

Supplement

COL4A3 is degraded in allergic asthma and degradation predicts response to anti-IgE therapy

Markus Weckmann, PhD¹, Thomas Bahmer, MD², Jannie Marie Bülow Sand, PhD³, Sarah Rank Rønnow, MSc^{3,4}, Martin Pech, PhD¹, Cornelis Vermeulen, PhD⁵, Alen Faiz, PhD^{5,6,7,8}, Diana Julie Leeming, PhD³, Morten Asser Karsdal, PhD⁴, Lars Lunding, PhD⁹, Brian George G. Oliver, PhD^{10,11}, Michael Wegmann, PhD⁹, Gudrun Ulrich-Merzenich, PhD¹², Uwe R Juergens, MD¹³, Jannis Duhn¹⁴, Yves Laumonier, PhD¹⁴, Olga Danov, PhD¹⁵, Katherina Sewald, PhD¹⁵, Ulrich Zissler, PhD¹⁶, Marnix Jonker, MS^{5,6}, Inke König, PhD¹⁷, Gesine Hansen, MD¹⁸, Erika von Mutius, MD, MSc¹⁹, Oliver Fuchs, MD, PhD^{20,1}, Anna-Maria Dittrich, MD¹⁸, Bianca Schaub, MD¹⁹, Christine Happle, MD, PhD¹⁸, Klaus F. Rabe, MD, PhD², Maarten van de Berge, MD⁵, Janette Kay Burgess, PhD^{6,7,21}, Matthias Volkmar Kopp, MD^{1,20} and the ALLIANCE Study Group as part of the German Centre for Lung Research (DZL)

1 Division of Pediatric Pneumology & Allergology, University Medical Center Schleswig-Holstein, Lübeck, Germany; Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Germany

2 Department of Pneumology, LungenClinic Grosshansdorf, Grosshansdorf, Germany; Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Germany

3 Nordic Bioscience A/S, Herlev, Denmark

4 University of Southern Denmark, The Faculty of Health Science, Odense, Denmark

5 University of Groningen, University Medical Center Groningen, Department of Pulmonary Diseases, Groningen, GRIAC (Groningen Research Institute for Asthma and COPD), The Netherlands

6 University of Groningen, University Medical Center Groningen, Department of Pathology & Medical Biology, Groningen, GRIAC (Groningen Research Institute for Asthma and COPD), The Netherlands

7 Woolcock Institute of Medical Research, The University of Sydney, Glebe, NSW, Australia

8 School of Medical and Molecular Biosciences, University of Technology Sydney NSW 2007 Australia

9 Division of Asthma-Exacerbation & -Regulation; Program Area Asthma & Allergy, Leibniz-Center for Medicine and Biosciences Borstel; Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Germany

10 School of Medical and Molecular Biosciences, University of Technology Sydney NSW 2007 Australia

11 Woolcock Institute of Medical Research, The University of Sydney, Glebe, NSW, Australia

12 AG Synergyresearch and Experimental Medicine, Medical Clinic III, University Hospital Bonn

13 Department of Pneumology, Medical Clinic II, University Hospital Bonn

14 Institute for Systemic Inflammation Research, University of Lübeck, Lübeck 23562, Germany

15 Fraunhofer Institute for Toxicology and Experimental Medicine ITEM, Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research (DZL), Nikolai-Fuchs-Strasse 1, 30625 Hannover, Germany

16 Center of Allergy and Environment (ZAUM), Technical University of Munich and Helmholtz Center Munich, German Research Center for Environmental Health (CPC-M), Munich, Member of the German Center of Lung Research (DZL), Germany

17 Institute for Medical Biometry and Statistics, University of Lübeck, Lübeck, Germany; Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Germany

18 Department of Pediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany; Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center of Lung Research (DZL), Germany

19 University Children's Hospital, Ludwig Maximilian's University, Munich, Germany; German Research Center for Environmental Health (CPC-M), Munich, Germany, Member of the German Center of Lung Research (DZL)

20 Department of Paediatric Respiratory Medicine, Inselspital, University Children's Hospital of Bern, University of Bern, Bern, Switzerland

21 Discipline of Pharmacology, Faculty of Medicine, The University of Sydney, NSW, Australia

Corresponding Author

Dr. Markus Weckmann

Division of Pediatric Pneumology & Allergology, University Medical Center Schleswig-Holstein, Campus Centrum Lübeck, Member of Airway Research Center North (ARCN) of the German Center of Lung Research (DZL), Ratzeburger Allee 160, 23538 Lübeck, Germany; T: 0049-451-500-50858; F: 0049-451-500-42814

E: markus.weckmann@uksh.de

Members of the ALLIANCE Study Group:

