Microfibrillar-associated protein 4 modulates airway smooth muscle cell phenotype in experimental asthma

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SUPPLEMENTARY MATERIAL

Source of reagents

HDM was from Greer (Lenoir, NC, USA). Alum was from Thermo Scientific (Waltham, MA, USA). CCL11 ELISA kit, CCL24 ELISA kit and recombinant human PDGF-BB were from R&D (Minneapolis, MN, USA). IL4 ELISA kit was from Biolegend (San Diego, CA, USA). IL5 ELISA kit and IL13 ELISA kit were from eBioscience (San Diego, CA, USA). Anti-IgE-HRP antibody was from Southern Biotech (Birmingham, AL, USA). Rabbit anti-FITC antibody, goat anti-rabbit Ig-HRP antibody and DAB+ chromogen were from Dako (Glostrup, Denmark). Hydroxyproline Colorimetric Assay Kit was from Biovision (Milpitas, CA, USA). TRIzol and amphotericin B reagent were from Invitrogen (Waltham, MA, USA). NucleoSpin RNA kit was from Macherey-Nagel (Hoerdt, France). Hemacolor staining kit, anti-integrin αvβ3 antibody and anti-integrin β1 antibody were from Millipore (Billerica, MA, USA). Anti-integrin αv antibody was from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY, USA). Protease and phosphatase inhibitors were from Roche (Basel, Switzerland). Pre-cast SDS-PAGE gels and nonfat dry milk were from Biorad (Hercules, CA, USA). ECL detection reagents were from Amersham (GE Healthcare, Chalfont St. Giles, UK). BSMCs were obtained from Lonza (Basel, Switzerland). DMEM, FBS, antibiotics and L-glutamine were obtained from Gibco (Waltham, MA, USA). Alfazyme was from PAA Laboratories (GE Healthcare, Chalfont St. Giles, UK). Vybrant Cell Adhesion Assay Kit was from Molecular Probes (Waltham, MA, USA). Anti-GAPDH antibody and anti-αvβ5 antibody were from Santa Cruz (Dallas, TX, USA). BrdU Cell Proliferation Assay Kit, anti-FAK antibody, anti-pFAK(T397) antibody, anti-integrin β5 antibody, PI3K inhibitor and MEK inhibitor were from Cell Signaling Technology (Danvers, MA, USA). Anti-MFAP4 monoclonal antibodies were produced in our laboratory. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of lung mechanics and AHR

24 h after the last challenge mice were anesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (10 mg/kg), tracheostomized and connected to computer-controlled small animal ventilator (Flexivent, SCIREQ, Montreal, Canada). Mechanical ventilation was set at 150 breaths/min with a tidal volume of 10 ml/kg and a positive end-expiratory pressure of 3 cm H_2O. Lung function parameters were measured in the steady state and after exposure to increasing doses of nebulized methacholine (MCh). For each parameter, a coefficient of determination of 0.90 was the lower limit for accepting a measurement.

Bronchoalveolar lavage (BAL)

Immediately after AHR measurements mice were sacrificed by cardiac puncture. Lungs were washed four times with 0.5 ml ice-cold PBS. BAL fluids were centrifuged at 1,000 g for 10 min at 4°C, and supernatants were stored in -80°C until further analysis. Cells were washed in red blood cell lysis buffer, resuspended in PBS, counted, cytospun and stained with Hemacolor. Differential cell count was performed in a blinded manner by two independent observers.

Preparation of lung homogenates

Frozen lungs were homogenized in 1 ml PBS with protease inhibitors. Homogenates were centrifuged for 10,000 g at 4°C. The supernatants were stored in -80°C until further analysis.

Measurement of specific IgE

Specific IgE was measured by ELISA. Briefly, wells were coated with 5 μg/ml OVA or HDM overnight at 4°C. Wells were blocked with PBS/0,05% Tween/1% BSA for 1 h. Serum samples diluted 1:20 in blocking buffer were then incubated for 2 h, after which wells were

washed and incubated with anti-IgE-HRP antibody. Results are shown as relative absorbance units (OD450).

