Eosinophils promote proliferation of pulmonary structural cells by stimulating extracellular matrix production in asthma

EOZINOFILAI SKATINA PLAUČIŲ STRUKTŪRINIŲ LĄSTELIŲ PROLIFERACIJĄ STIMULIUODAMI TARPLĄSTELINIO UŽPILDO PRODUKCIJĄ SERGANT ASTMA

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Summary. Introduction. Eosinophilic asthma can be characterized by intense infiltration of eosinophils into the airways, where they abundantly release various cytokines, chemokines and growth factors. Airway remodeling is closely related to increased airway smooth muscle (ASM) mass; however, another important feature could be changes in extracellular matrix (ECM) homeostasis, driven by the production of ECM proteins and metalloproteinases by lung structural cells. **Aim.** To evaluate and compare eosinophil adhesion to ASM cells and pulmonary fibroblasts or their secreted ECM and the effect on pulmonary structural cell autocrine proliferation adjustment in asthma. **Methods.** A total of 12 allergic asthma (AA), 8 severe eosinophilic asthma (SEA) patients and 11 healthy subjects (HS) were examined. Blood eosinophils were isolated using high density Ficoll centrifugation and magnetic separation. For each study individual combined cell cultures (co-cultures) between isolated eosinophils and pulmonary structural cells or their secreted ECM were prepared. ECM purification was performed using ammonium hydroxide (NH_4OH) based cell lysis. Eosinophils adhesion was evaluated by measuring eosinophils peroxidase activity in co-cultures; pulmonary structural cell proliferation was measured by AlamarBlue assay. **Results.** In all investigated groups eosinophil adhesion to ECM was significantly increased compared to eosinophil adhesion to ASM cells and pulmonary fibroblasts ($p < 0.01$). Eosinophil adhesion to ASM cells and pulmonary fibroblasts or their secreted ECM in asthma groups was significantly enhanced compared to HS group ($p < 0.05$). Isolated ASM cell or pulmonary fibroblast' produced ECM after co-cultures with AA, SEA and HS eosinophils promoted newly seeded ASM cell and pulmonary fibroblast proliferation ($p < 0.01$) with stronger effect in both asthma groups. ECM, isolated after combined cultures with AA, SEA or HS eosinophils and the blood serum of the study patient also significantly promoted the proliferation of ASM cells and pulmonary fibroblasts ($p < 0.01$). Isolated ASM cell or pulmonary fibroblast' produced ECM after co-cultures with AA and SEA eosinophils had a stronger effect to newly seeded pulmonary structural cell proliferation than after co-cultures with HS eosinophils. **Conclusions.** Eosinophils demonstrate enhanced adhesion to ECM components than to ASM cells or pulmonary fibroblasts, and adhesion further increases in asthma. Pulmonary structural cells promote their proliferation in autocrine manner via released ECM components after incubation with eosinophils in asthma.

Keywords: eosinophils, extracellular matrix, proliferation, airway smooth muscle, pulmonary fibroblast, asthma.

Santrauka. Įvadas. Eozinofilinei astmai būdinga intensyvesnė eozinofilų – svarbiausių uždegiminių ląstelių šios ligos patogenezėje – infiltracija į kvėpavimo takus, kur jie gausiai išskiria įvairius citokinus, chemokinus ir augimo faktorius. Kvėpavimo takų remodeliacija yra susijusi su padidėjusia bronchų lygiųjų raumenų (BLR) mase, tačiau kitas svarbus veiksnys gali būti BLR ląstelių arba plaučių fibroblastų tarpląstelinio užpildo baltymų ir metaloproteinazių gamybos ir funkcijų pokyčiai. **Tyrimo tikslas –** įvertinti bei palyginti eozinofilų adhezijos prie plaučių struktūrinių ląstelių arba jų išskirto tarpląstelinio užpildo skirtumus bei poveikį plaučių struktūrinių ląstelių autokrininės proliferacijos reguliavimui sergant astma. **Tyrimo metodai.** Tyrimo metu buvo ištirta 12 alerginės astmos (AA) ir 8 sunkios eozinofilinės astmos (SEA) pacientų bei 11 sveikų asmenų (SA). Kraujo eozinofilai išskirti naudojant centrifugavimą aukšto tankio Ficoll gradiente ir magnetinę separaciją. Kiekvienam tiriamajam buvo sudaromos kombinuotos kultūros tarp plaučių struktūrinių ląstelių arba jų pagamintų tarpląstelinio užpildo komponentų ir išskirtų kraujo eozinofilų. Tarpląstelinio užpildo komponentai išgryninti pritaikant osmotinį ląstelių lizės amonio hidroksidu metodą, kurio metu yra suardoma ląstelinė kultūros dalis, tačiau lėkštelės dugne išsaugoma stabili, netirpi tarpląstelinio užpildo struktūra. Eozinofilų adhezija prie plaučių struktūrinių ląstelių įvertinta matuojant eozinofilų peroksidazės aktyvumą kombinuotose kultūrose. Plaučių struktūrinių ląstelių proliferacija vertinta praėus 48 val. po naujų ląstelių užsėjimo ant tarpląstelinio užpildo komponentų naudojant Alamaro mėlio reagentą. **Rezultatai.** Visose tiriamųjų grupėse eozinofilų adhezija prie tarpląstelinio užpildo buvo reikšmingai didesnė, lyginant su adhezija prie BLR ląstelių ir plaučių fibroblastų ($p < 0,01$). Eozinofilų prisitvirtinimas astmos grupėse buvo reikšmingai intensyvesnis, lyginant su SA grupe ($p < 0,05$). Tarpląstelinio užpildo komponentai, išskirti po plaučių struktūrinių ląstelių inkubacijos su visų grupių eozinofilais, skatino naujai užsėtų BLR

