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Eosinophils and activation markers after allergen challenge - a pilot study for three dimensional analysis in the bronchial mucosa

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To the Editor,

The experimental procedure of segmental allergen challenge (SAC) in mild asthmatic subjects is an extremely valuable study tool to investigate mechanisms of bronchial asthma in patients in general and in particular for the role of eosinophils. In this procedure, BAL and bronchial mucosa can be analysed simultaneously after the induction of allergic inflammation. Older studies yielded data on different time points after the challenge with increasing numbers of eosinophils in the BAL¹. There are no data published on increased numbers of eosinophils within the bronchial mucosa 24 hours after SAC. Here, eosinophils were studied in mucosa and airway lumen of mild asthmatics undergoing segmental allergen challenge as described before^{2,3}. Eosinophils and their release products were investigated in thick sections of the bronchial biopsies using advanced three dimensional analysis. Subject data, methods and detailed results are given in the supplement. All subjects showed a clear eosinophilic response in the airway lumen and mucosa 24 hours after the challenge (Figure 1, S1). The data on neutrophils were inconclusive (Figure 1, S2). There was an increase of eosinophils in the mucosa and in the BAL. In the BAL the concentrations of IL-5 and ECP were elevated (supplement). In the mucosa ECP stained volumes were found elevated as well as signs of eosinophil activation and degranulation (Figure 1, B, C). In higher resolution the ECP expression was associated with either cellular structures or small granules or dispersed over a large area beneath the epithelium (Figure 2, A-E). In the presented study not cell associated, free

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ECP positive granules were seen at baseline but only to a small extent. However, after allergen challenge the ECP expressing volumes were massively increased (Figure 1, B, C). These results might suggest that in human tissue eosinophils degranulate after a single allergen challenge and release their inflammatory mediators into the surrounding tissue. This is in contrast to animal models of asthma where eosinophilic degranulation after allergen challenge does not occur extensively ^{4,5}. In the present study, subjects with a strong IL-5 reaction in BAL showed a strong eosinophilic response in the lumen. However, BAL IL-5 levels did not correlate with eosinophil numbers in the mucosa or volume of ECP positive surfaces in the mucosa (Figure S3). It is a long known fact that IL-5 levels in the BAL correlate highly with absolute numbers of eosinophils in the same compartment. In the present study the allergic reaction is comparable to those of other studies. The missing correlation between IL-5 in the BAL and numbers of tissue eosinophils showed that the relationship between both compartments is not as simple as assumed. Here, the data for the volume of ECP positive surfaces may give an important hint. Interestingly, the volume of ECP positive surfaces was inversely correlated with TNF- α and IL-8. TNF- α enhances migration of eosinophils from mucosa to lumen shown in an in vitro cell culture model ⁶. Therefore, one possible explanation is the more TNF- α is found in the BAL the less activated eosinophils are found in the mucosa. In conclusion, the findings in subjects with mild asthma are in alignment with other published results and suggest that 1) human tissue eosinophils release their granules in non-provoked state, and 2) toxic content of these cells is significantly released into the surrounding tissue after a single allergen challenge whereas the distribution and the degree of activation and degranulation of eosinophils differs widely between subjects. Many eosinophils in the airways indicate many eosinophils in the mucosa. Three-dimensional analysis in thick tissue sections using confocal microscopy is a valuable tool in the investigation of bronchial biopsies from patients suffering from bronchial asthma.

(Abbreviations: BAL=bronchoalveolar lavage, ECP=eosinophilic cation protein, IL=interleukin, MBP=major basic protein, NE=neutrophilic elastase, SAC=segmental allergen challenge, TNF=tumor necrosis factor)

References

1. Lommatzsch M, Julius P, Kuepper M, et al. The course of allergen-induced leukocyte infiltration in human and experimental asthma. *J Allergy Clin Immunol*. 2006;118(1):91-97.
2. Erpenbeck VJ, Hagenberg A, Dulkys Y, et al. Natural porcine surfactant augments airway inflammation after allergen challenge in patients with asthma. *Am J Respir Crit Care Med*. 2004;169(5):578-586.
3. Schaumann F, Muller M, Braun A, et al. Endotoxin augments myeloid dendritic cell influx into the airways in patients with allergic asthma. *Am J Respir Crit Care Med*. 2008;177(12):1307-1313.
4. Denzler KL, Borchers MT, Crosby JR, et al. Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J Immunol*. 2001;167(3):1672-1682.
5. Malm-Erfjält M, Persson CG, Erfjält JS. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. *Am J Respir Cell Mol Biol*. 2001;24(3):352-359.
6. Kikuchi I, Kikuchi S, Kobayashi T, et al. Eosinophil trans-basement membrane migration induced by interleukin-8 and neutrophils. *Am J Respir Cell Mol Biol*. 2006;34(6):760-765.

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Figure legends

Figure 1: Displayed representative images show 3D-reconstructions of triple-immunostaining in 100 μm biopsy sections for EG2⁺ surfaces as a marker for eosinophils (red surfaces), NE⁺ surfaces as marker for neutrophils (yellow surfaces) and cytokeratin (green surfaces) at baseline (A) and 24 hrs after segmental allergen challenge (B) of one subject; Settings for image acquiring: x25-water objective; argon laser (488 nm), helium/neon1 laser (543 nm) and helium/neon2 laser (633 nm); 90 optical slices with an interval of 1.1 μm ; grid spacing: 50 μm ; C-D: Depicted are the graphs of the quantification of volume of EG⁺ surfaces [$\mu\text{m}/100000 \mu\text{m}^3$] (C) and volume of NE⁺ surfaces [$\mu\text{m}/100000 \mu\text{m}^3$] (D) before (Baseline) and 24 hrs after segmental allergen challenge (Allergen). Each symbol represents one subject (for symbol legend refer to tab. 1). Per subject one 100 μm biopsy section comprised of 4 to 7 regions was analysed. The groups were compared using Wilcoxon matched-pairs signed rank test; **: $p < 0.01$, number of subjects: C: $n = 10$; D: $n = 10$; AL = Airway lumen; EG2 = antibody used to identify ECP; NE= neutrophil elastase; SAC = segmental allergen challenge.

