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Title: Tumstatin fragment selectively inhibits neutrophil infiltration in experimental asthma exacerbation

Short title: CP17 inhibits neutrophil activation and migration

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Abstract
Background: Asthma is a chronic inflammatory disease with structural changes present. Burgess and colleagues recently found tumstatin markedly reduced in adult asthmatic lung tissue compared to non-asthmatics. ECM-fragments such as tumstatin are named matrikines and act independently of the parent molecule. The role of Col IV matrikines in neutrophil inflammation (e.g. exacerbation in asthma) have not been investigated to date. Severe adult asthma phenotypes are dominated by neutrophilic inflammation and show a high frequency of severe exacerbations. Objective: This study sought to investigate the role of a novel active region within tumstatin (CP17) and its implication in neutrophil inflammatory responses related to asthma exacerbation. Methods: For reactive oxygen production, isolated neutrophils were pre-incubated with peptides or vehicle for 1h and stimulated (PMA). Luminescence signal was recorded (integration over 10 sec) for 1.5 h. Neutrophil migration was performed according to the SiMA protocol. Mice were sensitized to OVA/Alumn by intraperitoneal (i.p.) injections. Mice were then treated with CP17, vehicle (PBS) or scrambled peptide

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(SP17) after OVA exposure (day 27 and 28, polyI:C stimulation). All animals were sacrificed on day 29 with lung function measurement, histology and lavage. Results: CP17 decreased total ROS production rate to 52.44% (0.5µM, $p<0.05$ vs. SP17), reduced the in vitro directionality (vs. SP17, $p=1x10^{-6}$) and migration speed (5µM, $p =1x10^{-3}$). In vivo application of CP17 decreased neutrophil inflammation ~1.8-fold ($p<0.001$ vs. SP17) and reduced numbers of mucus producing cells (-29%, $p<0.05$). Conclusion: CP17 reduced the ROS production rate, migrational speed and selectively inhibited neutrophil accumulation in the lung interstitium and lumen. Clinical Relevance: CP17 may serve as a potential precursor for drug development to combat overwhelming neutrophil inflammation.

Introduction

Asthma is a chronic inflammatory disease where several structural changes decrease airflow and gas exchange. These changes include - but are not limited to - increased smooth muscle mass, increased vascularity, epithelial dysfunction and a notably altered extracellular matrix (ECM) composition (reviewed in [1,2]). The ECM is comprised of several classes of proteins to maintain integrity and balance between the elasticity and rigidity of the lung tissue. In adult asthma, collagen I, V, fibronectin and tenascin are increased [3] and col IV and elastin are decreased [4]. We recently found that the noncollagenous
domain-1 of col IV α3 (tumstatin) is markedly reduced in adult asthmatic lung tissue compared to non-asthmatics [5]. Col IV is a major ECM protein and it exists as 6 genetically distinct isoforms [6,7]. Its fibrils are not only found throughout the lung but also in the basal membrane of blood vessels and the epithelium [5,8]. Treatment of an experimental asthma mouse model with recombinant tumstatin resulted in a significant amelioration of bronchial hyperresponsiveness, infiltration with inflammatory cells, decreased vascular re-modelling and reduced mucus production [5], which was recently confirmed by a segmental allergen challenge model in a chronic house-dust mite sheep model [9]. Additionally, Harkness et al. identified that tumstatin may also play an important role in the control of neutrophil inflammation [10]. ECMfragments, which act independently of the parent molecule are collectively assigned to a class of molecules termed matrikines. Matrikines in the lung are innumerous and serve

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several functions (reviewed in [11–14]. The role of Col IV matrikines in neutrophilic inflammation (e.g. exacerbation in asthma), especially a novel active region in tumstatin [15] however, has not been investigated to date.