ląstelių arba plaučių fibroblastų proliferaciją ($p < 0,01$), o tiriamųjų kraujo serumas papildomai sustiprino šį poveikį ($p < 0,01$). Tarpląstelinio užpildo komponentai, išgryninti po BLR ląstelių ir plaučių fibroblastų inkubacijos su AA ir SEA grupių eozinofilais reikšmingai intensyviau skatino naujai užsėtų plaučių struktūrinių ląstelių proliferaciją ($p < 0,05$), lyginant su SA eozinofilais. **Išvados.** Eozinofilai intensyviau prisitvirtina prie tarpląstelinio užpildo komponentų negu prie BLR ląstelių ar plaučių fibroblastų, o sergant astma eozinofilų adhezija yra padidėjusi. Po sąveikos su eozinofilais plaučių struktūrinės ląstelės skatina savo proliferaciją autokrininiais mechanizmais, dėl pakitusios tarpląstelinio užpildo komponentų produkcijos sergant astma.
Reikšminiai žodžiai: eozinofilai, tarpląstelinis užpildas, proliferacija, bronchų lygieji raumenys, plaučių fibroblastai, astma.

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INTRODUCTION

Asthma can be characterized by a more intense eosinophil infiltration into the airways, where they abundantly release various cytokines, chemokines and growth factors. Eosinophils– are the most important inflammatory cell in asthma pathogenesis, though make up less than 5 % total peripheral blood leukocyte counts; however, the maturation of these granulocytes in bone marrow intensifies in response to the regulated inflammatory response, turning them into a major effector cells in inflammatory processes. In allergic asthma (AA) cases, T helper type 2 (Th2) cells release cytokines such as interleukin (IL) 4, IL-5 and IL-13, thus prompting type 2 immunity, which consists of excessive antibody titers leading to eosinophilia. On the other hand, non-allergic eosinophilic asthma patients have elevated numbers of type 2 innate lymphoid cells (ILC2s), which upon stimulation with the epithelial cytokines produce Th2-associated cytokines such as IL-5, which is important for eosinophil differentiation, maturation and migration [1]. Before blood eosinophils are sent to sites of inflammation, they need to be activated [2]. For the most part, it depends on cytokines IL-3, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines of eotaxin family [3, 4].

Chronic eosinophilic airway inflammation leads to airway hyperreactivity and structural airway changes, also described as remodeling [5, 6]. Airway remodeling can be described as structural epithelial changes, subepithelial fibrosis and increased bronchial smooth muscle mass due to cell hyperplasia and hypertrophy. However, new studies suggest [7] that another important airway remodeling reason could be the changes in extracellular matrix (ECM) homeostasis, induced by airway smooth muscle (ASM) cell and pulmonary fibroblast ECM protein production and disturbed ECM protein decomposition enzymes, also known as metalloproteinases function.

ECM is a non-cellular part of every tissue and organ, which is a dynamic complex of proteinglycans, glycosaminglycans, glycoproteins and collagens. Collagen makes up the majority of lung ECM protein. Fibrillary collagens (I, II, III, V, and XI) contribute to the broad structure of the lungs, and large elastic fibers provide elasticity to the lungs. Elastic fibers consist of two components: elastin – a highly crosslinked protein, and fibrillin containing microfibrils. While microfibrils serve as a scaffold for elastin, they are also important modulators of growth factor allocation and function [8]. Growth factors, such as transforming growth factor (TGF)-β1, are secreted as a dormant latent dimer bound to a member of the latent TGFβ-binding protein (LTBP) family [9]. This large complex can then covalently bind to fibrillin molecules in the microfibrils [10], creating a latent growth factor reserve in the ECM that can be activated and mobilized, when required, through interactions with αvβ6 or αvβ8 integrins, proteases, and other factors [9]. Essential cell differentiation and proliferation factors, such as fibroblast growth factor (FGF), TGF-β and various cytokines may accumulate in the ECM [11]. Altered ECM around ASM cells may affect their physiological activity, thus promoting the development of airway remodeling [12]. Miofibroblasts, the active form of fibroblast, secrete and organize ECM, which provides structural support for their adhesion, migration, and tissue organization, besides regulating cellular functions such as growth and survival. Miofibroblasts are the principal contributors to synthesis of ECM in most types of tissue, including the lung [13]. ECM structural and functional molecules create an environment, where nearby cells can communicate via cell-cell or cell-ECM interactions. The matrix structure, composition and amount can change cell behaviour, polarity, migration, differentiation, proliferation and viability [14]. Cell-ECM interaction is mediated by transmembrane cell adhesion receptors and integrins [15], which interact with cytoplasmic integrin tip and transmits bidirectional signals between ECM and intracellular signaling pathways. After migrating through blood vessel endothelium, eosinophils adhere to pulmonary structural cells or ECM proteins when their integrins recognise cell receptors or protein ligands [16]. Eosinophils are an important source of growth factors and cytokine, which could promote production of ECM proteins by pulmonary structural cell [17].

The protein composition of the ECM in the airways changes in asthma [11]. Fibroblasts and myofibroblasts are major producers of ECM components in the lungs,

but airway epithelial cells and bronchial smooth muscle cells are also important sources of these components [7, 18]. In response to lesions, fibroblasts migrate to the site of inflammation and differentiate into myofibroblasts [19], which specialize in ECM production. Myofibroblasts facilitate wound healing by additional secretion of ECM proteins – collagens I, III, and V, fibronectin, tenascin, and proteocyclans – lumican, versican, biglycan, and decorin [20]. Unusual ECM protein deposition is a characteristic feature of chronic asthma, causing airway stiffness and narrowing, and differences in ECM protein expression may reflect specific asthma endotypes [6].