Figure 2: Images of a granule filled cell positive for EG2 (red) displayed in the fluorescent channel (A), transmitted light channel (B) and as a merge of both channels (C). In the transmitted light channel (B) the cell with its nucleus could be detected: The circle labels the cell and the white disrupted circle the cell nucleus. This technique has been validated in separate experiments as shown in Figure S4 in the Supplement. The EG2⁺ staining within a tissue volume of a 20 μm thickness was shown in D. A 3D-reconstruction of the stack of images from D was calculated and shown in E: The EG2⁺ surfaces which resembled cell associated structures were indicated by stars. EG2 staining in free granules were indicated by circles and unbound EG2 staining as distributed within the tissue was labelled by arrows. F-H: Images show 3D reconstruction of 100 μm biopsy sections of three different subjects 24 hrs after segmental allergen challenge. The distribution of EG2⁺-staining (red surfaces) beneath the epithelial layer (green surfaces) is very diverse between the subjects. Settings for image acquiring A - E: x40-water objective; helium/neon1 laser (543 nm); grid: 10 μm ; scale bar: 10 μm ; F - H: x25-water objective; argon laser (488 nm) and helium/neon1 laser (543 nm); 90 optical slices with an interval of 1.1 μm ; grid spacing: 50 μm ; AL = airway lumen; EG2 = antibody used to identify ECP.

Figures

Figure 1

green: epithelium (cytokeratin) , red: eosinophils (EG2), yellow: neutrophils (NE)

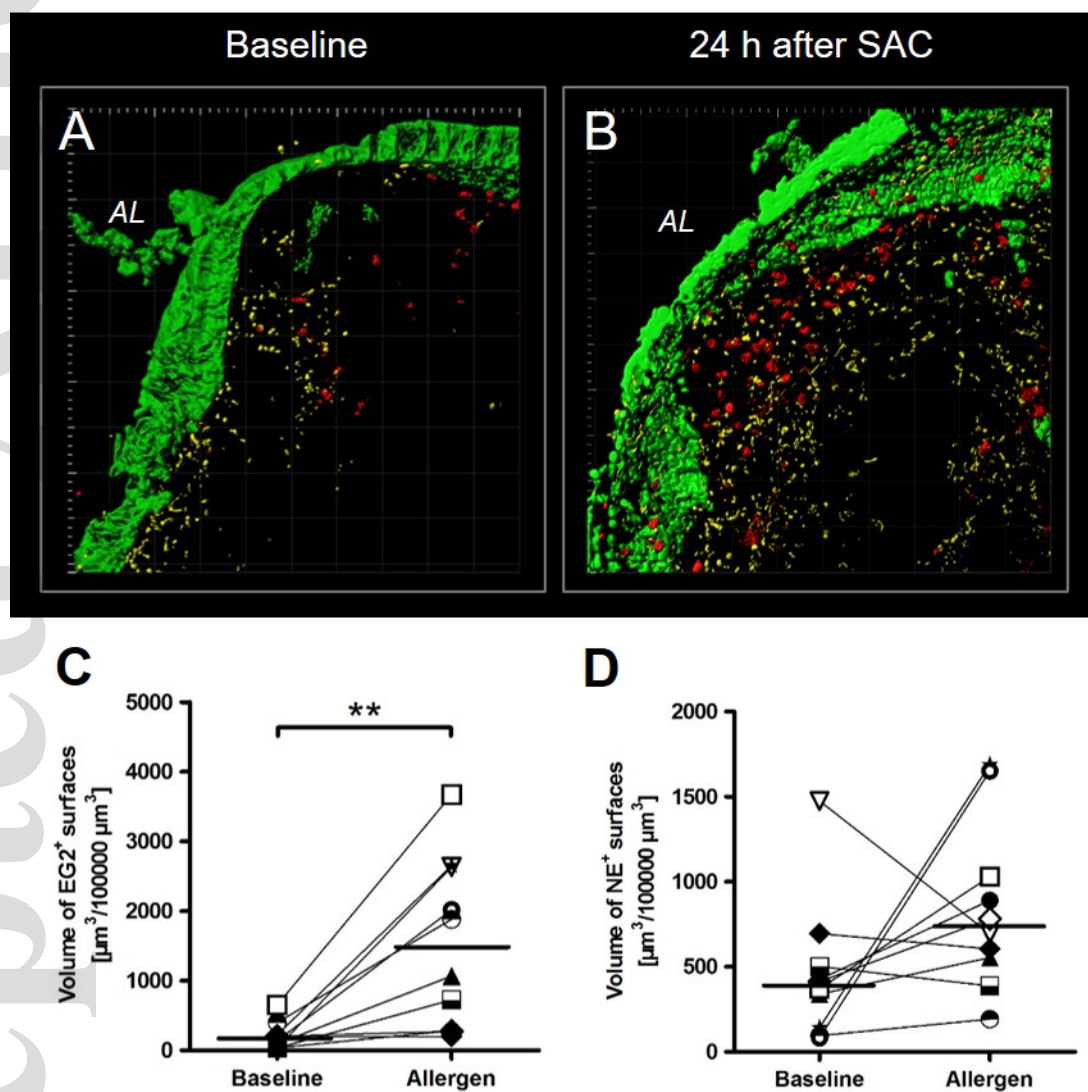
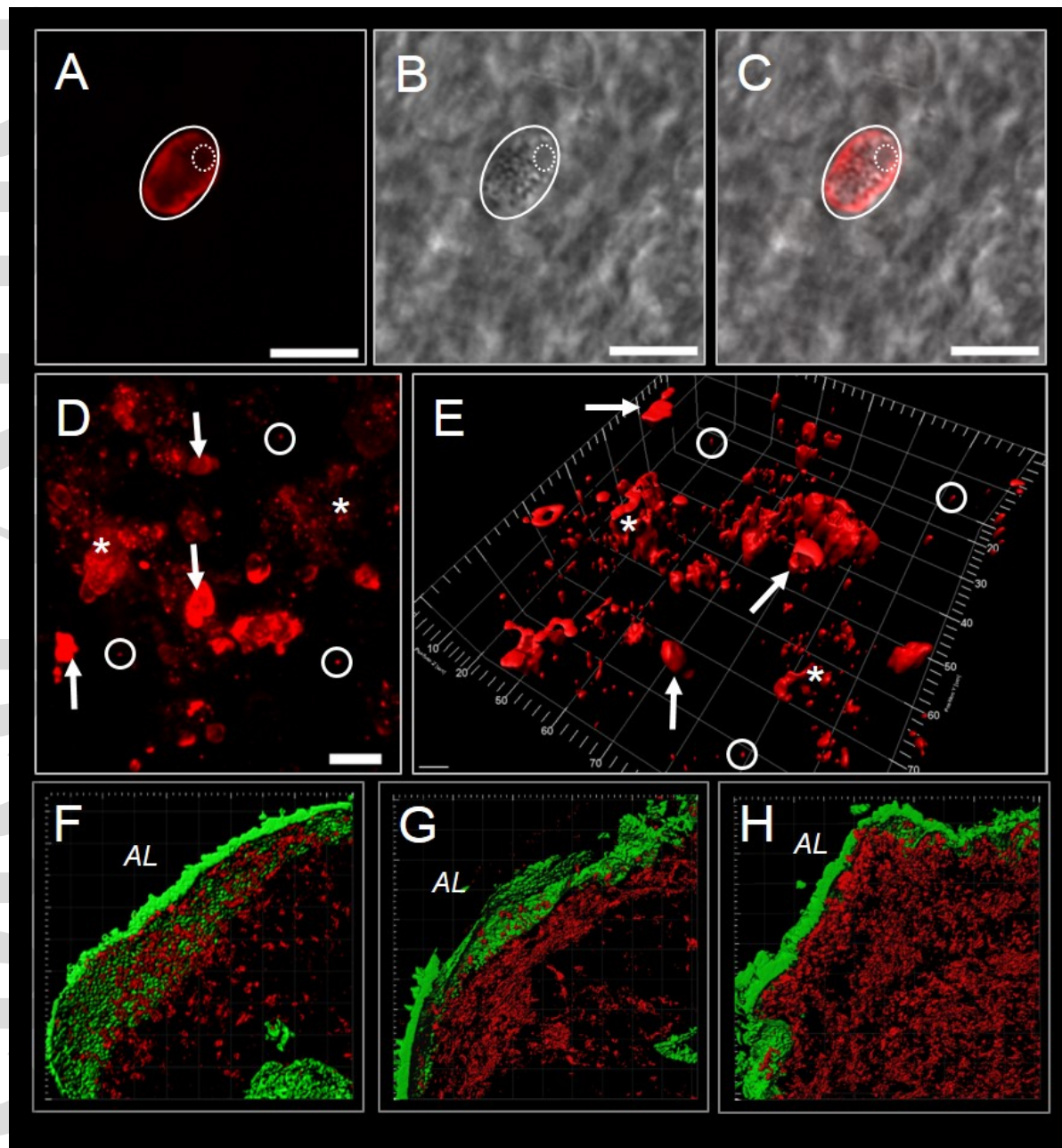


