ONLINE DATA SUPPLEMENT

The lysophosphosphatidic acid receptor LPA_1 promotes epithelial cell apoptosis following lung injury

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SUPPLEMENTAL MATERIAL AND METHODS

Lung immunohistochemical and immunofluorescence staining

Multiple paraffin-embedded 5 μm sections of the entire mouse lung were stained using the Apoptag® peroxidase *in situ* apoptosis detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Immunolabeling of p53 and p21 was performed with primary rabbit anti-mouse p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and primary rabbit anti-mouse p21 antibody (Thermo Scientific, Waltham, MA, USA), respectively. Appropriate biotinylated secondary antibodies were used, followed by detection with an ABC Development Kit (Vector Laboratories, Burlingame, CA, USA) and color development with DAB (Vector Laboratories). The numbers of $TUNEL^+$, $p53^+$ and $p21^+$ cells were determined by counting the number of positive cells in 10 randomly selected non-overlapping high-power fields in lung sections of WT and LPA_1 KO mice. For TUNEL co-staining with either T1α, a marker of type I alveolar epithelial cells (AECs) (E1), or pro-surfactant protein C (proSP-C), a marker of type II AECs, multiple paraffin-embedded 5 μm sections of the entire mouse lung were first stained using the Apoptag® fluorescein direct *in situ* apoptosis detection kit (Millipore) according to the manufacturer's instructions. Immunolabeling of T1α or pro-SPC on the same sections was then performed with primary hamster anti-mouse T1α antibody (eBioscience, San Diego, CA, USA) or primary rabbit anti-mouse pro-SPC antibody (Millipore), respectively. Appropriate biotinylated secondary antibodies were used and labeled with Strepta-Alexa 555 (Invitrogen, Carlsbad, CA, USA), and sections were mounted with VECTASHIELD® mounting medium with DAPI (Vector Laboratories).

NHBE, rat R3/1 and PMLF cell culture and apoptosis analysis

In different experiments evaluating normal human bronchial epithelial (NHBE) cell apoptosis, cells were transferred to Matrigel™ matrix thin layer 6 well multiwell plates (BD Bioscience, San Jose, CA, USA), Corning® 60mm not tissue culture (1)-treated culture dishes (low attachment dishes, Corning) or Corning® 6 well ultra low attachment culture plates (Corning). For NHBE cells transferred to matrigel, cells were cultured directly in bronchial epithelial cell growth medium (BEGM) with 0.1% fatty acid-free BSA (FAF-BSA, Sigma Aldrich, St. Louis, MO, USA) with or without 18:1 lysophosphatidic acid (LPA, Avanti Polar Lipids, Alabaster, AL, USA) for 48h prior to apoptosis analyses. For NHBE cells transferred to low attachment dishes, cells were cultured in BEGM without FAF-BSA or LPA for 24 h, and then in BEGM with FAF-BSA with or without either 18:1 or 18:0 LPA, and with or without AM095 (Amira Pharmaceuticals, San Diego, CA, USA) for 72h. For NHBE cells transferred to ultra low attachment dishes, cells were cultured directly in BEGM with FAF-BSA with or without 18:1 LPA for 24h. NHBE cells transferred to low attachment dishes were harvested with a cell lifter (Corning Costar) after incubation with 0.05% trypsin-EDTA (Cellgro, Manassas, VA, USA) for 1 min; cells transferred to matrigel were harvested after incubation with trypsin-EDTA without a cell lifter; cells transferred to ultra low attachment plates were harvested with pipetting only. Primary mouse lung fibroblasts (PMLF) for apoptosis assays transferred at passage 3 were transferred into MULTIWELL™ 6 well TC-treated polystyrene tissue culture plates (Falcon, BD Bioscience) or Corning® 6 well ultra low attachment culture plates. Transferred cells were cultured in DMEM with 15% FBS (DMEM-15%) for 24h, then washed twice with PBS, and then cultured in serum-free DMEM with 0.1% FAF-BSA with or without 18:1 LPA for 24h. PMLF were then harvested by incubation with 0.25% trypsin-EDTA for 1 min. Rat R3/1 cells

were cultured in supplemented DMEM (10%FBS) and used for experiments up to passage 10. Harvest and LPA treatment of rat R3/1 cells was similar to the treatment of PMLFs. For all experiments, cells were cultured at 37°C and 5% CO_2 in a humidified incubator. NHBE cells and PMLF harvested for apoptosis analyses were incubated with FITC annexin V (BD Bioscience) in buffer containing propidium iodide (PI, BD Bioscience) at room temperature for 15 min. Percentages of apoptotic cells, identified as annexin V-positive, PI-negative cells (E2), were determined by cytofluorometry performed using a FACS Calibur Cytometer (BD Bioscience) and analyzed using FlowJo® software (Tree Star, Ashland, OR, USA).

