LPA has been reported to prevent apoptosis in NIH 3T3, Swiss 3T3, and Rat-1 fibroblast cell lines (34), although the LPA receptor(s) mediating these anti-apoptotic effects were not identified. Such anti-apoptotic effects on fibroblasts could also contribute to LPA-induced fibroblast accumulation after bleomycin lung injury, and we therefore analyzed the effects of LPA on the apoptosis of primary mouse lung fibroblasts (PMLFs). PMLF apoptosis induced by serum deprivation for 24 hours was prevented by LPA concentrations as low as 1 μM (Figure 6A). Lower LPA concentrations produced trends toward reduced PMLF apoptosis, but these were nonsignificant (Figure E8). Of the established LPA receptors LPA_1 through LPA_5, we previously reported that PMLFs predominantly express LPA_1 (2). We consequently hypothesized that LPA's ability to prevent fibroblast apoptosis is mediated by LPA_1, the same receptor that mediates LPA's ability to promote epithelial cell apoptosis. To investigate this hypothesis, we first determined whether the LPA receptor antagonist Ki16425 inhibited LPA's ability to prevent PMLF apoptosis. This antagonist was originally reported to inhibit LPA-induced responses mediated by $LPA_1 \geqslant LPA_3 >> LPA_2$ (35), but more recent studies have demonstrated that the ability of Ki16425 to inhibit mouse LPA_1 is over 10-fold greater than its ability to inhibit mouse LPA_3, as assessed by calcium flux assays performed with transfected cells (personal communication, Gretchen Bain, Amira Pharmaceuticals, San Diego, CA). The ability of LPA to prevent PMLF apoptosis induced by serum deprivation was completely abrogated by Ki16425 at a concentration of 100 μM

LPA (μM) **-** **1**
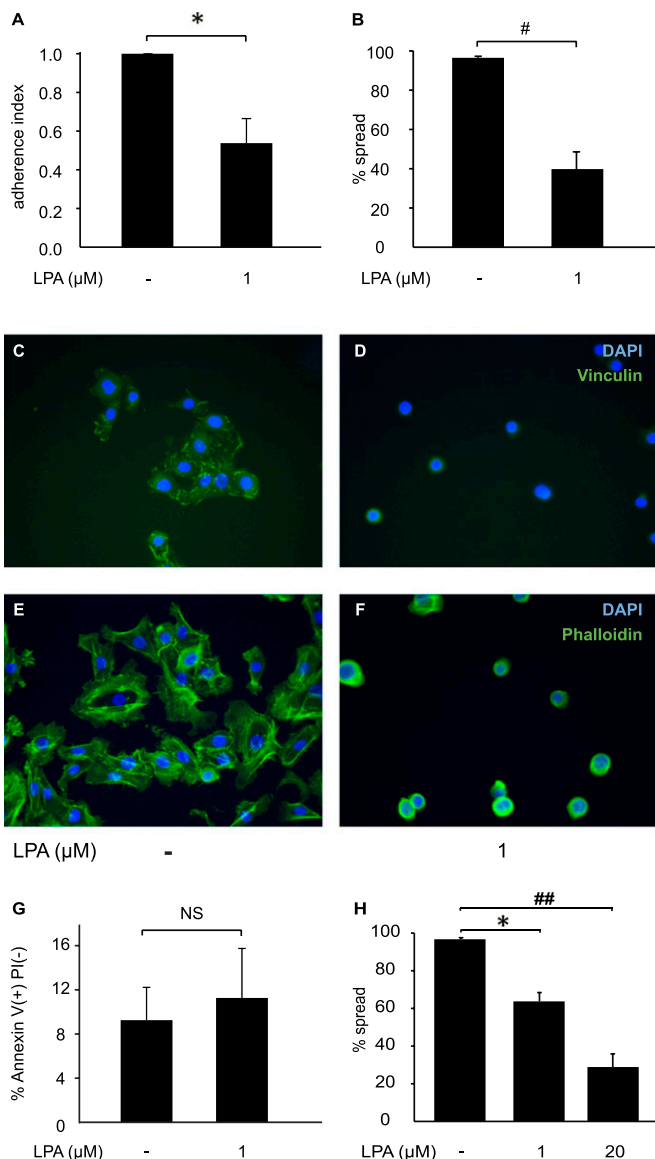
Figure 5. LPA promoted detachment and limited attachment of lung epithelial cells. (*A*) LPA promoted detachment of NHBE cells from low–attachment, untreated polystyrene. Six independent experiments were performed. Data represent the means of the adherence indices produced in the individual experiments ± SEM; the values produced in the individual experiments themselves were the means of *n* = 9 cultures per treatment condition. *$P < 0.05$, LPA-treated versus untreated NHBE cells. (*B*) LPA limited attachment of NHBE cells to high-attachment tissue culture–treated polystyrene. Four independent experiments were performed. Data represent the means of the percentages of spread cells produced in the individual experiments ± SEM; the values produced in the individual experiments themselves were the means of *n* = 3 cultures per treatment condition. Spread cells were identified visually by phase contrast microscopy as having flattened from their initial rounded shape such that the nucleus and cytoplasm could be differentiated. #$P < 0.001$, LPA-treated versus untreated NHBE cells. (*C, D*) LPA limited NHBE cell formation of focal adhesions. Representative NHBE cells labeled with anti–vinculin-fluorescein isothiocyanate 3 hours after transfer onto high-attachment tissue culture–treated permanox slides (*C*) without LPA treatment and (*D*) with LPA treatment. (*E, F*) LPA limited NHBE cell formation of actin stress fibers. Representative NHBE cells labeled with fluorescein-phalloidin 3 hours after transfer onto high-attachment permanox (*E*) without LPA treatment and (*F*) with LPA treatment. (*G*) LPA did not promote the apoptosis of NHBE cells transferred to ultra–low attachment polystyrene plates. Three independent experiments were performed. Data represent the means of the percentages of annexin V(+) PI(−) cells produced in the individual experiments ± SEM; the values produced in the individual experiments themselves were the means of *n* = 3 cultures per treatment condition. Differences between LPA-treated and untreated NHBE cells were not significant. (*H*) LPA limited attachment of R3/1 rat alveolar epithelial cell line cells to high-attachment tissue culture–treated polystyrene. Data represent the mean percentages of spread cells produced in a representative experiment ± SEM, with *n* = 3 cultures per treatment condition. Spread cells were identified as in (*B*). *$P < 0.05$ and ##$P < 0.0001$, untreated R3/1 cells versus R3/1 cells treated with 1 and 20 μM LPA, respectively.

