Ezrin, a membrane cytoskeleton cross linker protein, as a marker of epithelial damage in asthma

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ONLINE DATA SUPPLEMENT

Supplementary Materials and Methods

Ethics statement

The protocol was approved by the ethics committee of The First Affiliated Hospital of Nanjing Medical University (2013-SRFA-037). Written informed consent was obtained from all patients. Animal experiments were performed under a Project License from the British Home Office, UK under the Animals (Scientific Procedures) Act 1986.

Study subjects

The diagnosis of bronchial asthma and severity of asthma were based on the Global Initiative for Asthma (GINA) guidelines [29]. Briefly, the severity of asthma was based on the level of asthma symptom control in the past 4 weeks. Patients were separated into those who had: 1) daytime symptoms ≥ twice per week; 2) nocturnal symptoms; 3) need for reliever treatment ≥ twice per week or 4) limitations in activities. Those with (1) or (2) were considered partly controlled and those with (3) or (4) were designated uncontrolled. In total 23 healthy subjects, 21 patients with well-controlled asthma, 17 patients with partly controlled asthma and 23 uncontrolled asthma patients were studied (Table 1). No patients had a history of lung cancer or chronic cardiopulmonary diseases. Healthy subjects had no history of asthma or any other chronic disease. All subjects were non-smokers and free from pulmonary infection in the 4 weeks before the study. There were no significant differences in gender, ages and BMI among the subject groups (Table 1). We followed-up 6 patients who were treated with the combination of low dose ICS (budesonide) and rapid-onset LABA (formoterol) in a single inhaler and recorded their lung function and symptom control.

Exhaled breath condensate (EBC) and serum collection

Human EBC and serum samples were collected from respiratory outpatients with asthma of the First Affiliated Hospital of Nanjing Medical University, Nanjing Jiangning People's Hospital and Nanjing First Hospital and community healthy volunteers.

The EBC was collected by using an EcoScreen condenser (Jaeger, Wurzburg, Germany), which allowed for the noninvasive collection of nongaseous components of the expiratory air. Patients and control subjects were asked to breathe through a mouthpiece and a two-way non-rebreathing valve, which also served as a saliva trap, at a normal frequency and tidal volume, wearing a nose clip, for a period of 10 minutes (min). The condensate (at least 1 ml) was collected in ice at 4℃, transferred to 1.5ml polypropylene tubes, and immediately stored at -80℃ for the subsequent analysis [28]. Serum samples were isolated (by centrifugation at 3000 rpm, 10 min within 2 hours) after whole blood collection, and the sera were kept at -40℃ until they were assayed. Patient serum samples in (Figure 2C) were from the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED). Details on the selection of patients and data collection have been published previously [30]. The U-BIOPRED study was registered at ClinicalTrials.gov (identifier: NCT01976767) and was approved by all Medical Ethics Boards. All patients provided written informed consent.

Animal experiments

BALB/c mice (6-8 weeks old, 20±0.7g) were randomly divided into four groups: control, ovalbumin (OVA), anti-IgG +OVA and anti-IL-13 +OVA. Protocols for the OVA-induced acute asthma model as well as an anti-IgG and an anti-IL-13 (30μg/mouse) in allergic asthma model were as previously described [31-32] and were summarized in Figure S1A. Mice were

sensitized by intraperitoneal injection with 4 ml/kg of the OVA-alum suspension (2 mg/kg OVA and 80 mg/kg aluminum hydroxide) on days 0, 7, and 14. Animals were anesthetize and challenged intranasally with the 4 mg/ml OVA solution by administering 12.5 µl per nostril on days 21, 22 and 23. Saline challenge served as the control. The anti-IgG+ OVA and anti-IL-13+ OVA groups were pretreated with Goat anti-Rabbit IgG antibody (Cat. No. RD AP132P) and Goat anti-Mouse IL-13 antibody (Cat. No. RD AF-413-NA) (30µg/mouse) by intranasal injection 100µl 1hour (h) before challenge with OVA on 3 consecutive days (days 21, 22 and 23). AHR was measured on day 24. Haematoxylin and eosin (H&E) stained fixed lung tissues sections were used to assess inflammation: grade 0: no inflammatory cells; grade 1: few inflammatory cells; and grade 2,3,4: most bronchi or vessels which were surrounded by a thin layer (1-2 cells: grade 2), a moderate layer (3-5 cells: grade 3), or a thick layer (>5 cells: grade 4) of inflammatory cells, respectively, by microscope (Nikon, Tokyo, Japan) at 400 × magnification. The total inflammatory score was calculated as the average of all individual inflammatory scores. Bronchial alveolar lavage fluid (BALF) were collected within 24h after the last exposure and stored at -80°C.