Cell Detachment and Attachment Assays

For detachment assays, NHBE cells were transferred into a Corning® 96 well flat bottom polystyrene not TC-treated microplate (Corning) at 6,000 cells/well, and incubated in BEGM for 1h. FAF-BSA with or without 18:1 LPA was added to the wells, and the cells were incubated for an additional 2h. The media was then aspirated and the plate was centrifuged in an inverted position at 900 x g for 5min at 4°C. Cells remaining in the wells were then fixed with 5% glutaraldehyde and stained with crystal violet (Sigma Aldrich). Wells were washed with tap water followed by 10% acetic acid, and absorption of the remaining crystal violet was measured at 570nm. Adherence indices were determined as absorption in wells in which cells had been treated with FAF-BSA/LPA relative to wells in which cells had been treated with FAF-BSA only. For attachment assays, NHBE or rat R3/1 cells were transferred into a MULTIWELL™ 96 well TC-treated flat bottom polystyrene tissue culture plate (Falcon, BD Bioscience) at 5,000 cells/well, and cultured directly in BEGM with FAF-BSA with or without LPA. Attached cells

were then identified after 3h as those that had spread, or flattened, as described (E3), and counted in 10 randomly selected non-overlapping high-power fields.

SUPPLEMENTAL REFERENCES

E1. Swaney JS, Chapman C, Correa LD, Stebbins KJ, Broadhead AR, Bain G, Santini AM, Darlington J, King CD, Baccei C, et al. Pharmacokinetic and pharmacodynamic characterization of an oral, LPA1-selective antagonist. *J Pharmacol Exp Ther* 2011;336(3):693-700.

E2. Moore A, Donahue CJ, Bauer KD, Mather JP. Simultaneous measurement of cell cycle and apoptotic cell death. *Methods Cell Biol* 1998;57:265-278.

E3. Humphries MJ. Cell adhesion assays. *Methods Mol Biol* 2009;522:203-210.

Supplemental Figure E1

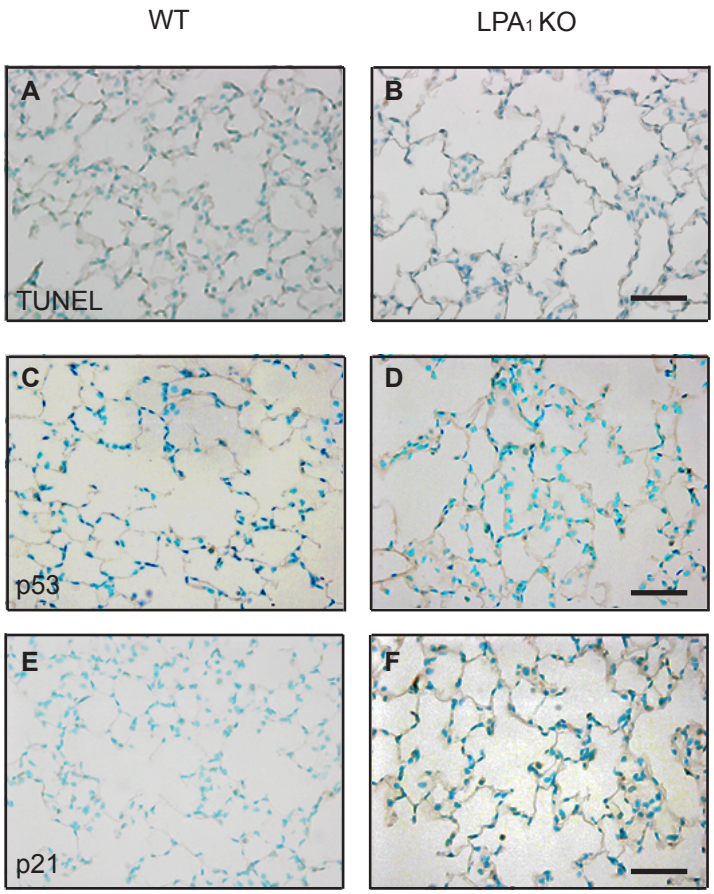

Figure E1. Minimal apoptosis was present in the lungs of WT and LPA$_1$ KO mice at baseline. Representative (A, B) TUNEL/peroxidase-stained, (C, D) p53/peroxidase-stained, and (E, F) p21/peroxidase-stained sections of WT and LPA$_1$ KO mouse lungs at baseline prior to bleomycin challenge (day 0). Scale bars = 50 μm.