We hypothesized that eosinophil adhesion to pulmonary structural cells might differ from adhesion to ECM proteins. Furthermore, recent studies suggest that eosinophils can promote pulmonary structural cell proliferation [21], however there is not enough data about eosinophil disturbed ECM protein production which could also affect cell behavior. We used a decellularization technique to mimic *in vivo* processes by allowing investigated ASM cells and pulmonary fibroblasts to proliferate and deposit their own ECM components rather than using commercial proteins to coat tissue culture plates for our experiments.

EXPERIMENTAL SECTION

Ethics

The research protocol was approved by the Regional Biomedical Research Ethics Committee of the Lithuanian University of Health Sciences (BE-2-13). The study was registered in the U.S. National Institutes of Health trial registry ClinicalTrials.gov with identifier NCT03388359.

Study population

The study included severe eosinophilic asthma (SEA) patients, free of steroid non-severe allergic asthma (AA) patients, and healthy subjects (HS), who comprised the control group. The participants in this study were adult women and men, aged between 18 and 75 years who signed written informed consent prior to enrolling in the study. Investigated participants were all patients from the Department of Pulmonology, Hospital of the Lithuanian University of Health Sciences.

Admission criteria for the SEA group were: diagnosed with asthma ≥12 months; blood eosinophil count $\geq 0.3 \times 10^9$/L *throughout* the screening visit or $\geq 0.15 \times 10^9$/L if with recorded eosinophil amount $\geq 0.3 \times 10^9$/L in the 1 year period prior the screening; no other indications that could cause poor control of asthma symptoms; recorded at least 1 year treatment of high doses of inhaled corticosteroids combined with long-acting beta-agonist ± episodic use of oral corticosteroids ± long-acting antimuscarinic agent prior to inclusion in the study; in the 1 year before the screening visit ≥ 2 exacerbations of asthma that required treatment with systemic glucocorticoids.

The non-severe AA group involved patients with recent and untreated mild AA, confirmed with symptoms and medical history no less than 12 months, positive skin prick test to clinically important allergen(s), and positive bronchial provocation with methacholine or bronchodilator reversibility test.

HS tested negative to clinically relevant allergen(s) and were without allergic and other chronic respiratory diseases.

Rejection criteria consisted of asthma exacerbation in less than a month before the study, clinically relevant permanent allergy symptoms, active airway infection 1 month before the study, use of oral steroids ≤ 1 month before the study, and treatment with targeted (biological) therapy (such as mepolizumab, benralizumab, omalizumab).

All participants were neither current nor former smokers.

Study design

In the beginning, all participants underwent spirometry, skin prick test, physical examination and a methacholine challenge test to confirm the admission and exclusion criteria. If participants fulfilled the criteria, they were introduced about the requirements for participation in the study and informed written agreement was acquired.

72 hours before peripheral blood extraction, pulmonary structural cells – ASM cells and pulmonary fibroblasts – were seeded into cell culture plates (Fig. 1). During the participants visit, peripheral blood was drawn and exhaled FeNO was measured. Separated blood eosinophils viability and amount was assessed, then eosinophils were immediately used in fixed amounts to create co-cultures with ASM cells and pulmonary fibroblasts or their secreted ECM proteins for eosinophil adhesion and cell proliferation experiments. If the eosinophil yield was $< 1.5 \times 10^6$/20 mL blood; purity < 96 %; viability < 98 %; the investigated subject was excluded from the study. All data presented in the manuscript were from participants who passed these criteria.

ASM Cell and Pulmonary Fibroblast Cultivation *in vitro*

During the study we used healthy human ASM cells, immortalized by stable expression of human telomerase reverse transcriptase as described [22], and a commercial MRC-5 (Sigma, Ronkonkoma, NY, USA) lung fibroblast cell line. Both cell lines were grown in cultivation conditions of 5 % CO_2 in air at 37 °C with culture medium renewal every 3 days. To avoid diminish-

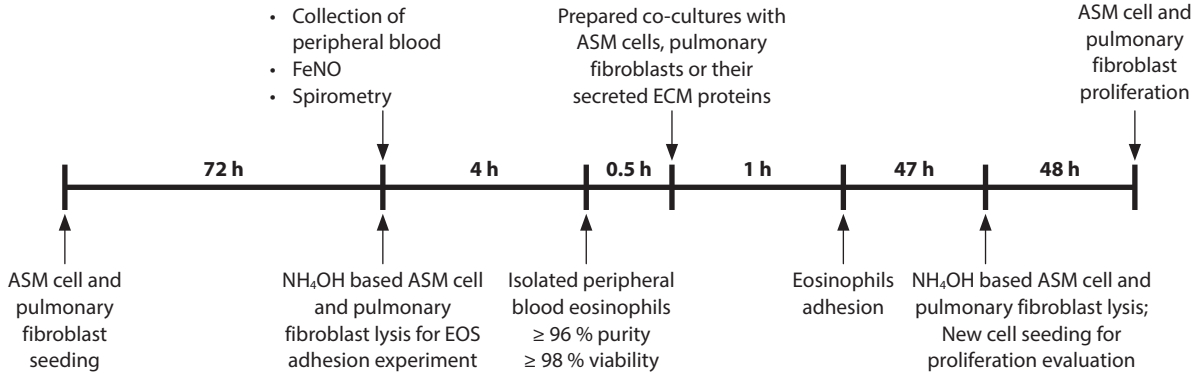

Fig. 1. Study design and experimental plan.