Immunohistochemical staining

Mouse lung tissues were stained with a rabbit monoclonal anti-mouse ezrin antibody (Cat. No. CST 3145S; Cell Signalling Technology Inc., Beverly, MA, USA) (1:100 dilution), a rabbit monoclonal anti-mouse E-cadherin antibody (Cell Signalling Technology Inc., Beverly, MA, USA) (1:100 dilution), a rabbit polyclonal anti-mouse ZO-1 antibody (Proteintech Group Inc, China) (1:100 dilution). The sections were developed using a DAB substrate kit (Thermo Fisher Scientific, Massachusetts, USA). The results were quantified with Image-Pro Plus.

Cell culture and lentivirus shRNA gene transfection

Primary human bronchial epithelial cells (PBECs) were purchased from the ScienCell Research Laboratories (Carlsbad, CA, USA), and were cultured in bronchial epithelial cell medium (BEpiCM; ScienCell Research Laboratories) containing bronchial epithelial cell growth supplement, 100 mg/ml penicillin and 100 U/ml streptomycin 1% antibiotics (ScienCell Research Laboratories) according to the manufacturer's instructions. PHBECs were serially passaged and used for experiments until passage 5 and seeded in cell culture plates with a seeding density of 1×10^5 cells/cm^2.

The human bronchial epithelial cell line (16HBE cells) was obtained from the Beijing Tumor Center. Cells were maintained in RPMI 1640 (ATCC) with 10% fetal bovine serum (FBS, ScienCell, San Diego, CA,USA) and 1% penicillin/streptomycin(Gibco, Grand Island, NY, USA) at 37℃/5%CO_2[22]. Cells were transfected with lentiviruses encoding for a control shRNA or human Ezrin-shRNA targeting GFP according to the manufacturers' instructions. The lentiviruses were diluted in enhanced infection solution (10mg/mL) containing polybrene (5ug/ml) and added to the cells at 37℃ for 24h and freshly prepared complete medium. A fluorescence microscope (Nikon, Tokyo, Japan) was used to observe the effect of transfection and cell morphology was recorded for the next 48h.

Exosomes isolation and Nanoparticle tracking analysis (NTA)

16HBE cell supernatants were centrifuged at 300×g for 10min, 2000×g for 10 min, and at 10000×g for 30min in turn with the supernatant being retained at each step. The supernatant was then centrifuged at 100000×g for 70min and the pellet retained. The pellet was resuspended in PBS before being centrifuged again at 100000×g for 70min. Particle

suspensions were diluted with PBS and were analyzed by nanoparticle tracking, using a ZETASIZER Nano ZS apparatus (Malvern Instruments, Worcestershire, UK).

Transmission electron microscopy (TEM)