Lung histology

Formalin-fixed, paraffin-embedded lung tissues were cut in 4 μm-thick slides and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or Picrosirius Red (PSR). For immunohistochemistry, sections were subjected to antigen retrieval with 1.5% hydrogen peroxide, stained with FITC-conjugated antibodies against α-smooth muscle actin (α-SMA) or MFAP4 (dilution 1:1000 and 1:100, respectively) for 1 h and secondary rabbit anti-FITC antibody for 20 min, and visualized with goat anti-rabbit Ig-HRP antibody and DAB+ chromogen.

Morphometric analysis

Lung inflammation was graded on H&E-stained slides by point counting using CAST 1 software. Briefly, 36-point grid was laid onto the field of vision. In each of 25 randomly selected fields, points hitting the inflamed area as well as all points hitting lung parenchyma were counted. The degree of inflammation was quantified as the ratio between the number of points hitting the inflamed area and the total number of points, and is presented as percentage of inflamed lung area.

Subepithelial fibrosis was quantified by color threshold analysis as Picrosirius Red-positive area and normalized to the length of the basement membrane. Goblet cell hyperplasia was assessed by counting PAS-positive cells and normalizing to the length of the basement membrane. Smooth muscle cell remodeling was quantified by measuring the thickness of the smooth muscle cell layer around airways. At least 5 same-sized bronchioles were counted in each slide. All analyses were performed in a blinded manner using ImageJ software [S1].

Cytokine quantifications

The levels of IL-4, IL-5, IL-13, CCL11 and CCL24 in BAL or lung homogenates were measured using commercial ELISA kits according to the manufacturers' instructions. The minimum detection limits were: 1 pg/ml for IL-4, 4 pg/ml for IL-5 and IL-13, and 3 pg/ml for CCL11 and CCL24.

Hydroxyproline assay

Levels of hydroxyproline in lung tissues, being a surrogate for collagen content, were measured using Hydroxyproline Colorimetric Assay Kit according to the manufacturer's instructions.

Surface coating with ECM proteins

Tissue culture plates were left uncoated or incubated with 5-20 µg/ml of poly-D-lysine (PDL) as a negative control, recombinant human MFAP4 [S2] or human plasma fibronectin as a positive control and incubated overnight at 4°C. Plates were washed and incubated with 10 mg/ml bovine serum albumin (BSA) for 1 h.

Real-time PCR

Total RNA was extracted from homogenized lung tissues or serum-starved BSMCs seeded for 24 h on ECM coating using TRIzol reagent according to the manufacturer's instructions. Total RNA from BSMCs derived from brionchial specimens was extracted using NucleoSpin RNA kit according to the manufacturer's instructions. One µg RNA was used for cDNA production. Reverse transcription was performed using M-MLV Reverse Transcriptase. Real-time PCR was performed using the TaqMan Universal PCR Master Mix and TaqMan Gene

Expression Assays specific for the given gene. The assay kits used were as follows: mGAPDH, Mm99999915_g1; mMFAP4, Mm00840681_m1; hGAPDH, Hs99999905_m1; hMFAP4, Hs00412974_m1; hCCL11, Hs00237013_m1.

In some experiments, cells were seeded in serum-free DMEM for 4 h to allow attachment. Subsequently, selective inhibitors of PI3K and MEK (LY294002 and PD98059, respectively) were added [S3-S4], and cells were collected after 24 h.

Adhesion assay

Cell adhesion was measured using the Vybrant Cell Adhesion Assay Kit (Molecular Probes). Briefly, cells were stained with calcein AM (1 µl/1 mln cells) in serum-free DMEM for 30 min at 37°C, seeded (100,000 cells/well) in uncoated or ECM-coated black 96-well plate and incubated for 1 h at 37°C. The plate was then washed with PBS and read. In some experiments, cells were pretreated with either RGD and DGR-containing peptides or anti-integrin antibodies for 30 min at room temperature prior to seeding. RGD-containing peptide recognizes and blocks the corresponding recognition motif on all αv integrins as well as α5β1, α8β1 and αIIbβ3 integrins. DGR-containing peptide is a non-blocking peptide used as a control. Integrin-blocking antibodies were anti-αv (clone L230), anti-β1 (clone P4C10), anti-αvβ3 (clone LM609) and anti-αvβ5 (clone P1F6). Isotype control antibody was used as a control for anti-integrin antibodies.