Barbara Roesler a, MD; Nils Welcherling a, MD; Naschla Kohistani-Greif a, MD; Katja Landgraf-Rauf a, PhD; Kristina Laubhahn a, MSc; Bianca Schaub a, MD; Markus Ege a, MD; Claudia Liebl, MSc a; Erika von Mutius a,c, MD MSc; Johanna Kurz a, b, MSc; Oliver Fuchs a,b,d, MD PhD; Isabell Ricklefs d, MD; Gesa Diekmann d, MD; Laila Sultansei d, MD; Markus Weckmann d, PhD; Gyde Nissen d, Matthias V Kopp d, MD; MD; Lena Liboschik d, MD; Xenia Bovermann d, MD; Alena Steinmetz d, MD; Gesche Voigt d, MD; Inke R. König e, PhD; Dominik Thiele e, MSc; Folke Brinkmann f, g, MD; Anna-Maria Dittrich f, MD; Christine Happle f, MD; Aydin Malik f, MD; Nicolaus Schwerk f, MD; Christian Dopfer f, MD; Mareike Price f, MD; Ruth Grychtol f, MD; Gesine Hansen f, MD; Michael Zemlin h,i, MD; Matthias Müller j, MD; Ernst Rietschel j, MD; Silke van Koningsbruggen-Rietschel j, MD; Thomas Bahmer k, MD; Anne-Marie Kirsten l, MD; Frauke Pedersen l, PhD; Henrik Watz k, MD; Benjamin Waschki k, MD; Klaus F. Rabe k, MD PhD; Christian Herzmann m, MD; Annika Opitz, MD m; Karoline I. Gaede m, PhD; Peter Zabel m, MD

a Department of Paediatric Allergology, Dr. von Hauner Children's Hospital, Ludwig Maximilians University, Munich, Germany, and Comprehensive Pneumology Center, Munich (CPC-M), Germany; Member of the German Center for Lung Research (DZL). b Department of Paediatric Respiratory Medicine, Inselspital, University Children's Hospital of Bern, University of Bern, Bern, Switzerland. c Institut für Asthma- und Allergieprävention (IAP), Helmholtz Zentrum Munich, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Munich, Germany. d University Children's Hospital, Luebeck, Germany, and Airway Research Center North (ARCN), Germany; Member of the German Center for Lung

Research (DZL). e Institute for Medical Biometry and Statistics, University Luebeck, University Medical Center Schleswig-Holstein, Campus Luebeck, Germany, and Airway Research Center North (ARCN), Germany; Member of the German Center for Lung Research (DZL). f Department of Paediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany, and Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Germany; Member of the German Center for Lung Research (DZL). g Department of Paediatric Pneumology, University Children's Hospital, Ruhr-University Bochum, Bochum, Germany. h University Children's Hospital Marburg, University of Marburg, Germany, and University of Giessen Marburg Lung Centre (UGMLC); Member of the German Center for Lung Research. i Department of General Pediatrics and Neonatology, Saarland University Medical School, Homburg, Germany. j Department of Paediatric Allergology and Pneumology, University Children's Hospital Cologne, University of Cologne, Germany. k LungenClinic Grosshansdorf GmbH, Grosshansdorf, Germany, and Airway Research Center North (ARCN), Germany; Member of the German Center for Lung Research (DZL). l Pulmonary Research Institute at LungenClinic Grosshansdorf, Grosshansdorf, Germany, and Airway Research Center North (ARCN), Germany; Member of the German Center for Lung Research (DZL). m Research Center Borstel – Medical Clinic, Borstel, Germany, and Airway Research Center North (ARCN), Germany; Member of the German Center for Lung Research (DZL).

Supplement methods:

Animal Experiments

Animal Treatment Protocol

HDM model:

Female 8-12 weeks old WT B57BL/6 mice were sensitized by i.p. injection of 10 µg HDM extract from *Dermatophagoides pteronyssinus* (Greer Laboratories Inc., Lenoir USA, Lot-#: 262538) in 100 µl PBS on day 0 and 7. This was followed by an intra-tracheal (i.t.) allergen challenge with 100 µg HDM in 50 µl PBS on day 14 and 21.¹ Mice were sacrificed 72 hours after the last allergen challenge for blood sampling and organ removal. The study was approved by the Schleswig-Holstein state authorities (V 242 - 24572/2018 (44-5/18)).

Recombinant human Chymase treatment

Balb/C mice were given 10µg recombinant human mast cell chymase (active, Sigma Aldrich, Germany) in 50µL PBS and sacrificed 24h later..