Neutrophilic inflammation in asthma occurs independent of infection and is a recognized phenotype of asthma (reviewed in [16]). The lack of response to therapeutics such as corticosteroids or β2-agonists [17] is typically associated with neutrophila in sputum or bronchoalveolar lavage samples [18,19]. Severe adult asthma phenotypes are very stable [20] and Haldar and colleagues identified two groups dominated by neutrophilic inflammation in secondary care refractory adult asthmatics [21]. A subgroup of these donors (11.7% of all donors tested) with high symptom expression had the highest frequency of severe exacerbations of all phenotypes tested (5.43 events per year) [21]. As a result, uncontrolled adult asthmatics are more likely to be hospitalized (severe exacerbations) and incur half of the costs associated to asthma therapy [22]. In addition, decreasing lung function and increasing lung tissue remodeling is found in these asthmatics. Asthma exacerbations are often a result of viral infections, most notably inducers of the common cold (Rhinovirus) and flu (Influenza strains) [23,24].

This study sought to investigate the role of a novel active region within tumstatin (CP17) and its implication in neutrophil inflammatory responses related to asthma exacerbation.

Methods

Donor Population

Blood from voluntary healthy donors (mean age 33.8 years) was taken via cubital venipuncture. Current infection was excluded by questionnaire and temperature measurement. Written informed consent was obtained from all participants and the study design was approved by the local Ethics Committee of the University of Lübeck, Germany (AZ 11-044) (table 1).

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Neutrophil isolation
Venous blood samples (cubital venipuncture) were treated with Lithium-Heparin and stored at room temperature (20°C) and processed within 1 hour. Granulocytes were isolated by density gradient centrifugation using Ficoll (PAA, Coelbe, Germany). Centrifugation was performed with 400 rcf (relative centrifugal force) for 30 min at 8°C (Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). Erythrocytes were lysed with pyrogen-free aqua bidest for 45 seconds and lysis was stopped with 10x PBS. Granulocyte collection was performed by centrifugation with 300 rcf for 10 min at 8°C. The final cell-pellet was resuspended in human AB serum (H4522 Sigma, Taufkirchen, Germany) and stored for 30 min at 37°C prior further use.
Neutrophil activation prior to stimulation or migration was excluded by CD62L labelling (cluster of differentiation) antibody (PN IM1231U, Beckman Coulter, Krefeld, Germany). CD62L (or L-selectin) is continuously produced by neutrophils and is shed of shortly after activation [25,26]. A loss of more than 20% of observed neutrophils was considered as a sample with premature activation and was not included in further examination.

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To investigate the effect of peptide treatment on migrational behavior, neutrophils ($3*10^6$ per mL in AB-serum, H4522 Sigma, Taufkirchen, Germany) were pre-incubated for 1 h with CP17 or SP17 at 5.0 or 50.0 $*10^{-6}$ mole/L at 37°C and 5% CO_2.
Quantification of in vitro chemotaxis
The assay was performed as previously described [27]. In brief, for time-lapse imaging

of neutrophil migration, neutrophils were placed into a µ-slide Chemotaxis 3D (IBIDI, Martinsried, Germany) and attracted towards IL-8 or fMLP. Neutrophils were resuspended and incubated for 60 min in human AB-Serum ($3x10^6$ per mL) with vehicle (MiliQ water), CP17 or SP17. Preincubated cells were mixed with placental matrix (human extracellular matrix, HEM final concentration: 400 ng/µL, BD Pharmingen, USA) and inserted into the channel of a µ-slide. After 5 min setting time at room temperature the right control reservoir was gently filled with RPMI medium with 0.1% BSA, HEPES, and peptides at respective concentrations to prevent additional gradients to form across the channel. For chemoattraction, left reservoirs were filled with medium as described above but containing 100 ng/mL IL-8 (BD Bioscience, Germany). Migration was recorded for 60 min every 30 s at 20°C using the bright-field microscopes EVOS®FL Cell Imaging System (life technologies, USA) with a monochrome CCD sensor or a CytoSMART LUX2 (CytoSMART Technologies, Eindhoven, The Netherlands). Images were recorded with a size of 1360x1024 or 720x1280 pixel and stored as 8-bit PNG or JPEG format.

Image Analysis and tracking of neutrophil granulocytes

All image data were collected on a USB flash drive and analyzed using the open source migration analysis[1] published in [27] implemented in MATLAB (R2016a, Mathworks, USA). This software uses a background subtraction and texture based image segmentation to detect neutrophil granulocytes. The integrated tracking module implements the validation algorithm developed by Rapoport & Becker et al. [28]. After

[1] https://github.com/cells2numbers/matlabMigrationAnalysis

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image segmentation and tracking, each trajectory of a neutrophil granulocyte is characterized using the mean speed and the directionality.