Supplemental Figure E2

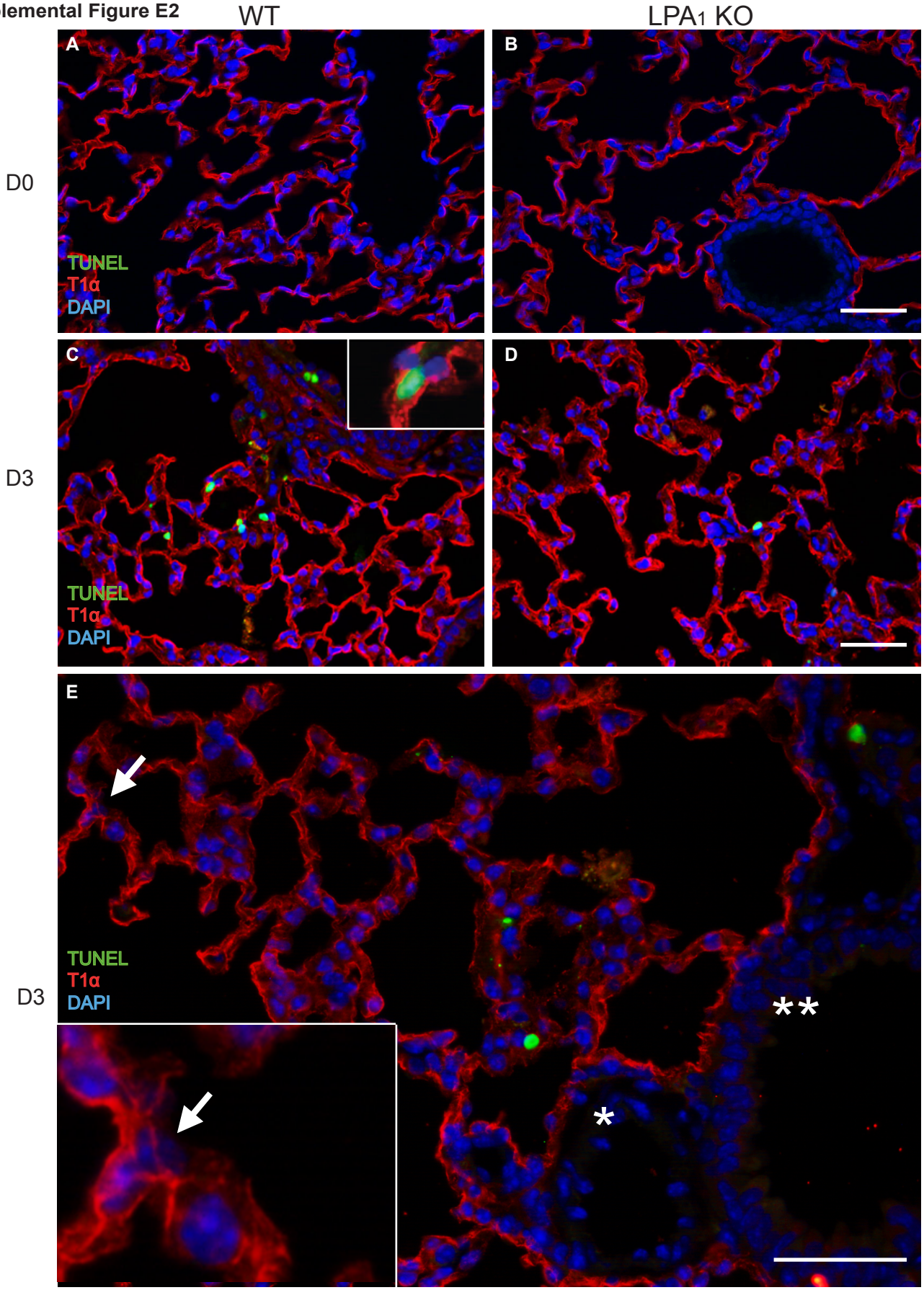

Figure E2. T1α staining for type I alveolar epithelial cells (AECs).
Panel A to E show representative WT and LPA1 KO lung sections with Alexa 555-labeled anti-T1α staining (red) and fluorescein-labeled TUNEL staining (green). Cell nuclei were visualized with DAPI staining (blue). Panel A and B show untreated WT and LPA1 KO lungs. Panel C and D show lungs 3 days post-bleomycin challenge, with fewer TUNEL+ cells in LPA1 KO lung sections. Some TUNEL+ cells were T1α+. Panel E demonstrates the specificity of anti-T1α staining for Type I AECs. The typical T1α labeling of type I AEC surfaces contrasts with the absence of T1α staining of the bronchial epithelium (*) and pulmonary endothelium (**). The main panel white arrowhead indicates a cell with the appearance of a type II AEC that also lacks T1α staining, as shown at higher magnification in the insert panel. Scale bars = 50 μm.