Precision-cut-lung-slices

Human lung slices

Human lung lobes were obtained from patients who underwent lobectomy for lung cancer. Experiments were approved by the ethics committee of the Medical School Hannover (MHH, Hannover, Germany) and are in accordance with The Code of Ethics of the World Medical Association (number 2701-2015). Human PCLS were sliced into approx. 300 µm thick lung sections as described before.² After PCLS preparation, two PCLS per well were incubated overnight with either 1% allergic plasma or culture medium. Allergic plasma was removed the next day and replaced by culture medium for 24h. Then collected supernatant was stored at -80°C for C4Ma3 analysis.

Mouse lung slices

Balb/c mice were sensitized by intranasal application of 25µg house-dust mite (HDM) in 50 µL of PBS (control group received PBS only) for four days per week, over four weeks, and used 24h after the last challenge for the preparation of precision-cut lung slices. Lungs from HDM-sensitized or PBS-treated mice were inflated using 1.5% agarose/medium solution and polymerized on ice. Slices of 350 µm were cut in 4°C cold EBSS, using an automatic oscillating tissue slicer (OTS 5000, Warner Instruments, CT, USA) and transferred into a medium filled petri dish. The medium was exchanged at least four times every 30 min for 2–3h to remove cell debris under cell culture conditions (37°C, 5% CO₂, and 100 % humidity). Two PCLS per well in duplicates were cultured in medium or stimulated

with 100 µg/mL polyI:C for 48h, supernatants of duplicates were pooled and stored at -80°C for C4Ma3 analysis.

Bronchoalveolar Lavage

Lungs were flushed with 1 ml of sterile ice-cold PBS containing protease inhibitor (Complete, Roche, Basel, Switzerland) via a tracheal canula, and obtained cells were counted using a Countess automated cell counter (Life Technologies, Darmstadt, Germany). 50 µl-aliquots of lavage fluids were cytospun, stained with Diff-Quick (DADE Diagnostics, Unterschleissheim, Germany) and cells were differentiated microscopically according to morphologic criteria.

Determination of Airway Responsiveness

24 h after the last OVA challenge airway responsiveness was assessed by performing a methacholine (MCh, acetyl-β-methyl choline, Sigma, St. Louis, MO, USA) provocation test, while central airway resistance was recorded using a Buxco FinePoint R/C system (DSI-Buxco Electronics, Sharon, CT, USA). Briefly, mice were anaesthetized with ketamin and xylazin and neuromuscular activity was blocked with pancuronium bromide (1 mg/kg; Sigma). Tracheostomized mice were ventilated mechanically through a tracheal canula that was attached to the FinePoint R/C system. Airflow and transpulmonary pressure were recorded continuously to calculate lung resistance (RL) (cm H₂O/mL/s) and dynamic lung compliance (mL/cm H₂O) in each breath cycle. Mice were allowed to stabilize for 5 min before MCh provocation testing with increasing concentrations of MCh (3.125, 3.25, 12.5, 25, 50, and 100 mg/mL) aerosolized for 5 min each. Baseline pulmonary parameters were assessed with aerosolized phosphate-buffered saline (PBS). Expressed results comprised the mean absolute values of the responses of lung resistance recorded during 5 min after the inhalation of each MCh aerosol. Mice received terbutaline (10 µg/kg; terbutaline-hemisulfate, Sigma) intravenously to solve MCh-induced broncho-spasm prior BAL and preparation of the lung.

Lung Histology

Lungs were fixed *ex situ* with 4% (wt/vol) paraformaldehyde (PFA) via the trachea under constant pressure, removed and stored in 4% PFA. Fixed lung tissues were embedded in paraffin. Subsequently, 2 µm tissue sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS), respectively. Photomicrographs were recorded by a digital camera (DP-25, Olympus, Tokyo, Japan) attached to a microscope (BX-51, Olympus) at 40- and 100-fold magnification using Olympus cell[^]A software. For mucus quantification, systematic uniform random samples of the lungs were prepared according to standard methods including the orientator technique. The surface area of mucin-containing goblet cells (Sgc) per total surface area of airway epithelial basal membrane (Sep) and the

volume of PAS-stained epithelial mucin (V_{mucin}) per Sep were determined using a computer-assisted stereology tool box (newCAST, Visiopharm, Hoersholm, DK) according to the following formulas:

$$\frac{S_{gc}}{S_{ep}} = \frac{\Sigma I_{gc}}{\Sigma I_{ep}} \text{ and } \frac{V_{mucin}}{S_{ep}} = LP \cdot \Sigma \frac{P_{mucin}}{2} \cdot \Sigma I_{ep}$$

where ΣI_{gc} is the sum of intersections of test-lines with goblet cells, ΣI_{ep} is the sum of all intersections of test-lines with the epithelial basal membrane, ΣP_{mucin} is the sum of all points hitting mucin and LP is the test-line length at final magnification.