(Figure 6B) and was partially blocked by lower concentrations of Ki16425 (Figure E9), consistent with the anti-apoptotic effects of LPA on fibroblasts being mediated by LPA$_1$. We next investigated whether LPA was able to prevent the apoptosis of PMLFs isolated from LPA$_1$ KO mice. LPA did not significantly reduce the apoptosis of LPA$_1$ KO PMLFs induced by serum deprivation for 24 hours, also suggesting that signaling through LPA$_1$ mediates the anti-apoptotic effects of LPA on fibroblasts (Figure 6C). In all the fibroblast experiments described above, PMLFs were grown on high-attachment tissue culture–treated plastic. To determine whether LPA's ability to prevent lung fibroblast apoptosis requires cell attachment, we investigated whether LPA was able to prevent apoptosis of PMLF transferred to ultra–low attachment plastic. Incubation with serum or LPA failed to reduce the apoptosis of PMLFs on ultra–low attachment plastic (Figure 6D). These results suggest that, as was the case for epithelial cells, LPA regulates the apoptosis of fibroblasts only when these cells are adherent and not when they are unattached.

DISCUSSION

We previously demonstrated that LPA signaling through LPA$_1$ contributes to the development of bleomycin-induced pulmonary fibrosis (2), finding that LPA$_1$ KO mice are dramatically protected from fibrosis and mortality in this model. In this prior work, we demonstrated that LPA-LPA$_1$ signaling contributes to fibroblast recruitment and vascular leak induced by bleomycin challenge. In the current study, we have described two additional mechanisms through which this signaling pathway may contribute to fibrosis induced by bleomycin lung injury: LPA signaling through LPA$_1$ promotes lung epithelial cell apoptosis but fibroblast resistance to apoptosis. LPA-LPA$_1$ signaling thus could contribute to the paradoxical apoptotic abnormalities observed in IPF, in which increased epithelial and reduced fibroblast apoptosis may play important roles in the development of fibrosis (36).

We found that the early wave of alveolar and bronchial epithelial cell apoptosis induced by bleomycin injury was attenuated in LPA$_1$ KO mice, suggesting that LPA signaling through LPA$_1$ promotes apoptosis in the alveolar and bronchial epithelia after lung injury. After an increase in BAL LPA concentration noted at Day 1 after bleomycin challenge, significantly increased numbers of TUNEL$^+$ alveolar and bronchial epithelial cells were present in WT compared with LPA$_1$ KO mice at Day 3 after bleomycin challenge.