Proliferation assay

Cells were serum-starved for 24 h, resuspended in serum-free DMEM, seeded (10,000 cells/well) in an ECM-coated 96-well plate and incubated for 24 h at 37°C. Cells were then stained with BrdU for another 24 h at 37°C. Proliferation level was measured using BrdU Cell Proliferation Assay Kit according to the manufacturer's instructions.

In some experiments, cells were seeded in serum-free DMEM for 4 h to allow attachment. Subsequently, anti-integrin antibodies or selective inhibitors of PI3K and MEK (LY294002 and PD98059, respectively) were added for the rest of the incubation period. Isotype control antibody and DMSO were used as controls for anti-integrin antibodies and inhibitors, respectively.

Western blotting

Cells were serum-starved for 24 h, resuspended in serum-free DMEM, seeded in ECM-coated 6-well plates (500,000 cells/well) and incubated for various time points. After incubation, cells were washed with PBS and lysed in RIPA buffer with protease and phosphatase inhibitors. Equal amounts of samples were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)/0,1% Tween (TBST) for 1 h at room temperature and incubated overnight at 4°C with antibodies against GAPDH, integrin αv (clone M9), integrin β5, pFAK (T397) or FAK. After washing with TBST, the blots were incubated with goat anti-mouse Ig-HRP or goat anti-rabbit Ig-HRP for 1 h at room temperature. Proteins were detected using ECL reagents.

Flow cytometry staining of cell surface integrins

Near-confluent BSMCs were gently detached and harvested with alfazyme. Cells (200,000/tube) were resuspended in PBS with 2% FBS, stained with 10 µg/ml of anti-integrin antibodies or isotype control antibodies (Table S2) for 1 h on ice, washed with PBS and incubated with corresponding secondary antibodies (Table S1) for 30 min on ice. Cells were

acquired on LSRII flow cytometer (BD). Analysis was performed in FlowJo (TreeStar Inc, Ashland, OR, USA).

Table S1. Patient characteristics.

Patient ID	Sex	Age	Asthma status	Smoking
134	M	63	Asthmatic	Ex-smoker
135	M	69	Asthmatic	Ex-smoker
136	F	67	Asthmatic	Ex-smoker
137	F	64	Asthmatic	No
138	F	54	Asthmatic	No
142	M	70	Asthmatic	No
143	F	63	Asthmatic	Ex-smoker
325	F	64	Nonasthmatic	Yes
326	M	66	Nonasthmatic	No
327	M	58	Nonasthmatic	Yes
348	F	60	Nonasthmatic	Ex-smoker
349	F	68	Nonasthmatic	No
350	M	77	Nonasthmatic	Ex-smoker
352	M	24	Nonasthmatic	Yes
353	M	76	Nonasthmatic	Ex-smoker

F, female; M, male.

Table S2. List of antibodies used for flow cytometry.

Antibody	Clone	Host	Supplier
Integrin β1	P4C10	Mouse	Merck Millipore
Integrin αvβ5	P1F6	Mouse	Santa Cruz
Integrin αvβ3	LM609	Mouse	Millipore
Integrin β3	PM6/13	Mouse	Santa Cruz
Integrin β6	H-110	Rabbit	Santa Cruz
Integrin β8	H-160	Rabbit	Santa Cruz
Mouse IgG1 isotype control	-	Mouse	Dako
Normal rabbit IgG isotype control	-	Rabbit	Santa Cruz
Goat anti-mouse Ig-FITC	-	Goat	Dako
Goat anti-rabbit Ig-FITC	-	Goat	Dako

Fig. S1. MFAP4 deficiency has no effect on IL-4 or IL-5 but reduces IL-13 production. IL-4 (A), IL-5 (B) and IL-13 (C) levels were measured in BAL (left panel) and lung homogenates (right panel) in OVA-treated mice. n = 5-15. *$p < 0.05$, calculated by one-way ANOVA.