Immunohistochemistry

Paraffin embedded mouse lungs were cut at 4 μ m. Antibody against MMP9 (1/200) (Abcam (ab38898)), antibody against mMCPT4 (1/200) (Biomol (M2414-20)) and antibody against Tryptase (1/100) (R&D (MAB1937)). Sections were deparaffinised and EDTA buffer (MMP9), Tris-HCL buffer (Chymase) and Tris-EDTA buffer (Tryptase) and heating (8-min boiling in microwave oven) were used as antigen-retrieval methods. Endogenous peroxidase activity was blocked by incubation with 0.3% (volume/volume) H_2O_2 for 30 min. Subsequently, sections were incubated with a primary antibody diluted in PBS supplemented with 1% (weight/volume) bovine serum albumin for 1 hour at room temperature. Sections were incubated with a secondary antibody (1/100) (DAKO) for 30 min at room temperature. After each incubation step, sections were rinsed in PBS for 5 min. Peroxidase activity was demonstrated by application of 3,3'-diaminobenzidine (Sigma, St. Louis, MO, USA) containing 0.03% H_2O_2 for 15 min. Sections were counterstained with Mayers haematoxylin, dehydrated and mounted with mounting medium (Merck, Darmstadt, Germany). A non-specific antibody as a negative control was used. The stained slides were scanned on a Hamamatsu scanner and analyzed on the Aperio ImageScope software. The positive pixel count v9 Algorithm was used under default settings.

Lung Lysate

Lungs were dissected from mice and snap-frozen in liquid nitrogen. Deep frozen lungs were homogenized with mortar and pestle. Lung powder was transferred into RIPA buffer, incubated for 40 minutes and centrifuged for 20 minutes at 4 °C. Protein containing supernatants were collected. Protein concentrations were determined with Pierce BCA Protein Assay Kit according to manufacturer's guidelines (Thermo Fisher Scientific, MA, USA).

Mesoscale Cytokine Assays

IL-4, -5, -6, -8, -13, Eotaxin, and IFN- γ concentrations were measured with U-Plex Assay Kit according to manufacturer's guidelines (Meso Scale Diagnostics, MD, USA). Cytokine concentrations

were normalized to protein concentrations as determined by Bicinchonic Acid (BCA, Pierce, ThermoFisher, Germany).

Serological determination of COL4A3 degradation

A competitive ELISA using a monoclonal antibody raised against a degradation fragment of the COL4A3 was used to assess C4Ma3 levels in human EDTA plasma or mouse serum by the following procedure: Streptavidin-coated microtiter plates (cat. no. 11940279, Roche Diagnostics, Hvidovre, Denmark) were coated with 100 μ L/well of 1.25ng/mL biotinylated peptide (PGDIVFRKGP-K-biotin) diluted in assay buffer (25mM PBS-BTB, 2g/L NaCl, pH 7.4) and incubated for 30 minutes. Standard peptide (PGDIVFRKGP), quality control samples, or samples of interest (20 μ L/well) were added in double determinations. Subsequently, 100 μ L/well of 100ng/mL HRP-labeled monoclonal antibody diluted in assay buffer was added, and plates were incubated for one hour. Following incubation, 100 μ L/well of 3,3',5,5'-tetramethylbenzidine (TMB) was added and plates were incubated for 15 minutes in the dark. To stop the enzyme reaction of TMB, 100 μ L 0.1% sulphuric acid was added and the absorbance was measured at 450nm with 650nm as the reference using an ELISA reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). All incubation steps were performed at 20°C with shaking at 300rpm and followed by five washes (20mM TRIS, 50mM NaCl, pH 7.2). The standard peptide had a starting concentration of 100ng/mL and was diluted 2-fold to create an 11 points calibration curve with the last point consisting of assay buffer only. A calibration curve was plotted using a 4-parametric mathematical fit model. Each ELISA plate included five quality control samples to monitor intra- and inter-assay variation.

BioRad Bioplex cytokine measurements

Serum levels of 27 cytokines (BioRad, Munich, Germany) were measured using Bio-Plex MAGPIX Multiplex Reader (Bio-Rad, Munich, Germany) according to manufacturer's recommendations. In brief, samples were defrosted at 4°C and 25 μ L was pipetted in doublets per patient. Concentration range varied for each cytokine and can be obtained from biorad.com. Standard curve fitting was performed using Bio-Plex Manager MP software (Bio-Rad, Germany, Version 1.0.0.03). A five parameter (5PL) fit with logistic weighting based on logarithmic coefficient of variance was used throughout. Panel of ten serum cytokines and chemokines reflective of type 1 (IL-12p70, IFN- γ , IL-17A, IL-6, IL-8) or type 2 (IL-4, IL-13, IL-5, eotaxin, IL-9) profiles. Only concentrations in range were used for further calculations. For statistics all cytokine data were log-transformed and rank based non-parametric tests were used (including correction for multiple comparison).

