Methods

**Western blot analysis**

Lungs isolated from mice were homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40) supplemented with a 1% protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO, USA). After 30 min on ice, the samples were centrifuged at 13,500 rpm for 20 min at 4 °C. The supernatants were recovered, and the protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific). The Laemmli buffer was added to the supernatants, and the samples were heated for 10 min at 70 °C. The protein extracts were loaded onto 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Mini-PROTEAN® TGX™ Precast Gels; Bio-Rad Laboratories), with 10 μg of protein loaded per lane. After electrophoresis, the proteins were electrotransferred onto a polyvinylidene fluoride membrane (GE Healthcare UK Ltd, Little Chalfont, UK). The membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h and incubated overnight with an anti-S100A9 rabbit mAb (#73425; Cell Signaling Technology, Beverly, MA, USA) or a rabbit β-actin antibody (#4967; Cell Signaling Technology). The membrane was then washed and incubated with a secondary antibody conjugated with horseradish peroxidase (HRP) (NA934; GE Healthcare, UK Ltd.). After further washing, the membrane was incubated with a chemiluminescent substrate (ImmunoStar Zeta; Wako, Osaka, Japan) and exposed using WSE-6100 LuminoGraph I (ATTO, Tokyo, Japan). Band intensity was analyzed densitometrically using the ImageJ software (National Institutes of Health).

**Immunohistochemical staining**

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Paraffin-embedded mouse lung tissue sections were stained with ENVISION+ Kit/HRP (Dako, Tokyo, Japan), as previously described (1). Briefly, after blocking endogenous peroxidase and proteins, the tissue sections were incubated overnight at 4 °C with an anti-S100A9 rabbit mAb (#73425; Cell Signaling Technology). The sections were then incubated with an HRP-labeled anti-rabbit IgG antibody; thereafter, the substrate chromogen was added and the sample was counterstained with hematoxylin.
References