Figure 2

green: epithelium (cytokeratin) , red: eosinophils (EG2)



List of abbreviations:

BAL=bronchoalveolar lavage, ECP=eosinophilic cation protein, IL=interleukin, MBP=major basic protein, NE=neutrophilic elastase, SAC=segmental allergen challenge, TNF=tumor necrosis factor

Materials and methods

Study Subjects

In this study, BAL and bronchial biopsies of asthmatic subjects were analyzed before and after segmental allergen provocation. The study population consisted of 12 non-smoking subjects (33.5 years, range: 23 – 53; 6 females, 6 males) with mild intermittent bronchial asthma. Clinical characteristics of the subjects are displayed in Table S1. The subjects only used β_2 -agonists occasionally. Subjects with a past or present history of allergen immunotherapy were excluded. Each subject showed an AHR to methacholine and a positive airway response to inhaled allergen challenges with grass pollen mix or Dermatophagoides pteronyssinus (both ALK-Scherax, Wedel, Germany) determined as previously described⁵. Two subjects had low serum IgE levels (30 IU/mL and 41 IU/mL) and ten subjects had elevated serum IgE levels (above 100 IU/mL). None of the subjects had infections of the respiratory tract during the last 4 weeks before the study. Females had a negative pregnancy test result before study procedures started. To determine the allergen dose for the segmental instillation, a skin prick test with a dilution series of the allergen extract in saline was performed as described before. The study protocol was approved by the Hannover Medical School ethics committee. Each subject gave written informed consent.

Bronchoscopy, sampling and procedures

The bronchoscopy was performed as described before (Ref. 1, 2 in the Letter). The bronchoscope (BF 160 P; Olympus Optical, Tokyo, Japan) was placed in a segment of the left lower lobe in wedge position for a baseline BAL. Three bronchial biopsies were taken from the lower lobes. Next, 10 mL saline solution was instilled into a segment of the right upper lobe bronchus as a control challenge. Allergen solution was instilled into three segments of the middle lobe and lingular bronchus, respectively. After 24 hours a second bronchoscopy was performed and BAL as well as one mucosal biopsy were collected from each challenged segment. Total nucleated cell count was determined using a Neubauer hemocytometer and differential cell counts were done after staining of cytospin slides by counting 400 cells per slide. Levels of the cytokines IL-5, IL-8 and tumor necrosis factor (TNF)- α were determined in BAL at baseline and after segmental challenge with saline and allergen. Cytokines were quantified using Meso Scale Discovery (MSD, Gaithersburg, USA) technology, which is based on the detection of electrochemiluminescence. The assay was performed according to the manufacturer's instructions. Data analysis was performed using the discovery workbench software. The measurement of ECP with the UniCap 100 kit (Phadia) was performed according to the manufacturer's instruction.