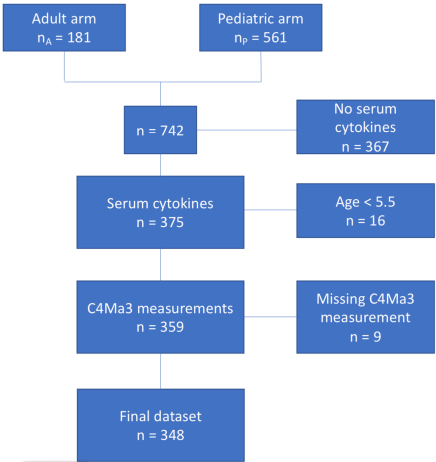
Supplementary references:

1. Raemdonck, K. *et al.* CD4⁺ and CD8⁺ T cells play a central role in a HDM driven model of allergic asthma. *Respir. Res.* **17**, 45 (2016).
2. Neuhaus, V. *et al.* Assessment of the Cytotoxic and Immunomodulatory Effects of Substances in Human Precision-cut Lung Slices. *J Vis Exp* e57042 (2018). doi:10.3791/57042
3. Lunding, L. P. *et al.* Poly(inosinic-cytidylic) acid-triggered exacerbation of experimental asthma depends on IL-17A produced by NK cells. *J Immunol* **194**, 5615–5625 (2015).

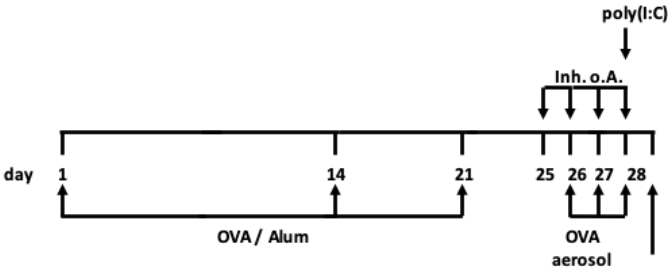
Supplemental Figure S1: A) ALLIANCE cohort flow chart B) Treatment protocol OVA/PolyI:C with and without Fulacimstat; Mice were sensitized to OVA by three intraperitoneal (i.p.) injections of 10 µg of OVA (OVA grade VI, Sigma, Deisenhofen, Germany) adsorbed to 150 µg of aluminum hydroxide (Imject alum, Thermo, Rockford, Illinois, U.S.) on days 1, 14 and 21 as previously described³. Mice were exposed three times to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in PBS) on days 26, 27 and 28 in order to induce acute allergic airway inflammation. PolyIC challenge was introduced on day 28 with 200µg in 50µL PBS via aerosol. Chymase Inhibitor treatment: 50µg Fulacimstat (BAY1142524, MCE, Germany) was administered intra-tracheally on days 25 (prior to OVA challenge), 26, 27 and 28 via aerosol. Fulcamistat was dissolved in dimethyl-sulfoxide (DMSO, Sigma, Germany) at a concentration of 500µg/mL and diluted in PBS for inhalation. Vehicle for inhalation: 10% DMSO in PBS. All animals were sacrificed by cervical dislocation under deep anesthesia on day 29. Negative control animals were sham-sensitized to PBS and subsequently challenged with OVA aerosol (PBS group). C) Levels of C4Ma3 in asthmatics were plotted against the effective airway resistance. Linear regression fit quality is represented by r_s (Spearman coefficient) D) C4Ma3 serum levels decreased in control individuals over age. Fitted line displays exponential decrease function with 99% confidence intervals for fitting, $r^2=0.48$, red bands indicate 99% CI.

Supplement Figure S1

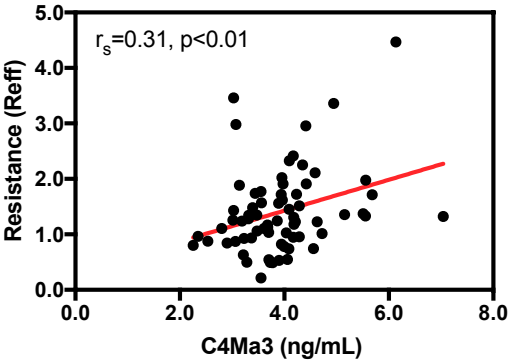
A)