Supplemental Figure E3

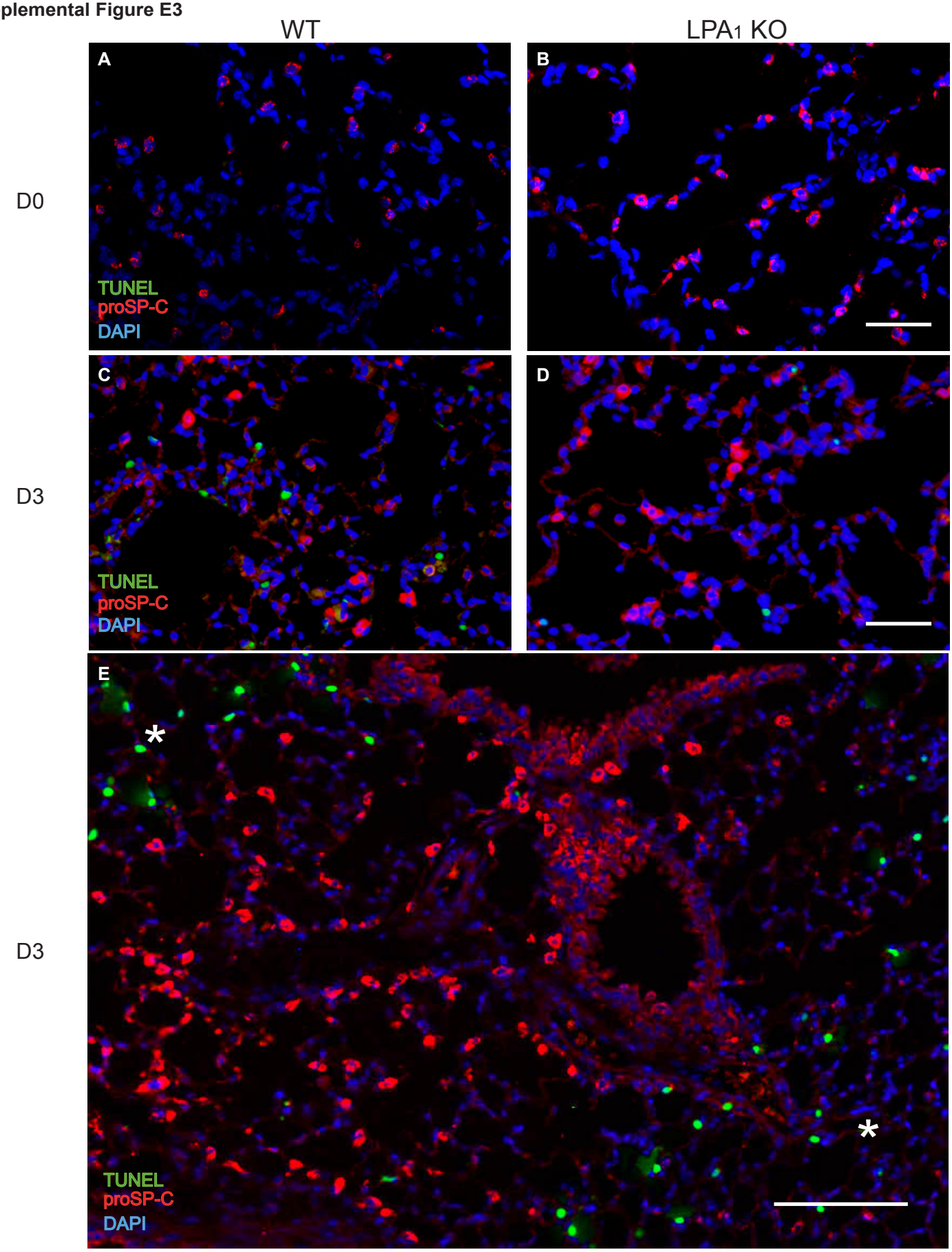

Figure E3. ProSP-C staining for type II alveolar epithelial cells (AECs).
Panel A to E show representative WT and LPA1 KO lung section with Alexa 555-labeled anti-proSP-C staining (red) and fluorescein-labeled TUNEL staining (green). Cell nuclei were visualized with DAPI staining (blue). Panel A and B show untreated WT and LPA1 KO lungs. Panel C and D shows lungs 3 days post-bleomycin challenge, with fewer TUNEL+ cells in LPA1 KO lung sections. Although TUNEL+ cells were not proSP-C+, panel E demonstrates reduced anti-proSP-C staining in areas of lung injury where apoptotic cells are present (*). Scale bars = 50 μm for all images.