ASM – airway smooth muscle. ECM – extracellular matrix. EOS – eosinophils. NH_4OH – ammonium hydroxide. FeNO - fractional exhaled nitric oxide.

ing in cell viability and activity after repeated number of cell passage, novel cells of the mainline were thawed each time after 6 passages. ASM cells were grown in "Dulbecco's modified Eagle's medium (DMEM)" (GIBCO by Life Technologies, UK), meanwhile pulmonary fibroblasts were grown in "Eagle's minimum essential medium (EMEM)" (GIBCO, Paisley, UK). Both cell culture mediums were supplemented with antibiotics penicillin/streptomycin (2 % *v*/*v*; Pen-Strep, GIBCO by Life Technologies, Paisley, UK), antifungal amphotericin B (1 % *v*/*v*; GIBCO, Paisley, UK), and cell growth supplement fetal bovine serum (FBS) (10 % *v*/*v*; GIBCO by Life Technologies). Before every experiment ASM and MRC-5 cells were serum-starved in respective medium, supplemented with penicillin/streptomycin, amphotericin B and insulin, transferrin, and selenium reagent (GIBCO by Life Technologies) to stop further cell proliferation and reduce possible errors due to the effect of serum mediators.

Blood Eosinophil Isolation and Purification

Patients' peripheral blood samples were collected into sterile vacutainers with dipotassium ethylenediaminetetraacetic acid (K2EDTA) (BD Bioscience, San Jose, CA, USA). Blood was then diluted with 1x phosphate-buffered saline (PBS) (GIBCO, Paisley, UK), layered on high density Ficoll-Paque PLUS reagent (GE Healthcare, Helsinki, Finland) and centrifuged at 400 × *g* force for 30 min at room temperature. Afterwards the upper layers were removed, and the bottom-most layer comprised of granulocytes and erythrocytes was collected, washed with sterile H_2O and 2x concentrated PBS then centrifugated at 300 × *g* force for 10 min at room temperature. This step was repeated until no red blood cells were left.

Then, enriched granulocytes amount and viability was evaluated using automatic cell counter ADAM (Witec AG, Switzerland). Eosinophil isolation and purification was performed by negative selection from the granulocyte layer using Magnetic-activated cell sorting (MACS) magnetically-labeled MicroBeads (Miltenyi Biotec, Somerville, MA, USA). The producer claim that eosinophil purification kits do not influence their viability, and that isolation efficiency is more than 96 %. Once eosinophils were separated, their amount and viability were assessed using automatic cell counter ADAM.

ASM Cell and Pulmonary Fibroblast' Secreted ECM Purification with NH4OH Based Cell Lysis

ECM production and harvesting protocols were adapted to laboratory conditions [13, 23]. To extract the insoluble ECM layer, a 50 mM ammonium hydroxide (NH_4OH) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) solution was used to lyse the cell cultures. NH_4OH solution was added to each well and incubated up to 10 minutes. During the incubation cells were observed under inverse light microscope (CETI, Inverso TC100, Medline Scientific, Chalgrove, UK) with installed XM-10-IR-2 camera (Olympus, Tokyo, Japan) while constantly gently shaking the plate to fully lyse all cells. When there were no visible cells left the liquid was aspirated and wells were washed 3 times with isotonic PBS and incubated 15 minutes with PBS in the thermostat to further wash any NH_4OH residue.

For adhesion assay, after the initial 3-day incubation the medium was removed and cells were washed once with PBS, and ECM was extracted as previously described. The insoluble ECM components attached to the bottom of the tissue culture plates were used for eosinophil adhesion experiments.

For ASM cell and pulmonary fibroblast proliferation assay, after the initial 3-day incubation the culture medium was removed and cells were washed once with PBS, ECM extracted as previously described, then supplemented with either DMEM or EMEM with 2 % FBS, respectively. After eosinophil enrichment from patients' peripheral blood, combined cell cultures were prepared and incubated for 2 days at standard conditions. After incubation, medium was removed and wells were washed once with PBS then lysed with 50 mM NH_4OH solution as previously described.

New ASM cells and pulmonary fibroblasts (1.5×10^4 in each well) were seeded onto ECM coated wells and incubated with 2 % FBS DMEM/EMEM for 2 days at standard conditions. After incubation cell proliferation was evaluated using AlamarBlue assay.

Eosinophil Adhesion to Pulmonary Structural Cells or Their Secreted ECM Evaluation

ASM cells and pulmonary fibroblasts were seeded into 24-well tissue culture plates (1×10^4 cells in each well) in DMEM for ASM cells or EMEM for pulmonary fibroblasts, both supplemented with 10 % FBS and cultivated for 3 days at standard conditions. Afterwards, the culture medium was aspirated, and each well washed once with warm isotonic PBS. The culture medium was changed 1 day before the experiments by supplementing cells with serum-free medium as described earlier. Half of the wells were lysed with NH_4OH to extract ECM components as described earlier. After eosinophil enrichment from patients' peripheral blood, combined cell cultures were prepared and eosinophil adhesion to pulmonary structural cells or their secreted ECM components was measured after 1 h of incubation, which is deemed a sufficient amount for stable eosinophils adhesion in co-culture. After incubation, non-attached eosinophils were aspirated, and wells were rinsed once with warm isotonic PBS. Eosinophil adhesion was evaluated by measuring residual eosinophil peroxidase (EPO) activity. To evaluate EPO activity, 116 µL of DMEM medium without phenol red and 116 µL of EPO substrate (1 mM *o*-phenylenediamine, 1 mM H_2O_2 and 0.1 % Triton X-100 in Tris buffer, pH 8.0) were supplemented to each investigated well. After 30 min of incubation at standard conditions, 58 µL of 4 M sulfuric acid (H_2SO_4) was supplemented to each investigated well to end the reaction and the absorbance was assessed at 490 nm by a microplate spectrophotometer. Results were represented as % of attached eosinophil number from total added, calculated from a control eosinophil amount (1.25×10^4) absorbance result.