Immunohistochemical detection of immune cells in paraffin sections

Biopsies were fixed in formalin, dehydrated with an alcohol series and embedded in paraffin. For immunohistochemical detection in bronchial biopsies, 3 μ m thin paraffin sections were prepared. The paraffin sections were stained with primary antibodies against major basic protein (MBP) (clone BMK13; Dunn Labortechnik GmbH), Cytokeratin Type I&II

(clone AE1/AE3; PROGEN Biotechnik GmbH, Heidelberg, Germany) and neutrophil elastase (polyclonal; Abcam; Cambridge, UK). Antigen retrieval was performed for MBP and cytokeratin. Slides were pre-incubated in normal goat serum (Vector Laboratories Inc., CA, USA) and then incubated with the primary antibody for 1 h at room temperature (RT). As secondary antibodies a biotin-SP-conjugated AffiniPure goat-anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch, PA, USA) or a peroxidase-conjugated AffiniPure goat-anti-mouse IgG, Fc γ subclass 1 specific (Jackson ImmunoResearch, PA, USA) were applied for 30 minutes incubation time at RT. A step-wise triple-labelling was done first performing an immunoreaction with the chromogen Histo Red, secondly with diaminobenzidine (DAB) and as a last step an immune reaction with Histo Green. The slides were finally counterstained with Mayer's hematoxylin (Linaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany). Coverslipping was performed using Eukitt quick hardening mounting medium (Fluka, Sigma-Aldrich, Steinheim, Germany).

Three-dimensional analysis

Biopsies were fixed with 2 % paraformaldehyde for several hours and washed in PBS overnight. They were embedded in low gelling agarose (Sigma-Aldrich Chemie GmbH, Munich, Germany) and cut into 100 μ m sections with a vibratome. After permeabilization with 0.3 % Triton X-100, blocking was performed with 5 % donkey serum. The 100 μ m sections were stained in three overnight incubation steps. Biopsy sections were incubated at 4 °C overnight with 1 μ g/mL mouse anti-human ECP antibody (clone EG2, Diagnostics Development; Uppsala, Sweden) combined with 0.2 μ g/mL rabbit anti-human neutrophil elastase (NE) (polyclonal, Abcam, Cambridge, UK). After several washing steps with 0.05% Tween/PBS, the sections were blocked again with 5 % donkey serum and subsequently incubated at 4°C overnight with 1.75 μ g/mL Cy3-conjugated F(ab')₂-fragments of donkey anti-mouse IgG (Dianova, Hamburg, Germany) and 1.75 μ g/mL Cy5-conjugated F(ab')₂-fragments of donkey anti-rabbit IgG (Dianova, Hamburg, Germany). The epithelium was stained in a third staining step with a mouse anti-human cytokeratin antibody (Dako Deutschland GmbH, Hamburg Germany) overnight at 4°C which was labelled beforehand with Zenon-fragments according to the manufacturer's instructions (Thermo Fisher Scientific, Hamburg, Germany). The appropriate isotypes were used as a control of staining specificity. The tissue sections were mounted with ProLong® Gold anti-fade (Invitrogen, Hamburg, Germany) to avoid bleaching of the fluorescent dyes. Staining of the markers was analysed by confocal laser microscopy (25x water immersion objective, 488 nm, and emission filter BP 505 – 550 nm, and 543 nm, emission filter 560 - 600 nm, thickness 100 μ m). The 3D-stacks were quantitatively analysed with IMARIS 6.2.1 (Bitplane AG, Zurich, Switzerland). Image analysis of the immunohistochemically stained slides was performed using images of the slides scanned with a Zeiss MIRAX®. Using the MIRAX® viewing software at 40-fold magnification, cells positively labelled were counted manually on a monitor showing the images in a format of 27.5 x 38.5 cm, resulting in evaluation of 8-12 screens for each biopsy slide. Images of complete biopsies slides were transferred to the ZEISS Axiovision® system and the area was located in every image including as much biopsy tissue as possible. The analysed tissue areas were calculated by the software. The number of positive cells was related to the area of the biopsy section. Three-dimensional fluorescence stack images of the slices of the biopsies were quantitatively analysed with IMARIS 6.2.1 software. Confocal data sets were processed via "surface rendering", which allowed an assessment of the volume of the EG2⁺ and NE⁺-staining. The volume of the EG2⁺ and NE⁺-surfaces was related to the

1 volume of total tissue which was detected by its autofluorescence. Subsequently, the volumes
2 of the EG2⁺- and NE⁺-surfaces were given as $\mu\text{m}^3/100000\mu\text{m}^3$ volume of total tissue.
3 Thresholds levels for the calculation of "surface objects" were set once for each channel and
4 the same thresholds were used for all datasets.

5 **Statistical analysis**

6 Statistical analysis was performed by Wilcoxon matched-pairs signed ranked test. Correlation
7 were performed using the correlation analysis according to Spearman (Software: GraphPad
8 Prism 4, version 4.03). Differences between pre/post allergen were considered statistically
9 significant at the level of $P < 0.05$.