Supplemental Figure E4

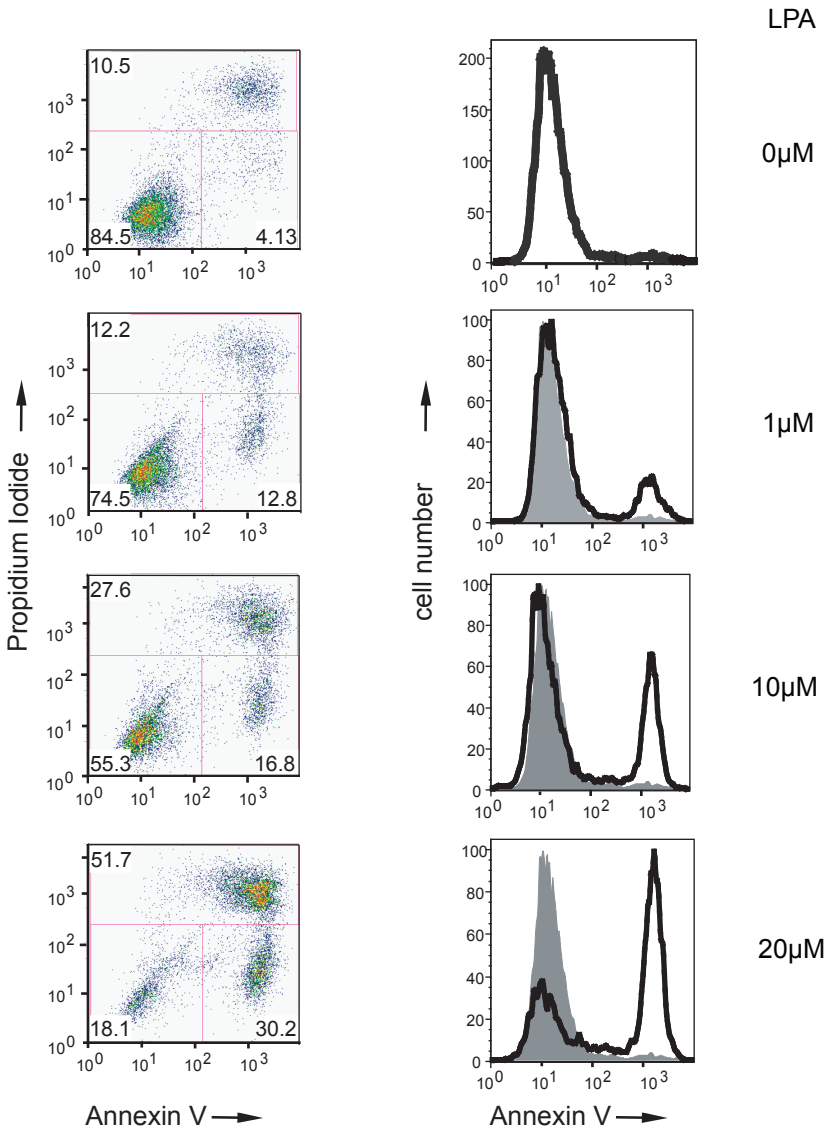

Figure E4. NHBE cell apoptosis induced by LPA was demonstrated by flow cytometry.
LPA dose-dependently induced the apoptosis of NHBE cells grown on low attachment untreated polystyrene. Apoptotic NHBE cells were identified in these experiments by flow cytometry following staining with annexin V and propidium iodide (PI), as annexin V-positive, PI-negative cells. These cells are displayed in the lower right quadrants of the representative dot plots presented (left column). The open black curves in the corresponding histograms (right column) display the numbers of annexin V-positive and -negative NHBE cells that were present among the PI-negative populations. The filled grey curves display control cells that were not stained with annexin V.

Supplemental Figure E5

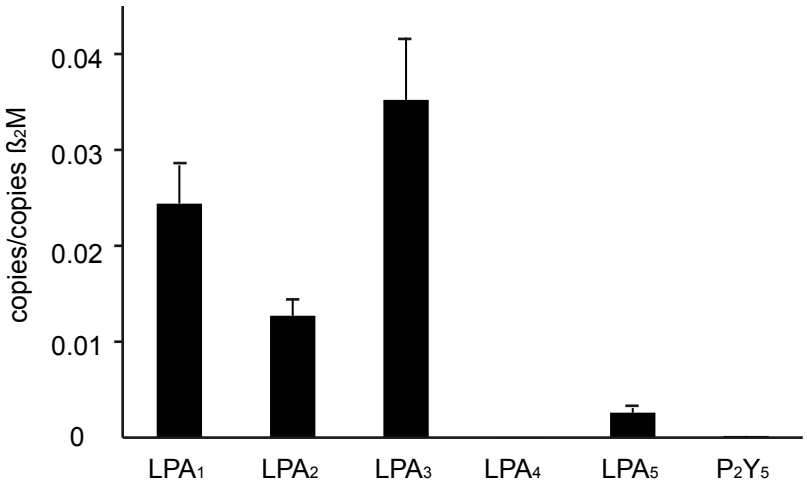