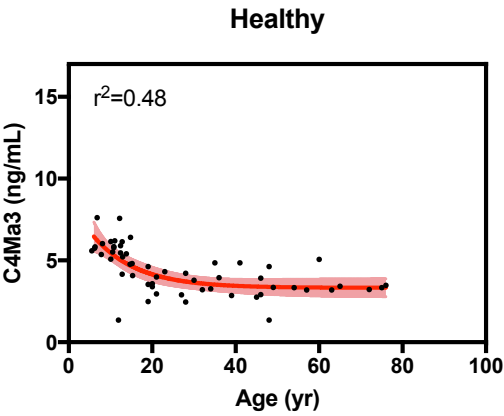
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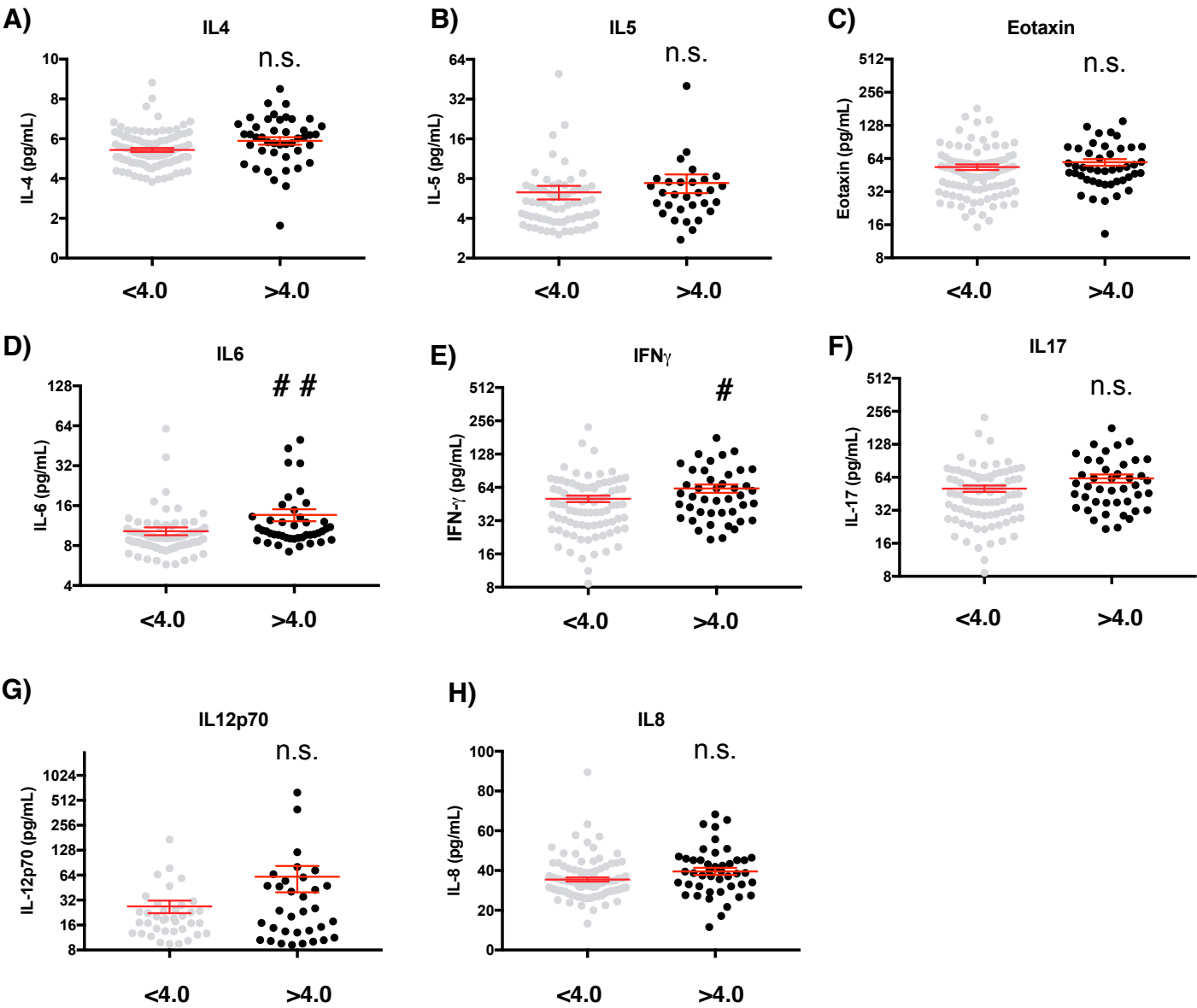


D)