AlamarBlue Cell Proliferation Asssay

Pulmonary structural cells for proliferation experiments were cultivated in 24-well plates (5×10^3 cells in each well) in conditions previously described. Cultivation medium was changed into the 2 % FBS medium before the experiments. ASM cells or pulmonary fibroblasts were co-cultured with a respective group of eosinophils isolated from AA patients, SEA patients, or the healthy subjects for 48 h. Some of the wells were supplemented with investigated subjets' serum (2 % *v/v*). After 48 h incubation, cells were lysed with NH_4OH to extract ECM proteins as described earlier. Afterwards new ASM cells or pulmonary fibroblasts were seeded into each well (1.5×10^4 cells/well) in 2 % FBS cultivation medium for 2 days. Cell proliferation was assessed by adding Hank's balanced salt and AmalarBlue reagent (10 % *v/v*; Invitrogen by Life Technologies, Paisley, UK). Conversion of AlamarBlue reagent resazurin to the reduced form known as resofurin was dependent on the ASM and MRC-5 cells metabolic activity, which was measured by dual-wavelength spectrophotometry at wavelengths of 570 nm and 600 nm. As stated by the manufacturer, the amount of AlamarBlue conversion is directly proportional to the number of viable cells. The results are expressed as the percentage increase or decrease in AlamarBlue reagent conversion by ASM or MRC-5 cells compared with the control pulmonary structural cells (co-cultured without eosinophils).

Statistical Analysis Methods

Statistical data analysis was conducted using GraphPad Prism 8 for Windows (ver. 8.0.1, 2018; GraphPad Software Inc., San Diego, CA, USA). Differences between two independent groups were evaluated using the Mann–Whitney two-sided U-test. The Wilcoxon matched-pairs signed-rank two-sided test was used for two dependent groups. A value of $p < 0.05$ was deemed statistically significant.

RESULTS

Characteristics of the Studied Participants

Table 1. Demographic and clinical characteristics of study population.

	AA patients (n = 12)	SEA patients (n = 8)	Healthy subjects (n = 11)
Age, years	27.3 ± 2.6	55.1 ± 6*#	31.4 ± 3
Sex (Male/Female), n	7/5	3/5	5/6
BMI, kg/m^2	22.9 ± 1.0	29.8 ± 1.3*#	24.6 ± 1.1
FEV_1, L	3.9 ± 0.3	1.8 ± 0.4*#	4.1 ± 0.3
FEV_1, % of predicted	88.8 ± 2.4*	54.4 ± 6.2*#	107 ± 4
Blood eosinophil count ($\times 10^9$)/L	0.54 ± 0.07*	0.7 ± 0.22*#	0.17 ± 0.04
IgE, IU/mL	394 ± 110*	168 ± 82.4*	23.4 ± 3.8
FeNO, ppb	47 ± 9.5*	67.7 ± 12.8*	14.3 ± 1.8

Data presented as mean ± standard error of mean; AA – allergic asthma; BMI – body mass index; FeNO – Fractional exhaled nitric oxide; FEV_1 – Forced expiratory volume in 1 s; IgE – immunoglobulin E; SEA – severe eosinophilic asthma; *p < 0.05 compared to healthy subject group; #p < 0.05 compared with AA group. Statistical analysis – Mann Whitney two-sided U-test between two independent groups.

All patients diagnosed with allergic asthma were susceptible to *D. ptereonyssinus* house dust mites. Blood eosinophils count, IgE and FeNO in patients with AA and SEA were significantly higher compared to the healthy subject group. In addition, the AA and SEA

groups showed a significant decrease in lung function compared to healthy subject group. Comparing asthma phenotypes, there is a tendency for SEA to occur in older age, more often in women. In our study, the SEA patients were significantly older than AA patients and healthy subjects as SEA has late-onset manifestation. Moreover, it has been suggested that eosinophil degranulation, but not adhesion, was higher in younger patients group compared to older asthma patients [24]. However, we state that the age distinctness did not influence our data as investigation relied on the severity of the illness but not on the age groups.

Eosinophil Adhesion to ASM Cells and Pulmonary Fibroblasts or Their Secreted ECM

We observed that 71.8 ± 1.9 % of AA patients' eosinophils adhere to airway smooth muscle (ASM) cells, meanwhile 87.2 ± 1.6 % eosinophils adhered to ASM cell secreted ECM ($p < 0.0001$). Similarly, 69.5 ± 3.2 % of SEA patients' eosinophils adhered to ASM cells *vs.* 88.3 ± 1.2 % to secreted ECM ($p < 0.0001$). In healthy subject group – 56.8 ± 2.6 % *vs.* 75.5 ± 1.3 % ($p < 0.0001$) (Fig. 2 A). In all groups eosinophil adhesion to ECM was significantly increased compared to eosinophil adhesion to ASM cells. There were no significant differences between asthma groups, however eosinophil adhesion to ASM cells or their secreted ECM in asthma groups was significantly increased compared to healthy subject group.

Eosinophils migrating into the airways can also interact with lung fibroblasts in connective tissue; thus we evaluated eosinophil adhesion to pulmonary fibroblasts and their secreted ECM components. We observed that 75.7 ± 1.9 % of AA patients' eosinophils adhere to pulmonary fibroblasts, meanwhile 88.2 ± 1.1 % eosinophils adhered to secreted ECM ($p < 0.0001$) (Fig. 2 B). Similarly, 72.1 ± 3.7 % of SEA patients' eosinophils adhered to pulmonary fibroblasts *vs.* 88.3 ± 1.6 % to secreted ECM ($p < 0.01$). In healthy subject group – 55.5 ± 3.4 % *vs.* 73.1 ± 1.8 % ($p < 0.001$). We found that the trends remained similar – in all groups eosinophil adhesion to ECM was significantly increased compared to adhesion to pulmonary fibroblasts. There were no significant differences between asthma groups; however, eosinophil adhesion to pulmonary fibroblasts or their secreted ECM in asthma groups was significantly increased compared to healthy subject group.