The directionality is calculated as the displacement (distance between the start-and endpoint of each trajectory) divided by the cumulative distance the neutrophil has traveled. An illustration of the migration patterns of directed and undirected cells are presented in Fig.2 A+B.

To assess the quality of the tracking results we followed the scheme presented in [27] and calculated the valid observation time (VOT) which is defined as the length of all valid trajectories divided by the sum of the length of all valid and invalid trajectories. The VOTs for all analyzed experiments reveal a high tracking quality with an average VOT of 0.84 for migration towards IL-8 and a VOT of 0.99 for a migration against fMLP. For statistical analysis, the trajectory information is exported as CSV and analyzed using R in version 3.4.3. p-Values are determined using a Welch Two sample t-test.

Normalization of the migration parameter

Migration patterns of the neutrophils are patient-specific and we normalized each CP17 and SP17 run against a vehicle control experiment using neutrophils from the same donor. For each patient, we calculated the mean and standard deviation of the speed and directionality of the control experiment. Next, we normalized the speed and directionality values of all trajectories by subtracting the mean of the vehicle control and by dividing by the standard deviation of the vehicle control experiment of this patient. The resulting values are denoted as differential speed and differential directionality in arbitrary units (AU). These units can be interpreted as the deviation from the vehicle control: a differential speed value of -0.3 is a reduction of speed by 30% as compared to the vehicle control run.

Reactive Oxygen Species (ROS)

Isolation of neutrophils was performed as described above. After isolation neutrophils were counted, adjusted to $5x10_6$ per mL and 50.000 cells were added per well of a 96-

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well plate containing peptides (CP17 or SP17) at 0.5, 5.0, 50 µmole/L or vehicle (MilliQ water) in phenol-red free RPMI 1640 medium (Gibco, Germany) with 25 mM HEPES (Gibco, Germany) and 0.1% BSA (Sigma, Germany). Neutrophils were then incubated 1h at 37°C and 5% CO_2 followed by the addition of 10 nmole/L PMA per well by automated injection together with 0.06 mM Luminol (or 1 mmole/L Lucigenin, both from Santa Cruz Biotechnology, USA) in a luminometer (FluoStar, BMG Labtech, Germany) and luminescence signal was integrated for 10sec per well every min for 1.5 h. Final accumulated luminescence signal was then used for analysis. Each condition was measured in triplicates and mean value was used to determine amount of produced ROS. Analysis was performed by MARS software (BMG Labtech, Germany).

Tumstatin derived peptides

Peptides were synthesized and provided by R. Bartels (Forschungszentrum Borstel, Germany) and sequence of CP17 (VCNFASRNDYSYWLSTP) was determined as described in Weckmann et al. [15] and compared to Monboisse et al. [29]. Scrambled peptides were generated using RandSeq from the Expasy web page (http://web.expasy.org/randseq/) and blasted against human protein sequences via the blastp web site (www.pubmed.com). Non-homologous sequences were further evaluated for solubility and cell toxicity (A549 and neutrophils, propidium iodide exclusion assay). Most similar, non-toxic behavior SP17-peptide was used for further experiments (sequence: PSFRDSAWNCYTNYLVS). CP17 dose was deducted from previous experiments (data not shown) using lipopolysaccharide stimulation.

Animals

Female, 6- to 8-week old C57BL/6 mouse (Charles River, Sulzfeld, Germany) were housed under specific pathogen-free conditions and received ovalbumin (OVA)-free diet and water ad libitum. All animal studies were approved by the animal ethics committee from the Department of State, Kiel, Germany (ethical approval number: V312-72241.123-3 (122-8/12).

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Animal Treatment Protocol

Exacerbation model was established as previously described [30]. Briefly, 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. Mice were exposed three times to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in phosphate-buffered saline, PBS) on days 26, 27 and 28 in order to induce acute allergic airway inflammation. [31] An amount of 1nmole CP17, vehicle (PBS) or scrambled peptide (SP17) was co-instilled after the OVA exposure and on day 27 and 28, followed immediately by a polyI:C stimulation on day 28. 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).