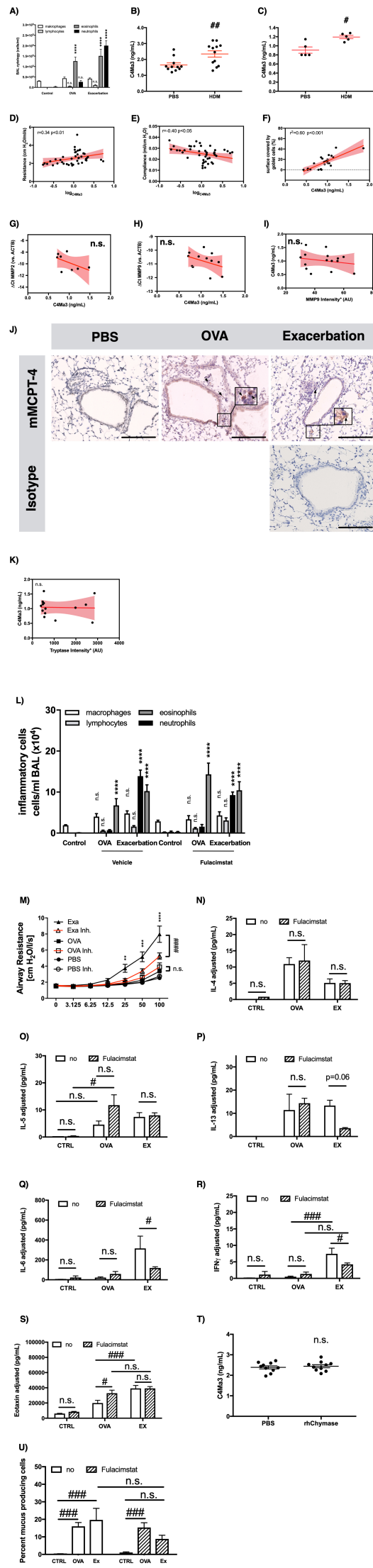
Supplemental Figure S2: Type 1 and type 2 serum cytokine levels of asthmatic patients with <4ng/mL or >4ng/mL C4Ma3 level. Y-axis log₂ transformed; Analysis was performed with Wilcoxon on ranks of log₁₀ transformed cytokine values with FDR p-value correction. A) Serum IL-4 levels, B) Serum IL-5 levels, C) Serum eotaxin levels, D) Serum IL-8 levels, E) Serum IL-12p70 levels, F) Serum IL-17 levels, G) Serum IL-6 levels, H) Serum IL-9 levels, I) Serum IL-13 levels, J) Serum IFN- γ levels. n.s. not significant, $p < 0.05$, $**p < 0.01$.

Supplement Figure S2



Supplemental Figure S3: A) BAL cytology of control, OVA, and exacerbation groups. Total cell numbers are displayed, macrophages (clear), lymphocytes (light grey), eosinophils (dark grey), neutrophils (black), n=16 per group. B) Serum of mice sensitized and challenged intra-nasally with house-dust mite allergen and compared to PBS challenged littermates was analyzed using the C4Ma3 neo-epitope assay for COL4A3 degradation. PBS n=8; n=9 HDM; Mean±SEM; Kruskal-Wallis with Dunn's post test ** p<0.01; C) Comparison of C4Ma3 release from allergic lung sections. Supernatant of 48h incubation of precision-cut lung slices from mice sensitized and challenged intra-nasally with house-dust mite allergen and compared to PBS challenged littermates. PBS n=5; HDM n=5; Mean±SEM; Kruskal-Wallis with Dunn's post test, * p<0.05 D) Lung function (resistance) measurement mice. C4Ma3 was log transformed (Spearman $r_s=0.34$, p<0.01). E) Compliance negatively correlates (Pearson correlation coefficient r) with increasing levels of C4Ma3 serum values in mice. Due to non-normal distribution C4Ma3 values were log transformed. F) Bronchial surface coverage with mucus producing cells as assessed by CAST ($r^2=0.60$, p<0.001). G) Total lung expression of MMP2 (-ΔCt vs. β-actin (ACTB) expression, lower -ΔCt values are representative of lower expression) in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. H) Total lung expression of MMP9 (-ΔCt vs. β-actin (ACTB) expression, lower -ΔCt values are representative of lower expression) in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. I) Immuno-histology for MMP9 in lung sections in correlation with C4Ma3 serum level in mice. No significant (n.s.) correlation was observed. J) mMCPT4 histology in mouse lung sections. Paraffin-embedded mouse lung sections were stained for mMCPT4. All sections counterstained with haematoxylin. Boxed areas are magnified and black arrows indicate mMCPT4 positive cells. Overall specific staining intensity was calculated according to positive pixel count algorithm v9 from Aperio ImageScope software. Scale bar: 20μm K) Immuno-histology for mast cell tryptase in lung sections in correlation with C4Ma3 serum level in corresponding mice. No significant (n.s.) correlation was observed. L) Broncho-alveolar cytology (macrophages, lymphocytes, eosinophils and neutrophils) of Fulacimstat treatment model. M) Airway resistance of Fulacimstat treatment model. Indicated in red: Treated animals; significant indicator * denotes comparison between PBS and exacerbation groups; # indicates comparison between treated and vehicle groups. N) IL-6 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # p<0.05 O) Recombinant human mast cell chymase was administered intra-tracheally to naïve mice and after 24h serum levels of C4Ma3 were measured. No significant increase detected. P) IL-4 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. Q) IL-5 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # p<0.05 R) IL-13 protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. S) Eotaxin protein concentration in lung lysate (pg/mL) adjusted to total protein content of sample. # p<0.05, ### p<0.001 T) IFN-γ protein concentration in lung lysate (pg/mL) adjusted to total protein content of