To investigate whether LPA-LPA$_1$ signaling could promote lung epithelial cell apoptosis by acting directly on these cells, we determined the effects of LPA on NHBE and R3/1 rat alveolar epithelial cell line cells in culture. We found in our *in vivo* studies that LPA-LPA$_1$ signaling promoted apoptosis in the alveolar and bronchial epithelia to similar extents and with
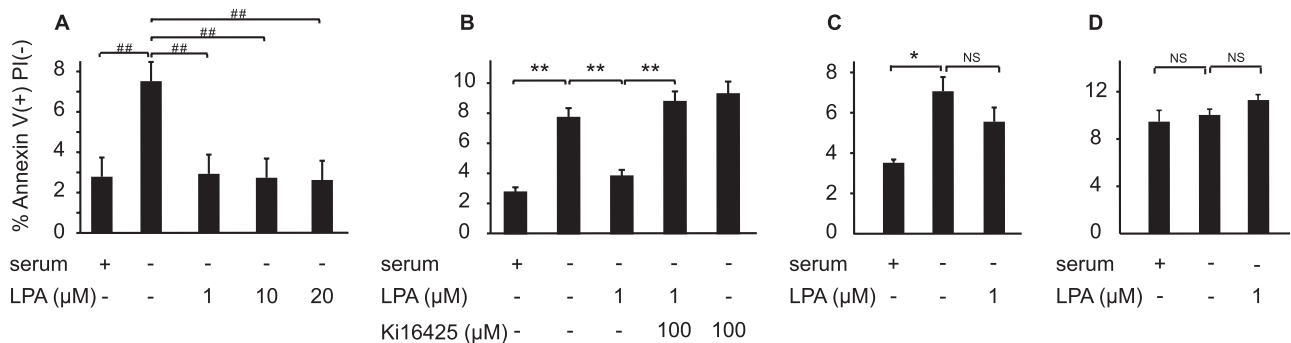
Figure 6. LPA signaling through LPA$_1$ promoted lung fibroblast resistance to apoptosis. (*A*) LPA promoted primary mouse lung fibroblast (PMLF) resistance to apoptosis induced by serum deprivation when these cells were grown on high-attachment tissue culture–treated polystyrene. Apoptotic cells were identified by flow cytometry after staining with annexin V and propidium iodide (8) as annexin V(+), PI(−). Three independent experiments were performed. In all panels of this figure, data represent the means of the percentages of annexin V(+) PI(−) cells produced in the individual experiments ± SEM; the values produced in the individual experiments themselves were the means of $n = 3$ cultures per treatment condition. $^{##}P \geqslant 0.0001$, serum-deprived PMLFs versus PMLFs in serum and serum-deprived PMLFs treated with 1, 10, or 20 μM LPA versus untreated serum-deprived cells. (*B*) LPA-induced resistance to apoptosis of PMLFs grown on high-attachment polystyrene was abrogated by Ki16425. Three independent experiments were performed. $^{**}P < 0.01$, serum-deprived PMLFs versus PMLFs in serum; serum-deprived, LPA-treated PMLFs versus untreated serum-deprived cells; and serum-deprived, LPA-treated PMLFs that were also treated with Ki16425 versus serum-deprived, LPA-treated PMLFs. (*C*) LPA did not promote resistance to apoptosis of PMLFs isolated from LPA$_1$ KO mice that was induced by serum deprivation when these cells were grown on high-attachment polystyrene. Three independent experiments were performed. $^{*}P < 0.05$, serum-deprived LPA$_1$ KO PMLFs versus LPA$_1$ KO PMLFs in serum. Differences between LPA-treated and untreated serum-deprived LPA$_1$ KO PMLFs were not significant. (*D*) LPA did not promote resistance to apoptosis of PMLFs transferred to ultra–low attachment polystyrene. Three independent experiments were performed. Differences between LPA-treated and untreated serum-deprived PMLFs were not significant.

similar kinetics, suggesting that primary bronchial epithelial cells are a relevant *in vitro* model in which to investigate the effects of LPA-LPA$_1$ signaling on epithelial cells. We found that LPA signaling through LPA$_1$ induced the apoptosis of subconfluent NHBE cells grown on matrigel, which was used as representative of basement membranes normally present in the lung before injury or fibrosis. We found evidence that LPA-LPA$_1$ signaling specifically mediates lung epithelial cell anoikis, the apoptosis of anchorage-dependent cells induced by their detachment (30). Mediators that promote anoikis can do so by promoting cell detachment or by promoting the apoptosis of cells once they have become detached. We found that LPA specifically induced NHBE cell detachment but did not influence the rate at which cells became apoptotic once they were detached. Consistent with this mechanism of apoptosis induction, we found that LPA inhibited NHBE cell formation of focal adhesions and actin stress fibers, both of which structures have been demonstrated to transmit survival signals in adherent cells (32, 33). We found that LPA had similar effects on R3/1 cell attachment as it did on NHBE attachment, suggesting that LPA negatively regulates the attachment of both alveolar and bronchial epithelial cells, although we have not investigated primary alveolar epithelial cells.