10

1 **Table S1: Clinical characteristics of the study subjects**

Subject	Age (y)	Sex	FEV ₁ (% predicted)	PC ₂₀ FEV ₁ Methacholine (mg/mL)	PC ₂₀ FEV ₁ allergen (BU)	IgE (IU/mL)	Allergen sensitivity*	Allergen Provocation† (S-QU)	Representing Symbols in Graphics
1	32	F	102	6.9	309	267	gp, Da, ti, Ry, efp, Mu, Ri, Ra, d	63	▽
2	32	F	878	0.04	162	266	gp, Da, ti, ry, tp, mu, ri, d, c	1000	★
3	45	M	115	16	574	118	gp, Da, ti, ry, tp, mu, ri, ra, Df, d, c, Al	63	▼
4	41	M	96	16	1765	852	gp, Da, ti, ry, efp, mu, ri, ra, d, c, Al	63	◯
5	37	F	86	1.7	54	161	gp, Da, ti, efp, Mu, Ra, Dp, Df	125	◆
6	31	M	84	0.5	406	300	gp, Da, ti, Ry, tp, Mu, Ri, Ra, d, c,	8	◇
7	53	M	115	11.1	1269	541	gp, Da, ti, Ry, Ri, Ra, Mu, tp, Dp,	31	■
8	42	F	120	0.4	111	154	gp, Da, ti, Ry, Ri, Dp, Df, c, h	250	▲
9	23	M	107	16	436	351	gp, Da, ti, ry, efp, mu, Ri, Ra, d, c, Al	125	◼
10	28	F	108	0.9	130	462	gp, Da, ti, ry, efp, Ri, Dp, Df, d, c,	63	◐
11	35	F	111	1.3	820	41	gp, Da, ti, Ry,	125	◻
12	23	M	92	1.5	375	30	Dp, c	500	●
Median	33.5	6F / 6M	104.5	1.6	390.1	266.5	-	125	-
Range	23 - 53	-	84 - 120.3	0.04 - 16	54.1 - 1765	30 - 852	-	7.8 - 1000	-

Dp = Dermatophagoides pteronyssinus; gp = mixed grass pollen; ry = rye pollen; tp = mixed tree pollen; mu = mugwort pollen; ri = ribwort pollen; Df = D farinae; d = dog hair; c = cat fur; f = mixed feather; h = horse; ra = ragweed pollen; efp = early flowering plants; ti = timothy grass pollen; fu = fungi II; Al = Alternaria alternate; Cl = Cladosporium herbarum. Bold allergen abbreviation indicates the used allergen for segmental challenge. *Allergen sensitivity was determined by means of skin prick testing. †Allergen challenge was performed with either grass pollen mix or D. pteronyssinus mix in the indicated amount diluted in 10 mL of saline solution.

Detailed results

Induced allergic airway inflammation – BAL

The emerging allergic airway inflammation was characterized by increased total cell numbers (median from 3 allergen challenged segments) in comparison to saline control (saline 4.7×10^6 cells/mL, range: 1.0 to 7.5 vs. allergen 23.6×10^6 cells/mL, range: 3.3 to 75.0). Subject 7 was excluded due to a high percentage of neutrophils in the baseline BAL indicating an ongoing inflammation of unknown aetiology.

Eosinophil numbers increased (allergen 14.6×10^6 cells/mL, range: 0.2 to 55.9) when compared to saline (0.0×10^6 cells/mL, range: 0.0 to 0.3) (Figure S1A). However, neutrophil numbers as a marker of unspecific inflammation were also significantly induced after saline instillation (0.2×10^6 cells/mL, range: 0.0 to 1.2) as well as after allergen instillation (1.2×10^6 cells/mL, range: 0.2 to 5.5) in comparison to baseline (0.0×10^6 cells/mL, range: 0.0 to 0.2) (Figure S1B). Total numbers of lymphocytes and macrophages were elevated after allergen challenge compared to baseline (Figure S1CD). One subject was excluded from the study due to a high percentage of neutrophils in the baseline BAL indicating an ongoing inflammation of unknown aetiology.

For further characterization of inflammation cytokine levels in the BAL were measured. The cellular influx in the allergen challenged segment was accompanied by enhanced levels of IL-5 (177 pg/mL, range: 56 to 1511) compared to baseline (0.0 pg/mL, range: 0.0 to 21) and saline (15 pg/mL, range: 0.0 to 38). ECP levels increased significantly from 1.1 µg/mL (range: 0.3 to 3.1) at baseline to 40.5 µg/mL (range: 8.3 to 59.4) after allergen challenge. Between saline control and allergen control the induction was 8-fold (saline: 5.2 µg/mL range: 0.4 to 13.9).

Induced eosinophil recruitment – airway mucosa

Representative images of bronchial biopsies at baseline and after allergen challenge are displayed in Figure 2. Only few MBP⁺ cells could be detected at baseline in the bronchial tissue (Figure S2A). After allergen challenge, increasing numbers of MBP⁺ cells were found in the mucosa (Figure S2B). In addition, MBP staining was distributed in the tissue reflecting MBP release by eosinophils. NE⁺ cells are increased after allergen challenge as well and show signs of NE release compared to NE⁺ cells baseline section. The quantification of eosinophil infiltration of the tissue revealed an increase in numbers of MBP⁺ cells after allergen challenge when compared to baseline and saline (Figure S2C). A significant increase in NE⁺ cells was only seen after saline challenge compared to baseline and allergen challenge (Figure S2D).

Extracellular location of EG2⁺-staining indicates degranulated eosinophils to different degrees between subjects

Eosinophilic degranulation was seen in most asthmatic subjects after segmental allergen provocation, identified as free EG2⁺-granules distributed throughout the subepithelial region. Microscopic images of non-degranulated and degranulated eosinophils are displayed in Figure 2. Using transmitted light, the granules were visible within the cell indicated with the white circle (A-C). These granules overlapped with EG2⁺ staining as shown in C. In case of degranulation the EG2⁺ staining could not be assigned to specific cells in most of the cases (D). The projection (D) and the three-dimensional reconstruction (E) of a confocal stack image revealed the extension of the cationic proteins release. EG2⁺ staining is located within the cells (*) and granules (circles) as well as distributed freely (arrows). After segmental allergen provocation EG2⁺ staining pattern varied between asthmatic subjects in 100 µm biopsy (Figure 2, F-H).