Fig. S2. MFAP4 does not contribute to IgE production. Allergen-specific IgE antibody levels do not differ between challenged groups in OVA or HDM model. n = 7-19.

Fig. S3. Integrin expression profile on BSMC surface. Figure shows relative expression of integrin β1, αvβ5, αvβ3, β3, β6 and β8 (solid histograms) together with isotype control signals (shaded histograms). Representative histograms of at least 3 independent experiments are shown. RFU, relative fluorescence units.

Fig. S1.

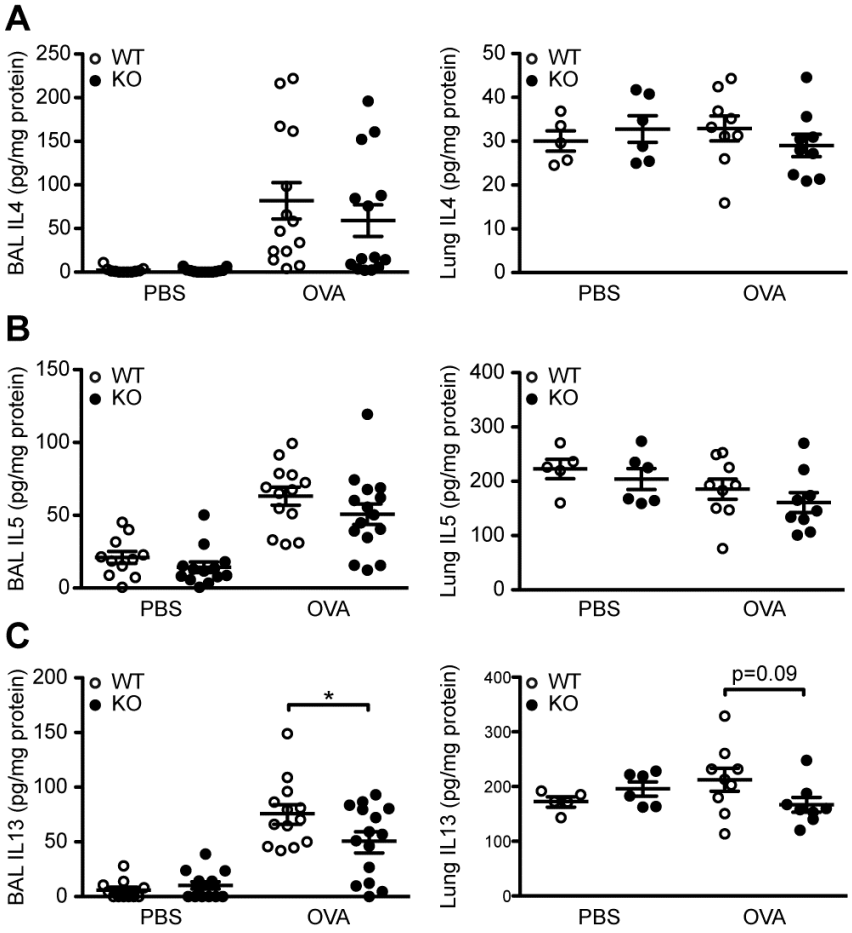
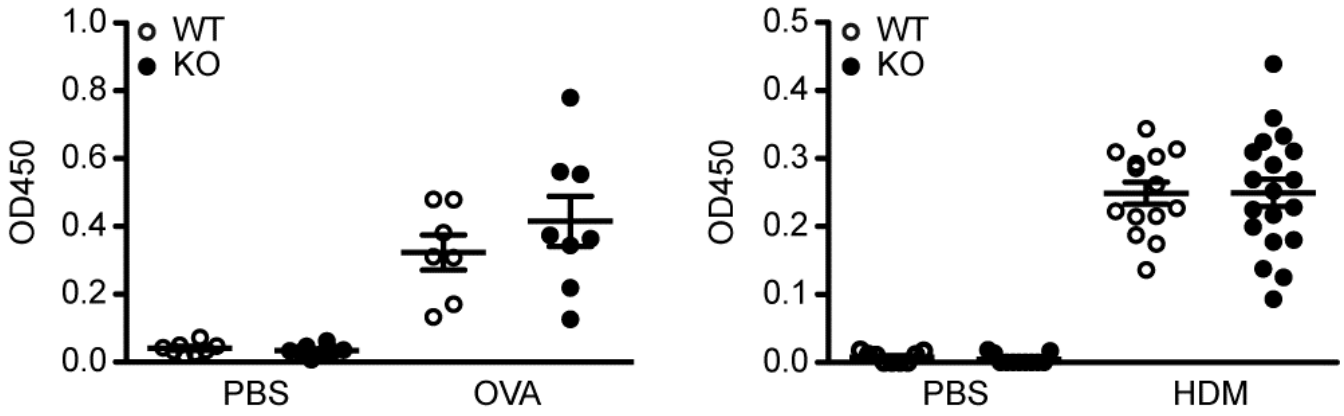