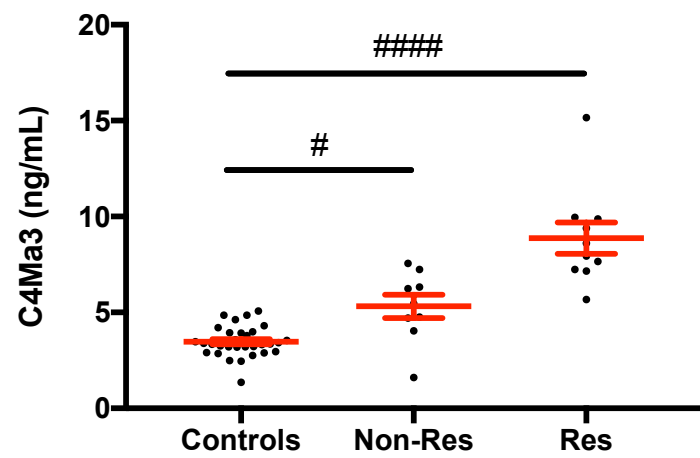
sample. # $p < 0.05$, ### $p < 0.001$ U) Percentage of mucus producing cells covering basal membrane of bronchus. ### $p < 0.001$. Unless otherwise specified: Mean \pm SEM. Kruskal-Wallis with Dunn's post test, n.s. = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****, ##### $p < 0.0001$.



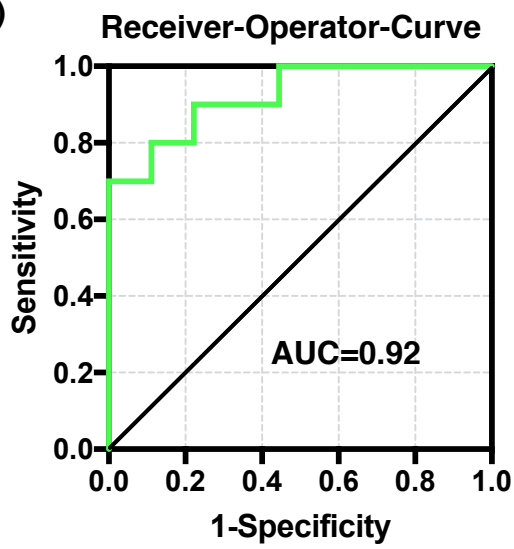
Supplemental Figure S4: C4Ma3 serum levels at baseline in asthmatics vs. controls prior anti-IgE therapy and Receiver-Operator-Curve of C4Ma3. A) Baseline serum level of C4Ma3 of asthmatics, who did (Res) or did not (Non-Res) respond to Omalizumab therapy as measured by an increase of the asthma control test score of 3 points or more after six month. Controls (n=31) were compared to Res (n=10) and Non-Res (n=9) with Kruskal-Wallis on ranks and Dunn's post test; # $p < 0.05$, ##### $p < 0.0001$ B) Receiver-Operator-Curve of C4Ma3 for prediction of responders (increase of 3 points or more) after a six month intervention with anti-IgE therapy. Sensitivity vs. 1-Specificity. AUC=0.92; C) Chymase activity leads to degradation of COL4A3 in asthma. In non-asthmatic airway, the basal membrane composition is maintained by the airway epithelial cells and adjacent mesenchymal cells. Typically, very few if any mast cells are found in the vicinity of the mucosal surface. In allergic asthma, the remodeling of the airway encompasses the airway epithelial cell response to damage which fundamentally changes the balance of extracellular matrix proteins in the basal membrane. This is further accompanied by an allergen-specific activation of mast cells found more frequently in the mucosa of asthmatics. The activation of mast cells then leads to the release of chymase, a protease able to degrade COL4A3. Levels of COL4A3 fragments corresponding to the level of chymase activity are eventually detected in blood. Since the COL4A3 fragments are a direct consequence of chymase release and activity (mast cell degradation) they are tightly linked to disease activity, e.g. asthma exacerbations.

Supplement Figure S4

A)



B)



C) Chymase degradation of COL4A3