Increase in volume of EG2⁺ surfaces after challenge

Quantification of the volume of EG2⁺ surfaces showed a significant increase one day after challenge compared to baseline (Fig. 1ABC). The volume of NE⁺ surfaces was not significantly influenced (Figure 1D).

Inverse correlation between IL-8 and TNF- α with volume of EG2⁺ surfaces

Eosinophil numbers in the BAL and in biopsy sections were increased after segmental allergen provocation without significant correlation. The volume of EG2⁺ surfaces did not correlate with either cell numbers. Levels of IL-5 measured in BALF did not correlate with either BMK-13⁺ cells (Figure S3A) or volume of EG2⁺ surfaces (Figure S3B) in biopsy sections of asthmatic subjects 24 hrs after challenge. The volume of EG2⁺ cells was inverse correlated with levels of TNF- α (RS = -0.89, P = 0.001) and IL-8 (RS = -0.75, P = 0.02) (Figure S3C and 5D).

Figure legends

Figure S1: Absolute numbers of eosinophils (A), neutrophils (B), lymphocytes (C) and macrophages (D) in the bronchoalveolar lavage before and after challenge with saline or allergen, respectively. Symbols represent different subjects; Statistical test: The groups were compared using Wilcoxon matched-pairs signed rank test; * = P $\leq$ 0.05; ** = P $\leq$ 0.01; number of subjects: n=10.

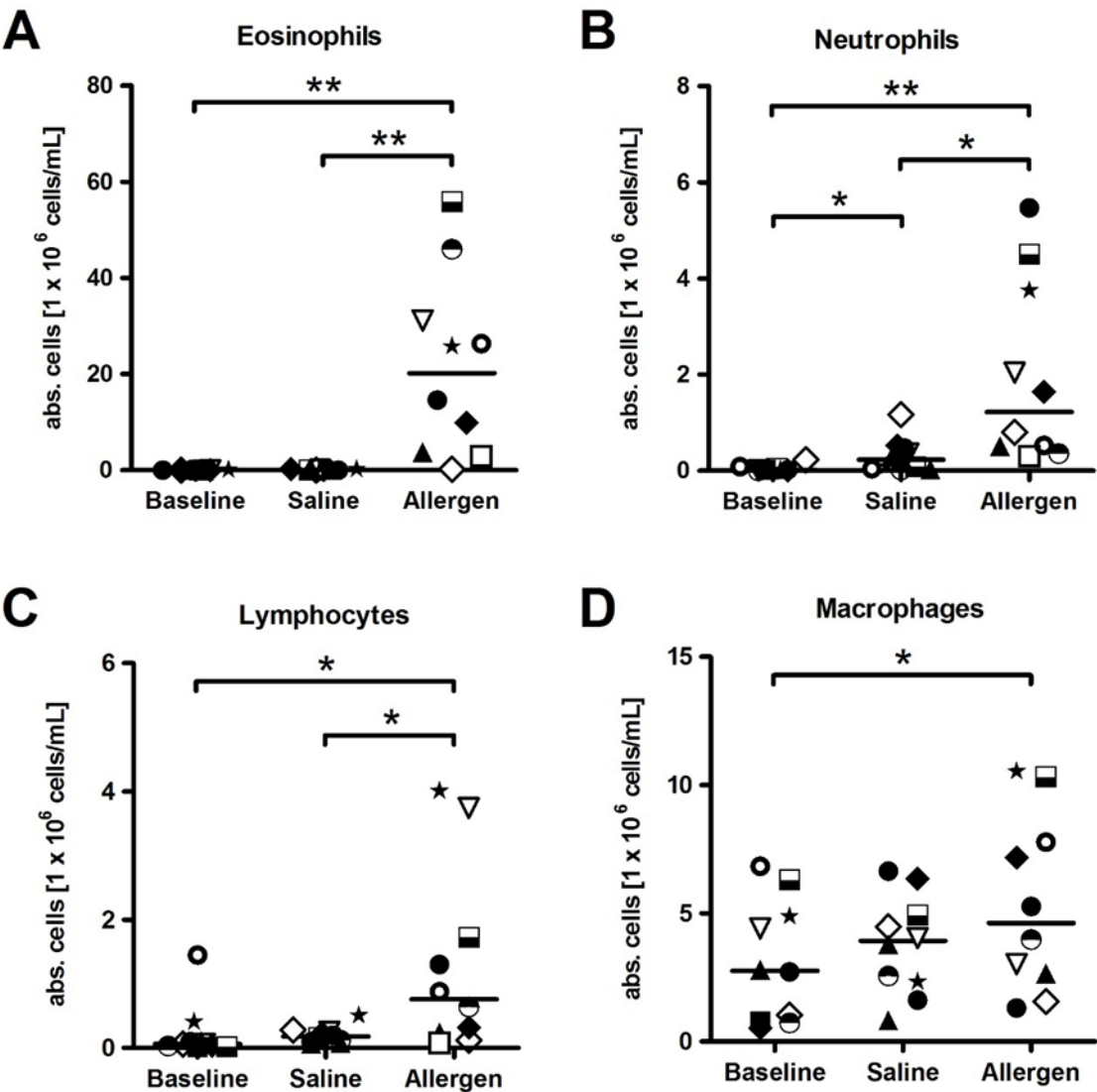
Figure S2: Images represent triple-staining against major basic protein (BMK-13, brown), neutrophil elastase (NE, red) and cytokeratin (green) in 3 μ m biopsy sections before (A) and 24 hrs after segmental allergen provocation (B). The cell nuclei were counterstained with Mayer's haematoxylin (blue). Magnifications of these stains were used to quantify the number of stained BMK-13⁺ and NE⁺ cells in biopsy sections. The images were acquired using a Zeiss® Miramax Viewer, scale bar: 100 μ m. C, D: The depicted graphs show the quantification of the BMK-13⁺ (C) and NE⁺ cells (D) per area in paraffin sections before (Baseline) and 24 hrs after challenge with saline or allergen. One or two biopsy sections were evaluated for each asthmatic subject. Per section 8 to 12 FOV were evaluated. The table beneath gives the medians and ranges for each analysed group. The groups were compared using Wilcoxon matched-pairs signed rank test, (baseline: *: P < 0.05; **: P < 0.01; saline: #: P < 0.05; ##: P < 0.01), number of subjects: C: n = 10; D n = 9; BMK-13 = antibody detects major basic protein (MBP); FOV = field of view; NE = neutrophil elastase; SAC = segmental allergen challenge.