A
BAL IL4 (pg/mg protein)
Lung IL4 (pg/mg protein)
B
BAL IL5 (pg/mg protein)
Lung IL5 (pg/mg protein)
C
BAL IL13 (pg/mg protein)
Lung IL13 (pg/mg protein)
WT
KO
PBS
OVA
*
p=0.09

Fig. S2.


OD450
WT
KO
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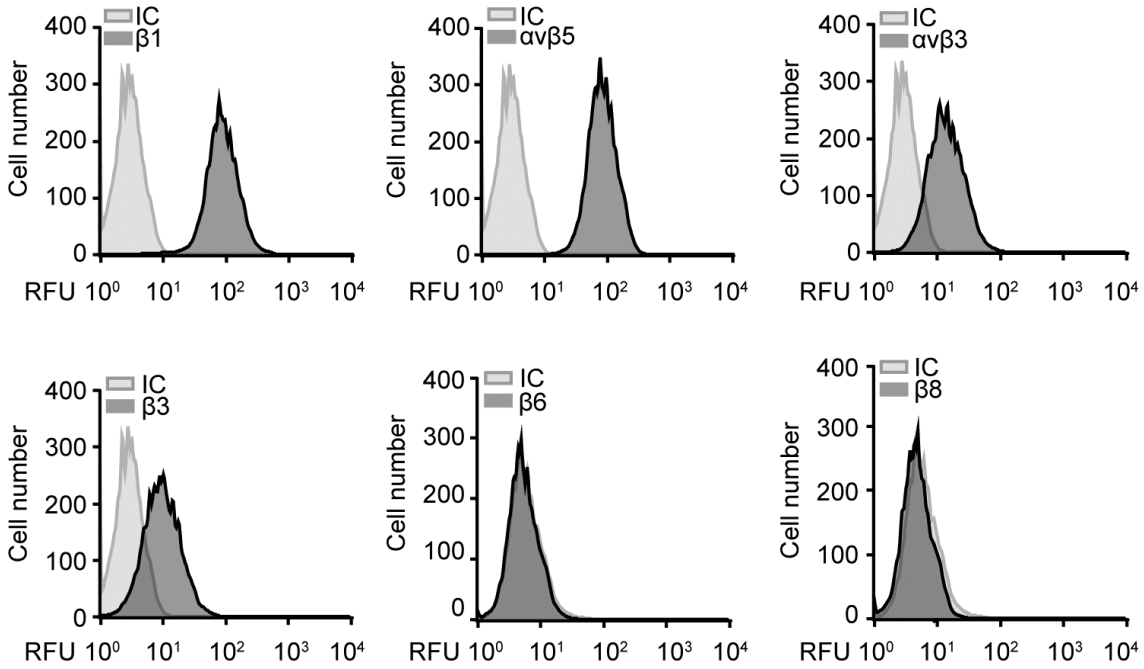
HDM

Fig. S3.



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