Figure E5. NHBE cell LPA receptor expression. Data from one of two independent experiments are presented as mean copies of receptor mRNA relative to copies of β2-microglobulin (β2M) mRNA ± SEM, as determined by quantitative PCR performed on mRNA isolated from n = 3 cultures of NHBE cells in each experiment.

Supplemental Figure E6

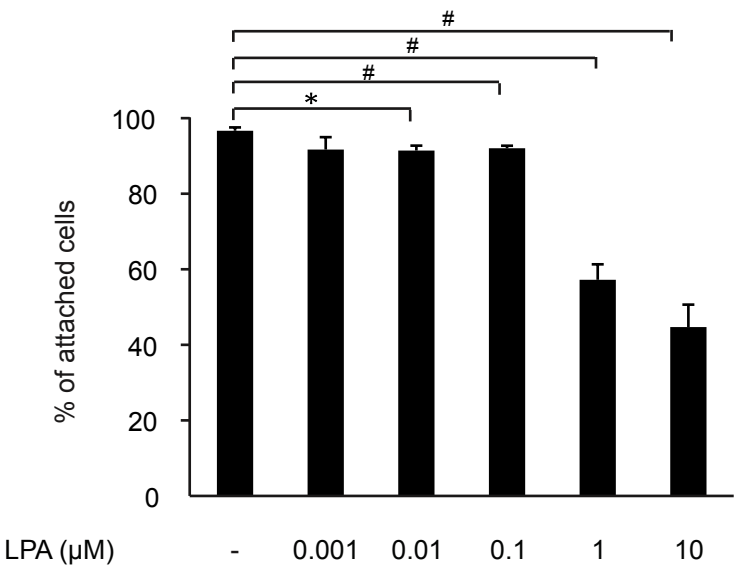

Figure E6. LPA inhibited NHBE cell attachment dose-dependently.
LPA limited the attachment of NHBE cells to high attachment tissue culture-treated polystyrene in a dose-dependent manner.
Data represent the means of the percentages of attached cells ± SEM;
n = 3 cultures per treatment condition. Attached cells were identified visually by phase contrast microscopy as having spread, i.e. flattened from their initial rounded shape such that their nucleii and cytoplasm could be differentiated.
*P < 0.05 and #P < 0.001, LPA-treated vs. untreated NHBE cells, as indicated.