ASM Cell and Pulmonary Fibroblasts Proliferation Autocrine Adjustment via ECM

Isolated ASM cell produced ECM components after co-cultures with AA, SEA and HS eosinophils promoted newly seeded ASM cell proliferation, ASM cell count after 48 h was 10.4 ± 0.8 % in AA, 11.8 ± 1.2 % in SEA and 3.7 ± 0.5 % in healthy subject group higher ($^{\&}p < 0.01$) compared to ASM cells grown on ECM which was produced by incubating ASM cells without eosinophils (Fig. 3 A).

Meanwhile, ECM proteins, isolated after co-cultures with AA, SEA or HS eosinophils and patients blood serum supplement also significantly promoted the proliferation of newly seeded ASM cells by 14.8 ± 1.2 %, 17.0 ± 1.1 % and 5.4 ± 0.4 % respectively ($^{\#}p < 0.01$) compared to ASM cells grown on ECM which was produced by incubating ASM cells with patients' blood

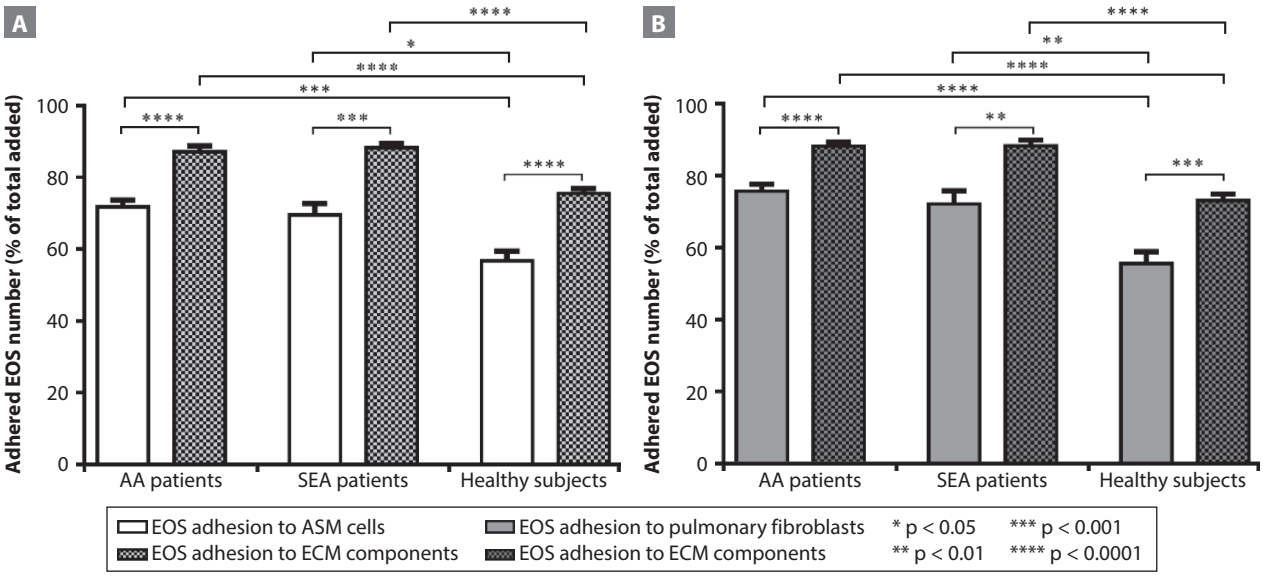

Fig. 2. Eosinophil adhesion to ASM cells and pulmonary fibroblasts or their secreted ECM components: (A) Eosinophil adhesion to ASM cells or their secreted ECM; (B) Eosinophil adhesion to pulmonary fibroblasts or their secreted ECM.

AA (allergic asthma) n = 11; SEA (severe eosinophilic asthma) n = 8; Healthy subjects n = 11. Results are shown as mean ± standard error of mean. ASM – airway smooth muscle. ECM – extracellular matrix. EOS – eosinophils. Statistical analysis – Mann–Whitney two-sided U-test between two independent groups, Wilcoxon matched-pairs signed-rank two-sided test for dependent groups.