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 light microscope (Leica, Wetzlar, Countess automated cell counter (Life Technologies, Darmstadt, Germany). Fifty µL-aliquots of lavage fluids were cytospined, stained with Diff-Quick (DADE Diagnostics, Unterschleissheim, Germany) and cells were differentiated microscopically according to morphologic criteria.

Determination of Airway Responsiveness

Twenty-four h after the last OVA challenge (with polyI:C and peptide treatment) 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 DSI FinePoint R/C system. Airflow and transpulmonary pressure were recorded

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continuously to calculate lung re
compliance (mL/cm H_2O) in each b
before MCh provocation testing w
(3.125, 3.25, 12.5, 25, 50, and 100 m
5 min each. Baseline pulmonary
Expressed results comprised the
resistance recorded during 5 min
received terbutaline (10 µg/kg; ter
MCh-induced bronchospasm prior B

Lung Histology

Lungs were fixed ex situ with 4%
under constant pressure for 20min
were embedded in paraffin. Subseq
hematoxylin and eosin (HE)
Photomicrographs were recorded b
attached to a microscope (BX-51,
Olympus cell^A software. For m
samples of the lungs were prepare
orientator technique [3]. The surfac
surface area of airway epithelial ba
epithelial mucin (V_{mucin}) per S_{ep} we
toolbox (newCAST, Visiopharm, H
formulas: and
lgc
all points hitting mucin and LP is th
esistance (R_L) (cm H_2O/mL/s) and dynamic
breath cycle. Mice were allowed to stabilize for
with increasing concentrations of aerosolized
mg/mL) followed by a responding time of aeros
parameters were assessed with aerosolized
mean absolute values of the responses o
n after the inhalation of each MCh aerosol
rbutaline-hemisulfate, Sigma) intravenously to
BAL and preparation of the lung.
% (wt/vol) paraformaldehyde (PFA) via the tr
n, ligated and stored in 4% PFA. Fixed lung t

quently, 2 µm tissue sections were stained wit
or periodic acid-Schiff (PAS), respec
by a digital camera (DP-25, Olympus, Tokyo, J
Olympus) at 40- and 100-fold magnification
mucus quantification, systematic uniform ra
ed according to standard methods [2] includi
ce area of mucin-containing goblet cells (S_{gc}) pe
asal membrane (S_{ep}) and the volume of PAS-s
ere determined using a computer-assisted stere
Hoersholm, DK) [4, 5] according to the foll
d
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Results

1. CP17 reduces predominantly intracellular reactive oxygen species

PMA induced ROS production was measured using two substrates, luminol and lucigenin. Luminol permeates the cell membrane and detects total ROS (tROS), whereas lucigenin only reacts with extracellular ROS (eROS) [32]. Using a scrambled peptide (SP17), we found no significant difference in the final amount of eROS in an endpoint assay (data not shown). Kinetic analysis of the tROS production over 60min revealed that tROS could be elevated by either of two entirely different kinetic patterns (supplement figure 1a). We measured accumulated ROS at peak production rate (637 sec in vehicle or medium control) and found a significant reduction of ROS with CP17 at 0.5µM (vs. SP17, $p<0.0001$) and 5.0 µM (vs. SP17, $p<0.001$). This reduction resulted solely from a decreased production rate (see supplement figure 1a and schematic suppl. figure 1b) and we therefore determined the normalized production rate at the halfmaximal tROS level (for formula see methods), which is an indicator of the slower production rate. CP17 decreased neutrophil tROS production to 52.44% after incubation with 0.5µM ($p<0.05$) and reached its maximal effect at 5µM (reduction to 30.40%, $p<0.001$). This suggests that CP17 is a potent inhibitor of neutrophil total ROS production as measured by luminol.