Figure S3: The displayed graphs show correlations of IL-5-levels (A and B), IL-8 (C) and TNF- α levels (D) with BMK-13⁺ cells (A) and volume of EG2⁺ surfaces (B, C, D) 24 hrs after segmental allergen provocation. The coefficient of Spearman (RS) for parametric correlations was calculated for each pair. Symbols represent values for one subject. Number of subjects: A: n = 8; B-D: n = 9. IL = interleukin; MBP = major basic protein, ECP = eosinophil cationic protein; TNF- α = tumour necrosis factor alpha; Rs = correlation coefficient of Spearman.

Figure S4: These two images show an eosinophil granulocyte stained with EG2 antibody (red) and counterstained with the nuclei stain To-Pro 3 (blue) as a single confocal plane (A) and a 3D-reconstruction of a stack image in human endobronchial biopsies of an allergic asthmatic subject. The bilobed-shaped nucleus (blue) can be clearly identified within the

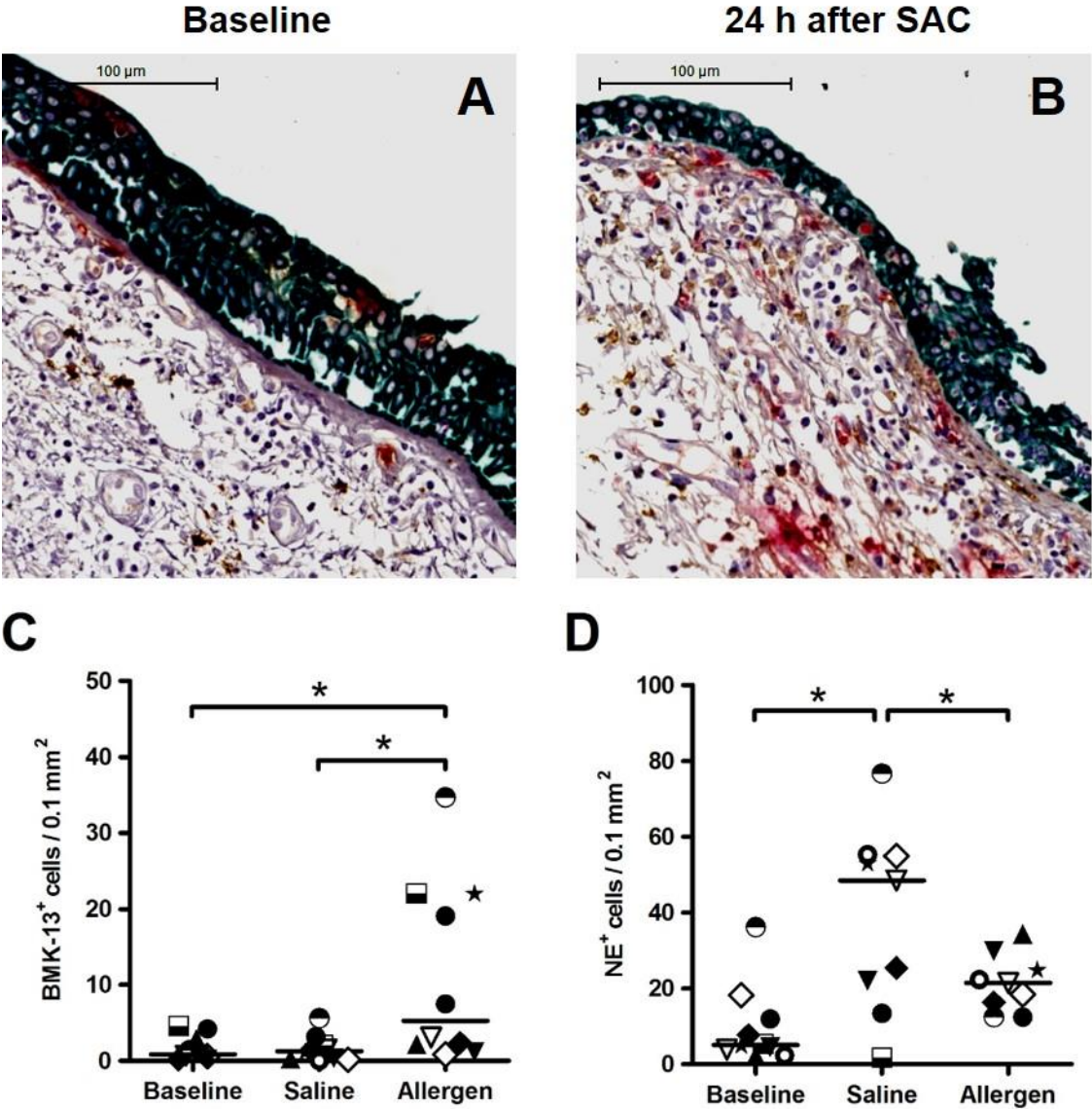
- 1 EG2-stained cytoplasm (red) in both images. Scale: 10 μm (A) and 5 μm (B); EG2 = antibody
- 2 against eosinophil cationic protein (ECP); 3D = three-dimensional.
- 3