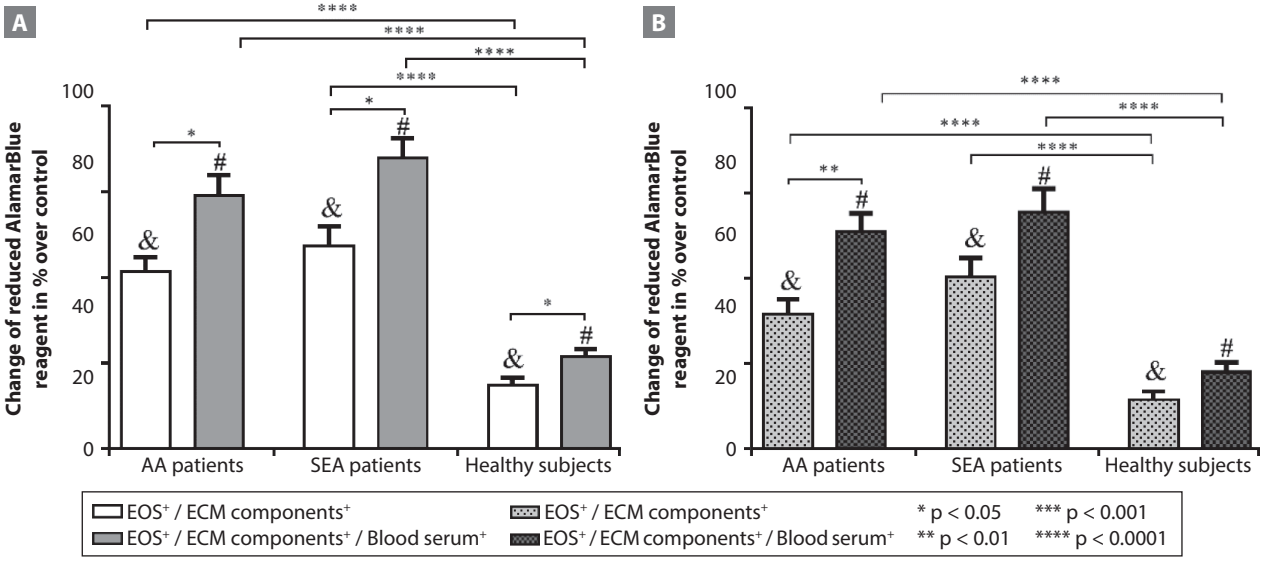

Fig. 3. Newly seeded pulmonary structural cell proliferation on ECM, purified from ASM cell or pulmonary fibroblast co-cultures with eosinophils: (A) Eosinophil disturbed ECM production effect on ASM cell proliferation; (B) Eosinophil disturbed ECM production effect on pulmonary fibroblast proliferation.

AA (allergic asthma) n = 12; SEA (severe eosinophilic asthma) n = 8; Healthy subjects n = 9. Results are displayed as mean±standard error of mean. EOS – eosinophils. ECM – extracellular matrix. Statistical analysis – Mann–Whitney two-sided U-test between two independent groups, Wilcoxon matched-pairs signed-rank two-sided test for dependent groups.
& – $p < 0.01$ compared to pulmonary structural cells grown on ECM which was produced by incubating pulmonary structural cells without eosinophils.
# – $p < 0.01$ compared to pulmonary structural cells grown on ECM which was produced by incubating pulmonary structural cells with patients' blood serum, without eosinophils.

serum supplement but without eosinophils (Fig. 3 A). No significant differences were found between the asthma groups, however, ASM cell proliferation was more intense in both asthma groups compared to the healthy subject group.

Similarly, pulmonary fibroblast produced ECM, isolated after co-cultures with AA, SEA and healthy subject eosinophils promoted newly seeded pulmonary fibroblast proliferation by 7.9 ± 0.9 % in AA, 10.1 ± 1.1 % in SEA and 2.9 ± 1.5 % in HS group ($^{\&}p < 0.01$) compared to pulmonary fibroblasts grown on ECM which was produced by incubating pulmonary fibroblasts without eosinophils (Fig. 3 B). Meanwhile, pulmonary fibroblast produced ECM proteins, isolated after co-cultures with AA, SEA and healthy subject eosinophils and patients' blood serum supplement also promoted lung fibroblast proliferation by 12.7 ± 1.1%, 13.9 ± 1.4 % and 4.5 ± 0.6 % respectively ($^{\#}p < 0.01$) compared to pulmonary fibroblasts grown with the subjects' blood serum but not affected by eosinophils (Fig. 3 B). Similarly to ASM cells, pulmonary fibroblast proliferation was more intense in the AA and SEA groups compared with the healthy subject group.

DISCUSSION

This study showed that the adhesion of eosinophils to ECM in all study groups was found to be significantly higher compared to adhesion to ASM cells and pulmonary fibroblasts. In addition, eosinophils from AA and SEA patients adhered more actively to lung structural cells produced ECM compared with healthy subjects' eosinophils. Isolated ASM cell or pulmonary fibroblast' produced ECM after combined cultures with AA, SEA and HS eosinophils, promoted newly seeded ASM cell and pulmonary fibroblast proliferation. ECM, isolated after co-culturing with AA, SEA or HS eosinophils and patients' blood serum supplement also significantly promoted the proliferation of newly seeded ASM cells and pulmonary fibroblasts. ASM cell and pulmonary fibroblast proliferation was more intense in both asthma groups compared to healthy subject group.

Increased eosinophil adhesion in asthma may be explained by an increase in the expression of outer membrane integrins or a different state of integrin activation [2], which results in eosinophils adhering more rapidly to ECM component ligands. The cytokines produced by the airway epithelium, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), also known as alarmins, are thought to be released in response to different bronchial epithelial stimuli (such as allergens, atmospheric pollutants, infectious agents), thus inducing eosinophils differentiation, maturation and migration into the airways. In our previous publications it was shown that AA patients' eosinophils displayed increased surface integrin gene expression compared to healthy subject group [25, 26]. The results in this study further suggest that eosinophils adhere more actively to ECM than to pulmonary structural cells possibly due to the expression of more ECM component-specific integrins and adhesion molecules

on the eosinophil membrane surface. Another reason might be due to the presence of more specific ECM component integrins in the active phase than active integrins specific for pulmonary structural cells, and these processes are further enhanced in asthma. This could be due to the fact that eosinophil recruitment from the bloodstream depends on peripheral blood eosinophils becoming "activated", which leads to their arrest on endothelium, extravasation and continued migration through the bronchial tissue by surface adhesion molecule interaction with the ECM. It is therefore possible that eosinophils are more adjusted to interact with ECM components, especially in chronic inflammation background cases, as displayed in this study by increased eosinophil adhesion to ECM, compared with adhesion to pulmonary structural cells.