We were further interested to see if other neutrophil functions such as CXCL8 (IL-8) secretion, degranulation and NETosis could be affected. CXCL8 secretion and neutrophil elastase release were stimulated with R848 (a toll-like-receptor 7 / 8 ligand, [33]) in the presence of SP17 and CP17, but no significant effect on these functions was observed (supplement figure 1c, d). We also investigated the possibility of an effect on neutrophil extracellular DNA trap formation (NET) as it has been shown that ROS production plays a critical role in NET formation [34–36]. We did not observe a significant difference of NETosis after treatment with CP17 (supplement figure 1e). These data suggest, that albeit CP17 altered ROS production kinetics, neither secretion, degranulation nor NETosis are affected.

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2. CP17 inhibits the in vitro migration of neutrophils

To assess the in vitro inhibitory effect of CP17 on the migration of neutrophils we used the SiMA migration assay described in [27]. In this assay an IL-8 and a fMLP gradient induces a chemotaxis, i.e. a directed movement of the neutrophiles towards the chemokines. To quantify the inhibitory effect of CP17 in this controlled environment, we first measured the speed and directionality of neutrophils in the absence of any peptide. This experiment is denoted as the vehicle control experiment and serves as a donor

specific baseline. Next, we measured the migration under the inhibitor CP17 or the control peptide SP17 and normalized the speed and directionality with respect to the vehicle (see methods "Normalization of the migration parameter" for details). The resulting differential speed and differential directionality values in arbitrary units directly reflect the induced changes: neutrophils in an experiment with a differential speed of 0 have the same speed as the neutrophils in the vehicle experiment, while a differential speed of -0.5 denotes a reduction of speed by 50%.

The migration profiles of different trajectories are illustrated in figure 2a,b while the inhibition induced by CP17 and SP17 in a dose of 5µM and 50µM is summarized in figure 2c-f. Under both stimulations (IL-8 and fMLP) we observe an inhibitory effect of CP17 characterized by a significant reduction of speed and directionality compared to the control peptide SP17. In IL-8-induced chemotaxis, decrease of directionality and speed was strongest at 5µM CP17 ($p<0.05$ and $p<0.0001$ respectively, figure 2c,e) while SP17 only induced minute changes from the vehicle baseline (represented by differential directionality of 0.0). These effects were inverted at the 50µM concentration for differential directionality ($p<0.05$ for CP17 vs. SP17, figure 2c) but not for differential speed ($p<0.05$, figure 2e). To test whether inhibition effects are specific to IL-8 we used fMLP-induced chemotaxis to confirm that 5µM CP17 reduces differential directionality and speed compared to SP17 ($p<0.0001$ and $p<0.01$ respectively, figure 2d,f). Overall, fMLP induced migration showed a higher divergence from vehicle baseline (represented by differential speed or directionality of 0.0) in the presence of peptides. These data suggest, that the specific order of amino acids within tumstatin (and represented by CP17) harbors a potent inhibitory effect for neutrophil migration speed and trajectory straightness.

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3. CP17 reduces neutrophil influx in mouse models of asthma exacerbation

The observation of decreased neutrophil migration in vitro let us to hypothesize that CP17 would inhibit neutrophil accumulation in experimental asthma exacerbation. We chose a model previously published by Lunding et al. [31], in which a viral infection is modeled by local application of the TLR-3 agonist polyI:C resulting in acute aggravation of already established experimental allergic asthma features (airway hyperresponsiveness, airway inflammation, mucus hypersecretion). Two instillations of 1 nmole of CP17 or SP17 preceded polyI:C treatment, 24h and 30min, respectively. Animals were given 24h to develop full exacerbation phenotype, lung function was measured and samples (e.g. BAL, lung specimen) collected for further analysis. Animals receiving polyI:C presented with a strong neutrophil influx to the airway lumen as