Anoikis is thought to play important roles in normal development and tissue homeostasis, and abnormalities of this apoptotic pathway have been implicated in several disease processes (37). Development of resistance to anoikis is a crucial step during tumorigenesis that is required for tumor cell anchorage–independent growth and metastatic spread. In contrast, evidence for increased apoptosis due to disruption of survival signals transmitted from ECM proteins has been reported in the development of renal fibrosis in glomerulosclerotic diseases such as lupus nephritis and IgA nephropathy (38). Aberrant induction of mesangial cell apoptosis after renal injury appears to contribute to the development of progressive fibrosis in these diseases and has been hypothesized to result from injury-induced changes in ECM composition, in which ECM proteins unable to suppress mesangial cell apoptosis are up-regulated (39).

Since the discovery that LPA is a potent signaling molecule involved in a wide variety of basic physiological and pathological processes, multiple investigators have reported that LPA can promote the survival or apoptosis of many different cell types (4). Consistent with our finding that LPA promotes lung epithelial cell anoikis, LPA$_1$ signaling has previously been shown to promote anoikis of other cell types, including ovarian epithelial cancer cells (40). In contrast to our results with epithelial cells, we found that LPA signaling through LPA$_1$ promoted the survival of primary mouse lung fibroblasts, consistent with previously reported findings that LPA prevents apoptosis of NIH 3T3, Swiss 3T3, and Rat-1 fibroblast cell lines (34) as well as other cell lineages such as Schwann cells (41). Whereas we found LPA's ability to promote epithelial cell apoptosis was associated with inhibition of focal adhesion and actin stress fiber formation in these cells, LPA's ability to prevent Schwann cell apoptosis was associated with promotion of Schwann cell focal adhesion assembly and actin rearrangement (42).

There is evidence to suggest that lung fibroblasts in IPF are abnormally resistant to apoptosis (12). Fibroblast resistance to apoptosis coupled with increased epithelial cell apoptosis has been referred to as an "apoptosis paradox" in IPF (36). The molecular pathways responsible for the divergent susceptibilities of epithelial cells and fibroblasts to apoptosis in IPF have yet to be fully identified, but our data suggest that LPA signaling through LPA$_1$ may contribute. There is precedent for epithelial cell apoptosis and fibroblast resistance to apoptosis being induced by the same mediator, as we propose for LPA. TGF-β has been shown to produce divergent apoptotic behaviors in different cell types and stimulation contexts (43). As we found with LPA, TGF-β induces apoptosis of lung epithelial cells (44, 45) but induces resistance to apoptosis in lung fibroblasts (46, 47). Recently PGE$_2$ has also been shown to mediate divergent effects on lung epithelial cell and fibroblast apoptosis but in the directions opposite to LPA and TGF-β. PGE$_2$ was found to promote the apoptosis of primary IPF lung fibroblasts but to protect primary fibrotic lung type II cells against apoptosis (48). Therefore, increased LPA levels (2), increased TGF-β levels

and activity (49), and decreased PGE$_2$ levels (50) in the lung in IPF may contribute to the increased epithelial cell but reduced fibroblast apoptosis observed in this disease.

In this study, we have demonstrated that LPA signaling through its receptor LPA$_1$ promotes epithelial cell apoptosis induced by lung injury. Mechanistically, LPA appears to induce epithelial cell anoikis by promoting the detachment of these cells. In contrast, LPA-LPA$_1$ signaling promotes fibroblast resistance to apoptosis. In addition to the reductions in fibroblast recruitment and vascular leak that we previously observed in bleomycin-challenged LPA$_1$ KO mice, concurrent reductions in epithelial cell apoptosis and fibroblast resistance to apoptosis may further explain the dramatic protection of these mice from fibrosis and mortality in the bleomycin model of pulmonary fibrosis. These new findings may have implications for therapeutic targeting of LPA-LPA$_1$ signaling in IPF. In addition to slowing the development of new areas of fibrosis by reducing epithelial cell apoptosis, antagonism of LPA$_1$ may also be able to reverse the progression of established areas of fibrosis by promoting fibroblast apoptosis. Contributions of LPA and LPA$_1$ to the "apoptosis paradox" of IPF therefore highlight the potential of this signaling pathway to be a new therapeutic target for this devastating disease.

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