Activated eosinophils may secrete proinflammatory mediators such as cytokines, chemokines, leukotrienes, matrix metalloproteinases and granule proteins [27], aswell as bind to ASM cells and promote ASM to release cysteinyl leukotrienes, which in turn enhances nearby ASM proliferation. Eosinophils express seven integrins: α4β1, α4β7, α6β1, αDβ2, αMβ2, αLβ2, αXβ2 [28], which can mediate their adhesion to adhesion molecules VCAM-1 (vascular cell adhesion molecule 1) and ICAM-1 (intercellular Adhesion Molecule 1), but also to ECM proteins fibronectin through α4β1 and α4β7; laminin through α6β1 and vitronectin through αMβ2 [2]. One study conducted in 2013 showed that inhibition of eosinophil adhesion to ASM cells using specific antibodies against eosinophil adhesion receptors was associated with inhibition of ASM cell proliferation [29]. However, the ability of eosinophils to adhere to pulmonary structural cells secreted ECM has not been evaluated, which means eosinophils could degranulate to release the same compounds into the microenvironment and act on adjacent cells. Based on the results of the study, new ways could be found in the future to block certain eosinophil integrins responsible for adhesion to the ECM and to reduce the accumulation of eosinophils in the airways, thus inhibiting the development of airway remodeling during asthma.

Airway remodeling is a crucial feature of asthma which includes hypertrophy and hyperplasia of the ASM and adjustments in the ECM component profile around the ASM. In our previous publications it was found that eosinophils from AA patients' increased COL1A1 and FN gene expression in ASM cells [30]. During this study it was found that eosinophil-disrupted ECM production promoted new pulmonary structural cell proliferation. One possible mechanism could be enhanced production of eosinophils promoted pro-proliferative ECM components, such as collagen I and fibronectin [31], by pulmonary structural cells. Pulmonary structural cells secrete ECM to maintain their microenvironment, however during asthma, inflammation promotes eosinophil migration, accumulation and degranulation in the airways, which leads to subsequent ASM proliferation and ECM deposition. Further investigations should aim to confirm pro-proliferative ECM protein deposition after incubation with eosinophils via immunohistochemistry staining methods.

Although one of the major causes of airway remodeling is known to be hyperplasia and hypertrophy of ASM [5], and eosinophils may promote these processes [21]; however, not enough research has been done to evaluate the effects of ECM components on these processes. It has been discovered that ASM, derived from asthma patients, had increased collagen I, perlecan [32] and fibronectin [33] production. Such changes in the altered matrix could be able to feedback reverse signal to the cells in an autocrine manner within their environment to influence other cellular functions. Considering that the response of lung structural cells to changes in their surrounding ECM components may contribute to airway remodeling in asthma, in this study we focused on investigating potential effect of asthma patients' eosinophils on lung structural cell proliferation via autrocrine ECM adjustment.

Cell proliferation is linked with a diverse set of pathways, which control differentiation, cell quiescence, senescence and responses to a variety of stresses [34]. A proposed primary mechanism to increased ASM mass is ASM cell proliferation, induced by various cytokines, growth factors, inflammatory mediators and allergens [35], and various amount of these mitogens can be found in blood serum. During the study it was found that patients' blood serum addition significantly promoted the proliferation of newly seeded ASM cells compared to control cells. Fully differentiated ASM cells can also produce various cytokines and growth factors such as IL-1β, IL-5, IL-6, GM-CSF, eotaxins, platelet derived growth factor-β, vascular endothelial growth factor into the airways [36] and further promote nearby cell proliferation and migration in a paracrine or autocrine manner. Regarding pulmonary fibroblasts, increased ECM production can be partly explained by the ability of eosinophils to secrete various cytokines, chemokines, and growth factors such as TGF-β, which is an important factor in the differentiation of fibroblasts into myofibroblasts that actively produce ECM proteins [7, 37]. ECM proteins can also bind soluble growth factors and regulate their distribution, activation and presentation to cells [8]. It has been shown that eosinophils contribute to oxidative and nitrosative stress in mild asthma [38]. Eosinophil adhesion to ECM could cause local oxidative stress damage to surrounding ECM network and

release the bound growth factors into the microenvironment, affecting nearby pulmonary structural cells proliferation. Identification of pro-proliferative ECM components and their production-promoting factors may contribute to the reduction of asthma-induced airway remodeling processes.

Previous experiments utilizing commercial ECM components such as collagen and fibronectin, have indicated an increase in the proliferation of airway epithelial cells and lung fibroblasts on these protein matrices in a mouse model [39], but this method imprecisely recreates the human tissue. The location of the functional and structural components that make up the ECM has not been completely investigated; thus it is not yet possible to completely synthesize these matrices under laboratory conditions. Recently, decellularization procedures have become favored substitutes to tissue culture plate coating with ECM components because the aim of the decellularization method is to reduce any adverse effect on the arrangement, biological activity, and mechanical disorder of the remaining ECM after efficiently eliminating all cellular and nuclear materials [13, 23]. A new methodology was utilized in this study based on the feature of adhesive cells ability to produce ECM. The ECM matrices obtained during the experiments maintain crucial structural components, but may lack the related modulatory proteins or soluble components required to promote tissue-specific cellular functions. However, freshly seeded cells partially regenerate missing ECM components [40]. In addition, the NH_4OH used to remove the cell portion in the study may have cytotoxic effects on newly seeded cells, but this effect was reduced by washing the ECM components several times with warm PBS and further incubating tissue culture wells in PBS before seeding new cells.

CONCLUSIONS

Eosinophils demonstrate enhanced adhesion to ECM components rather than to ASM cells or pulmonary fibroblasts, and adhesion further increases in asthma. Pulmonary structural cells promote their proliferation in autocrine manner via released ECM components after incubation with eosinophils in asthma. Based on the results of the study, new ways could be found in the future to block certain eosinophil integrins responsible for adhesion to the ECM components and to reduce the accumulation of eosinophils in the airways, thus inhibiting the development of airway remodeling during asthma. Finally, visualisation and identification of pro-proliferative ECM components and their production-promoting factors may contribute to the reduction of asthma-induced airway remodeling processes.

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