measured by cytospin analysis (figure 3a). Sensitized and OVA challenged animals showed little neutrophil infiltration (25.484 cells/mL) whereas stimulation with polyI:C increased cellular influx to 143.516 neutrophils/mL ($p<0.001$). Treatment with 1 nmole CP17 profoundly decreased neutrophilic inflammation ~1.8-fold (78.500 cells/mL, $p<0.001$) compared to the unchanged elevated level of SP17. To confirm CP17's effect on neutrophils, we replicated our treatment in a mouse model of neutrophilic asthma [37]. Corroborating our hypothesis, CP17 significantly ($p<0.05$ vs. SP17) reduced neutrophil cell numbers (~43%) in the BAL cytology of this model (supplement figure 2e). The observed effect was highly selective for neutrophils in both models, as no other cell type (lymphocytes, macrophages, eosinophils) in the lumen was affected significantly by peptide treatment (figure 3b, supplement figure 2f).
Mucus production is a hallmark feature of exacerbations in our model and was reduced in mice, which received CP17, when compared to SP17 (figure 3c, upper panel, PAS). Utilizing the newCAST imaging system for analyzing numbers of mucus producing cells, a significant reduction by 29% (SP17: 22.95±2.42 vs. CP17: 16.27±2.06, $p<0.05$, n=15-16) was detected (figure 3d) in the exacerbation but not in the OVA groups (supplement figure 2g). We confirmed the effect on mucus producing cells in a neutrophilic asthma mouse model, were CP17 reduced the number of mucus producing cells 2.4-fold ($p<0.01$ vs. SP17, supplement figure 2h). Overall, the degree of influx of inflammatory cells was markedly reduced in CP17 treated animals (figure 3c, lower panel, H&E) as evident by the reduced infiltrates in the interstitial space between blood vessel and airway. No

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significant changes were found in lung resistance and compliance (figure 3e,f and supplement figure 2a,b). Also, we did not observe a reduction of epithelial derived proinflammatory
chemokine expression (CXCL1, CCL20) with CP17 treatment (supplement figure 2c,d) nor direct effects on A549 cell activity [15], suggesting little effect of this peptide on epithelial cells. This data indicates that CP17 is highly specific for inhibition of neutrophilic inflammation in murine models of neutrophilic asthma and asthma exacerbation.

Discussion

The significant reduction of tumstatin in lung tissue from asthmatic patients has been suggested to contribute to the pathogenesis of this disease by allowing for increased neovascularization, eosinophil influx and mucus production [5,9,10,38]. This study shows for the first time a distinct role of tumstatin (CP17) in moderating experimental asthma exacerbations by alleviating neutrophil inflammatory profiles in vitro (ROS

production, migration) and *in vivo* and adds to the accumulating body of evidence of col IV matrikines to play a vital role in ECM-inflammatory cross-talk.
Effects of col IV NC1 domain fragments on leukocyte behavior have been known for more than 20 years [29,39]. Recently, the significant reduction of tumstatin in asthmatic lung biopsies has spurred further investigations by Burgess and colleagues on the exact function of tumstatin in mouse and sheep models of allergic asthma [5,9,38]. Tumstatin itself and various active fragments contained in it conferred significant biological activity by reducing eosinophil infiltration, reduced mucus production, decreased levels of IL-13 and VEGF and amelioration of bronchial hyperresponsiveness [5]. Tumstatin's activity on microvascularization was extensively explored in the context of tumor growth, however little to no evidence was available for asthma exacerbations [40–42]. Here we show that CP17, an active fragment of tumstatin, reduces the rate at which the ROS is produced. Monboisse *et al.* [29] reported earlier that a peptide termed a3(IV) aa 72-89 (which contains a part of CP17) had no significant effect of overall ROS production as measured by a stimulation with 8nM PMA and an end-point ferrycytochrome assay. Instead, Monboisse and colleagues showed more convincing data with peptide a3(IV) 185-203, which produced a significant reduction of total ROS

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by ~49% compared to medium alone. Neither kinetic patterns of ROS production nor control peptides were used in their study. To overcome the limitations of end-point measurements and to eliminate artificial effects from the presence of larger concentrations of peptides we compared to a scrambled peptide with similar aa content, yet randomized aa order. Firstly, by comparing similar concentrations of active and inactive peptide we observed a general reduction of ROS with increasing concentrations of peptide (data not shown). This may in part be explained by anti-oxidative capabilities of these class of peptides. [43–45]. CP17 is predicted to be an antioxidant protein (confidence 0.945) with SP17 showing almost identical capabilities (confidence 0.935) (table 2) [46]. This indicates a certain capacity for these peptides to act as ROS scavengers. Secondly, and highly supportive of our initial hypothesis, the CP17-specific reduction of the ROS production rate indicates that this function is conferred in the sequence of CP17 (acting via a yet unknown receptor). In addition, our use of PMA as a strong yet artificial stimulus may further suggest that a fundamental cellular pathway might be involved. Further research is warranted to identify a possible CP17 receptor and the mechanism by which it interferes with ROS production.
Anti-migratory effects of matrikines on tumor cells *in vitro* and *in vivo* are well documented [11,47,48]. Reports on inhibitory effects on neutrophil migration however