Supplemental Figure E7

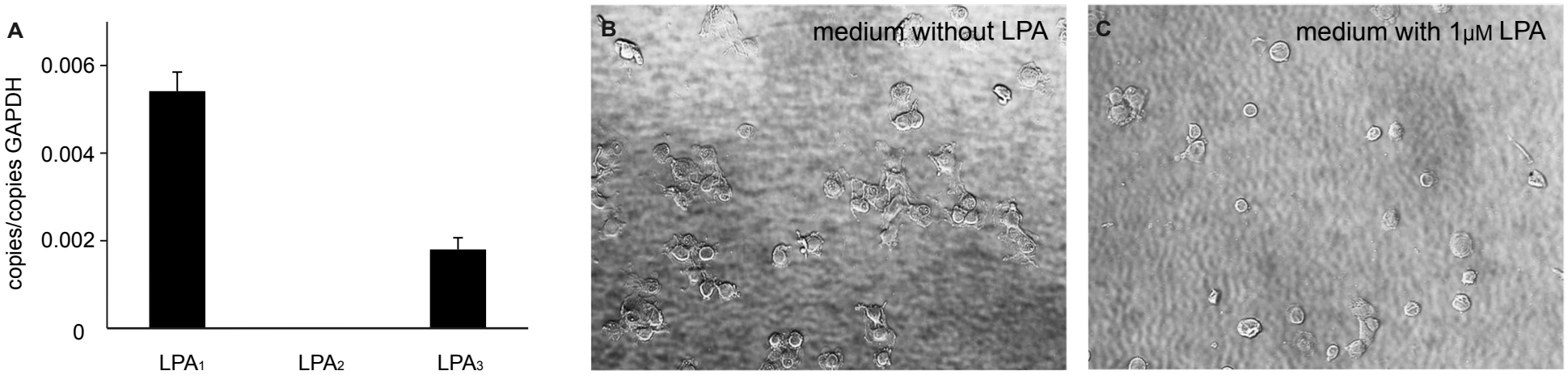

Figure E7. R3/1 cell LPA receptor expression and the effect of LPA on R3/1 cell attachment. (A) Mean copies of LPA receptor mRNA relative to copies of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA ± SEM, as determined by quantitative PCR performed on mRNA isolated from n = 3 cultures of R3/1 cells. (B and C) LPA limited the attachment of rat R3/1 cells to high attachment tissue culture-treated polystyrene. Representative images of the spreading of these cells (B) in the absence of LPA and (C) in the presence of 1μM LPA. Spread cells were identified visually by phase contrast microscopy as having flattened from their **in**itial rounded shape such that the nucleus and cytoplasm could be differentiated.