- 1 Figures
- 2 Figure S1



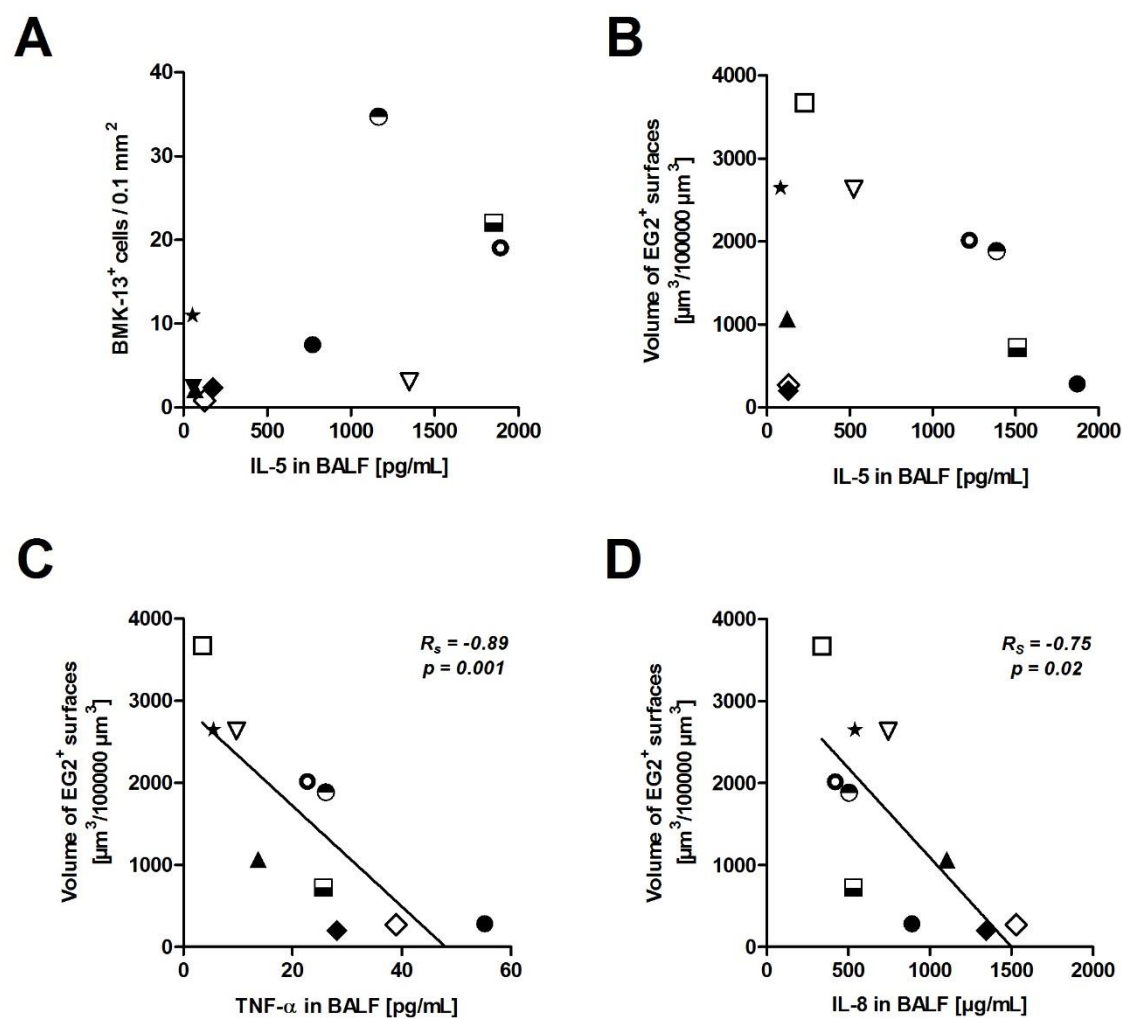
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1 **Figure S2**

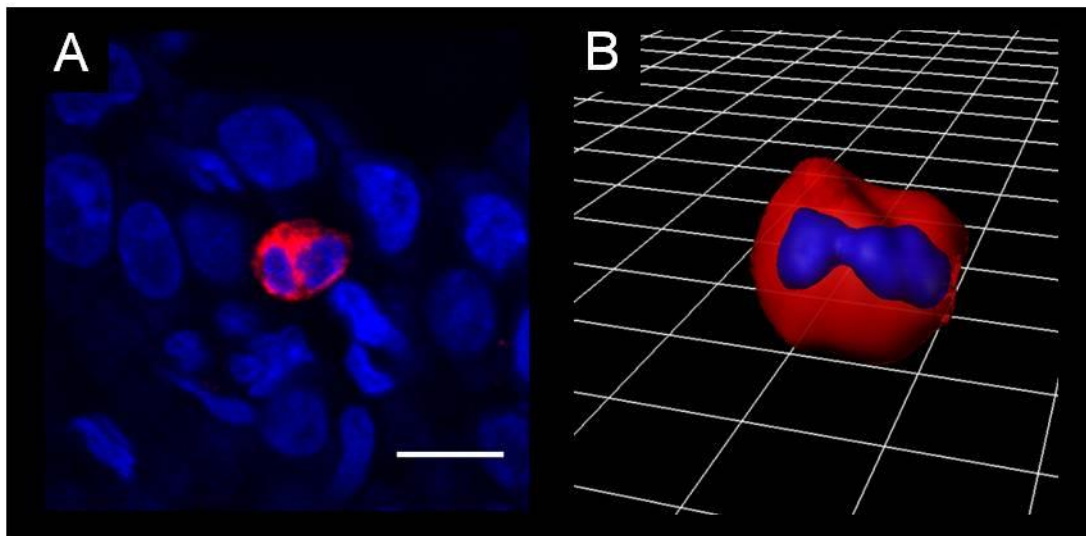


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1 **Figure S3**



1 **Figure S4**



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