Samples (exosomes and ultra-thin sections of mouse lung tissue) were prepared as described previously [35-36] and were photographed under a TEM (JEOL-1010, Jeol, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-4 (M400B), IL-5 (M5000), and IL-13 (DY413) in mouse BALF were measured by ELISA kit according to manufacturer's instructions (R&D, Minneapolis, MN, USA). The levels of ezrin in human samples were detected by human ELISA kits (SEB297Hu, Cloud Clone Corp, Wuhan, China). The detection range was 0.312ng/ml-20ng/ml with the minimum detectable concentration of ezrin differentiated from zero being 0.117ng/ml. Ezrin levels in murine BALF were detected by specific anti-mouse ELISA (CSB-EL007914MO, Cusabio, Wuhan, China). The detection range was 62.5pg/ml-4000pg/ml with minimum detectable concentration of ezrin compared to zero being 15.6pg/ml. Human serum IL-13, periostin and IgE were measured respectively using human IL-13 ELISA kit (CSB-E0460, Cusabio, Wuhan, China), human periostin ELISA kit (SEH339Hu, Cloud Clone Corp, Wuhan, China) and human IgE ELISA kit (BMS2097, Raybiotech, USA) according to manufacturer's protocols. All samples were within the detectable range of each assay. The absorbance was measured at 450nm using a model 550 microplate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Cell proteins were extracted using RIPA lysis buffer with protease inhibitors (Beyotime, China). Nuclear protein was extracted using NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher, Waltham, USA). 20μg protein was separated by 10% SDS-PAGE and transferred to PVDF-membranes (Millipore, Bedford, MA). The primary rabbit antibodies used were anti-ezrin (1:1000) (Cell Signaling Technology, US), anti-p-JAK2 (1:1000) (Proteintech Group Inc, China), anti-P-STAT6 (Proteintech, Group Inc, China), anti-GAPDH (1:1000) (Cell Signaling Technology, US) and anti-Histone (Cell Signaling Technology, US). Membranes were blocking in 5%BSA for 1h at room temperature before incubation with primary antibody at 4°C overnight. Membranes were washed three times in TBST and then incubated with HRP-linked anti-Rabbit IgG secondary antibody (1:5000) (Bioworld, Shanghai, China) at 20°C for 1h. The blots were visualized with an ECL plus reagent (ECL, Thermo Scientific, Waltham, USA).

RNA extraction and real time PCR (RT-qPCR)

RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA (1μg) was reverse transcribed using PrimeScript ™ Reverse Transcriptase (Takara, Kyoto, Japan) and quantitative real-time PCR was performed using SYBR qRCR premix (Takara, Kyoto, Japan). The specific sequences of forward (F) and reverse (R) primers used are as follows:

Human GAPDH: Forward Primer 5'-AGAAGGCTGGGGCTCATTTG-3'

Reverse Primer 5'-GGGGCCATCCACAGTCTTC-3';

Human Ezrin: Forward Primer 5'- ACCAATCAATGTCCGAGTTACC-3'

Reverse Primer 5'- GCCGATAGTCTTTACCACCTGA-3';

Cycling conditions were an initial denaturation at 95°C for 30 seconds (sec), followed by 40cycles at 95°C for 5sec, 60°C for 30sec, and 72°C for 10min. Data was normalized using the ΔΔCT method.

Measurement of transepithelial electrical resistance (TER) and epithelial permeability

16HBE cells were seeded in 24-well Transwell (Corning Costar, NY, USA) inserts and allowed to grow until fully integrated into a single layer. TER was measured daily using the Millicell-ERS system (Millipore Co., Bedford, MA, USA) [37]. 100ul RPMI-1640 containing 0.5mg/mL fluorescein isothiocyanate (FITC)-labelled dextran (Sigma Chemical Co.; molecular weight 40K Da) was added to the apical compartments (luminal side). 500uL RPMI-1640 without FITC-labelled dextran was added to the basal compartments (non-luminal side), and the plates were incubated at 37°C for 90 min. Samples from the apical and basal compartments were analyzed in black 96 well plates by multimode reader. Excitation and emission wavelengths were 490 and 520 nm, respectively.