Supplemental Figure E8

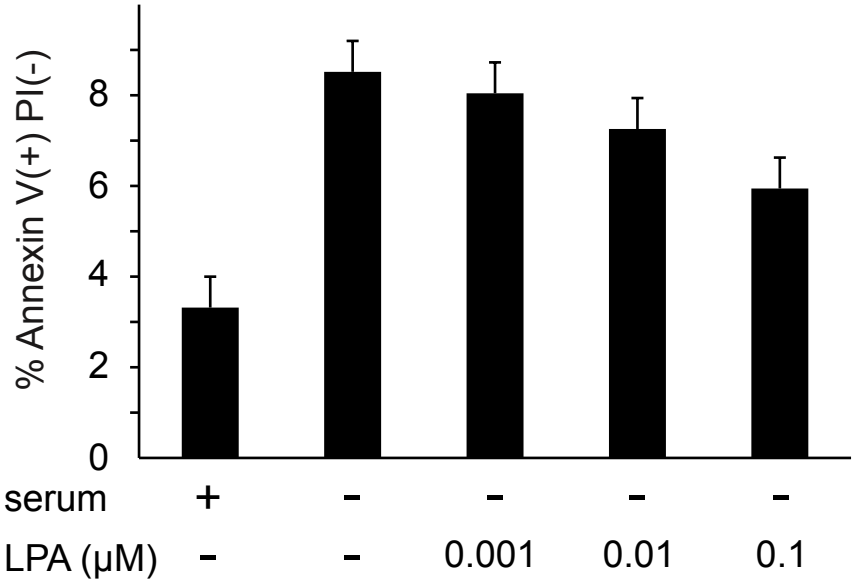

Figure E8. Effects of low LPA concentrations on PMLF apoptosis.As presented in Figure 6A of the manuscript, PMLF apoptosis inducedby serum deprivation for 24 hours was completely prevented by LPA concentrations as low as 1 mM. Lower LPA concentrations produced trends of reduced PMLF apoptosis when these cells were grown on high attachment tissue culture-treated polystyrene, as indicated above, but these trends were non-significant. One-way ANOVA rejected the hypothesis of equality of the group means, but none of the pairwise comparisons between untreated PMLF and PMLF treated with these low LPA concentrations were significant after making Bonferroni post-test corrections.
Apoptotic cells were identified by flow cytometry following staining with annexin V and propidium iodide (PI), as annexin V-positive, PI-negative cells.
Data represent the means of the percentages of annexin V(+) PI(-) cells ± SEM; n = 3 cultures per treatment condition.

Supplemental Figure E9

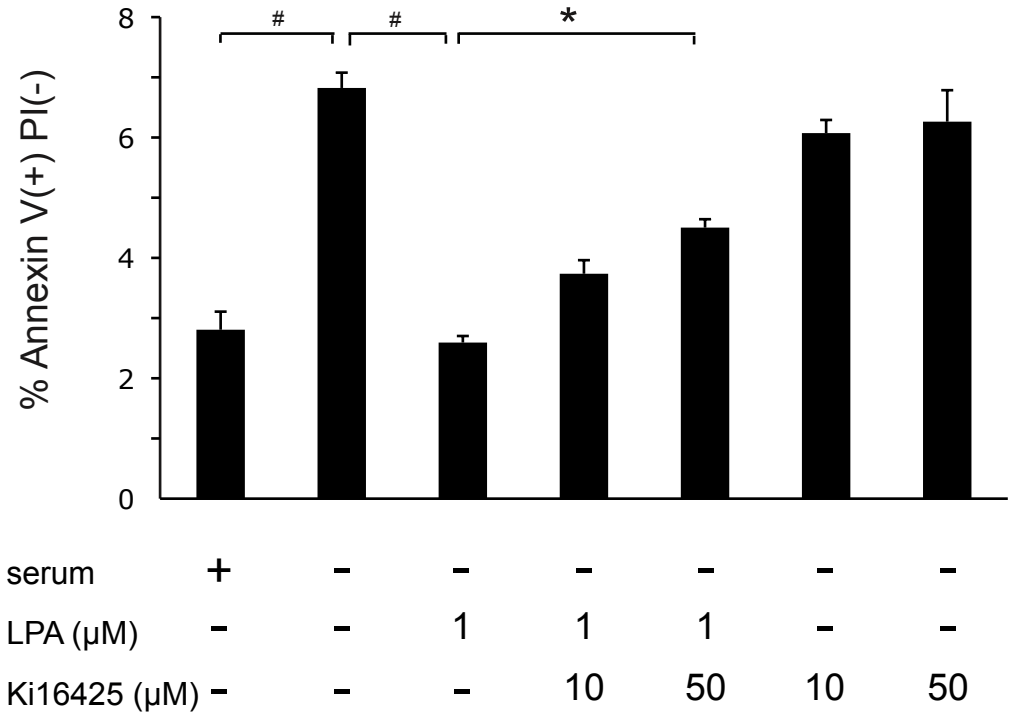

Figure E9. Effects of low Ki16245 concentrations on LPA's ability to prevent PMLF apoptosis. As presented in Figure 6B of the manuscript, LPA-induced resistance of PMLF to apoptosis was completely inhibited by 100 mM Ki16425. Lower Ki16425 concentrations partially inhibited LPA's ability to prevent PMLF apoptosis induced by serum deprivation when these cells were grown on high attachment tissue culture-treated polystyrene, as indicated above. #P < 0.001, serum-deprived PMLF vs. PMLF in serum, and serum-deprived PMLF treated with LPA vs. serum-deprived cells not treated with LPA. *P < 0.05, serum-deprived PMLF treated with LPA that were also treated with 50 μM Ki16425 vs. serum-deprived PMLF treated with LPA but not Ki16425. Apoptotic cells were identified by flow cytometry following staining with annexin V and propidium iodide (PI), as annexin V-positive, PI-negative cells. Data represent the means of the percentages of annexin V(+) PI(-) cells ± SEM; n = 3 cultures per treatment condition.