Statistical analysis

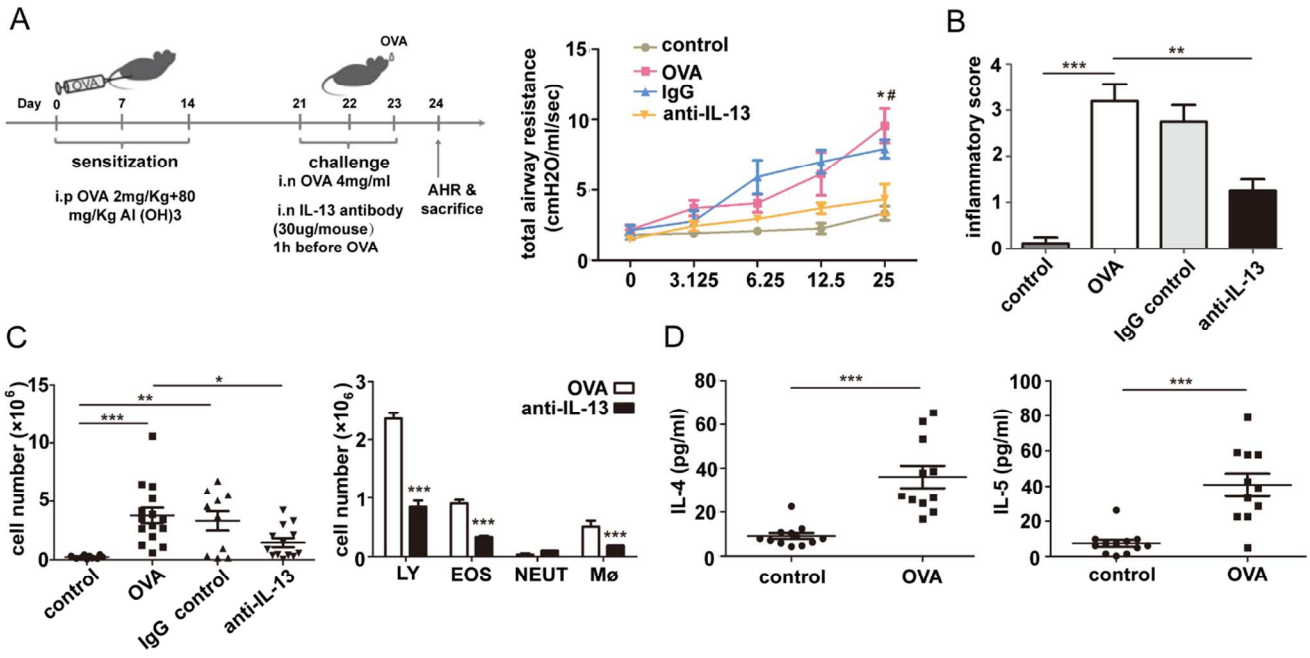
All data are presented as mean ± SEM and $P < 0.05$ was considered significant. The statistical analyses were performed using GraphPad Prism software v5.0 (GraphPad Software, Inc., San Diego, California, USA). Experiments with multiple comparisons were evaluated by one-way

ANOVA followed by Student-Newman-Keuls post-test or Bonferroni's post-test (normally distributed parameters) and Kruskal-Wallis test (non-normal distributed parameters) for multiple data sets. Comparisons between two groups were performed with an unpaired Student's t test for normally distributed parameters and with Wilcoxon rank-sum test for non-normal distributed parameters.

Supplemental Figure Legend

Figure S1. The establishment of an ovalbumin (OVA) induced allergic asthma and an anti-IL-13 in allergic asthma model. (A) A scheme of the development of the mouse model and the airway resistance of saline-exposed control animals (control), OVA-treated animals (OVA), OVA+anti-IgG antibody animals (anti-IgG) and ONA+anti-IL-13 antibody treated animals (anti-IL-13). **(B)** An overall inflammatory score was calculated to indicate the degree of inflammatory cell infiltration (n≥6/group). **(C)** The total cell number and cell type in BALF of OVA-treated 'asthma' mice (n=10) and OVA+anti-IL-13 antibody treatment mice (n=10). **(D)** Levels of IL-4 (control mice, n=12; 'asthma' mice, n=11) and IL-5 (control mice, n=8; 'asthma' mice, n=8) in BALF of asthma mice by ELISA. Data is shown as mean ±SD, **(B)** and **(C)** were analyzed using one-way ANOVA followed by Bonferroni's post hoc analysis. **(D)** were analyzed using Student's t-test. *P<0.05(OVA VS control), $^{\#}$P<0.05 (OVA VS anti-IL-13) in **(A)**. *P<0.05, **P<0.01 and ***p<0.001 compared to respective controls.

Figure S1


A
OVA
Day 0 7 14 21 22 23 24
sensitization
i.p OVA 2mg/Kg+80 mg/Kg Al (OH)3
challenge
i.n OVA 4mg/ml
i.n IL-13 antibody (30ug/mouse) 1h before OVA
AHR & sacrifice
control
OVA
IgG
anti-IL-13
total airway resistance (cmH2O/ml/sec)
B
inflammatory score
control
OVA
IgG control
anti-IL-13
C
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OVA
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LY
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IL-4 (pg/ml)
IL-5 (pg/ml)
control
OVA