are scarce. In our study, CP17 significantly decreased the directionality with which neutrophils migrated towards IL-8. This indicates that neutrophils encountering CP17 may experience a loss of sensing of the gradient or an inability to translate the gradient's signal accordingly. This is accompanied by a stark reduction of migrational speed in vitro in those neutrophils. Basal membranes of postcapillary venules and lung as well as the lamina propria are rich in col IV [5,49]. Furthermore, it has been noted that the passage of basal membranes represents a significant hurdle for neutrophils. However, the role of col IV, proteases and col IV fragments is inconclusive (reviewed in [50]). Col IV isoform 3 fragments have been shown to interfere with neutrophil function such as ROS production and $\alpha_v\beta_3$-integrin has been identified to be crucial for this interaction, but it is unclear if $\alpha_v\beta_3$-integrin plays any role in interstitial migration of neutrophils [29,51].

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Limiting the scope of our work, we did not use full length tumstatin in comparison to CP17 in our in vitro and in vivo experiments. We recombinantly produced tumstatin for previous studies [10,15,52], however remaining endotoxin levels did not allow the direct use on neutrophils. The 17 aa sequence of CP17 is located in the protrusion region (aa 65-82) of tumstatin [53,54], suggesting a high likelihood of this region being an exposed epitope, as compared to the theoretical internal sequences within the folded molecule. Further research is warranted with full length tumstatin to verify its effects on neutrophils in vitro and in vivo.

In the context of a neutrophil inflammation superimposed on an allergic airway inflammation we observed a significant reduction of neutrophil infiltration into the lumen of the lungs after the application of CP17. In addition, the interstitial space between blood vessel and bronchus also showed a marked reduction of infiltrates. This may suggest, that exogenous CP17's inhibitory effects may already be present at the level of the basal membrane of the blood vessel in vivo and offers the possibility that integrins such as $\alpha_M\beta_2$ or $\alpha_4\beta_1$ are binding partners of CP17. Burns et al. showed that neutrophils can utilize various α-subunits (α_4=CD49D, α_5=DC49E) in conjunction with the β_1-subunit for recruitment after endotoxin stimulus to the lung in vivo [55,56]. However, we could not induce a full reduction of neutrophil infiltration. We also found in our in vitro migration assay that not all neutrophils seem to respond equally to CP17, leaving subpopulations migrate as they would normally do. This observation may point at a concept that distinct levels of maturity in circulating neutrophil populations exist. As early as 1993, Lund-Johansen et al. identified selective integrin expression on neutrophils during granulopoiesis reflective of the state of maturity [57]. Recently, the

concept of mature neutrophil subpopulations in circulation was further investigated and detailed by Uhl and colleagues. The group identified a distinct set of neutrophils with prolonged time in circulation, which preferentially respond to endotoxemic inflammation [58]. In their experiments the lung accumulated over 50% of those mature neutrophils high in CD44, $\alpha_4\beta_1$ and CXCL4 expression. These matured neutrophils represented a highly phagocytic population in the lung [58]. It remains unclear if CP17 has a specific effect on this aged group of neutrophils and what consequence would arise from their inhibition. Further research is required to understand the exact mechanism of CP17 on migrating neutrophils.

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In conclusion, we characterized a 17 aa fragment (CP17) of the non-collagenous domain 1 of col IV α 3 (tumstatin) in vitro and in vivo. CP17 reduced the production rate of intracellular ROS and significantly reduced migrational speed in a µ-fluidic migration assay. In a mouse model of asthma exacerbation, CP17 selectively inhibited neutrophil accumulation in the lung interstitium and lumen. CP17 as part of the interstitial ECM may therefore represent an endogenous tissue-specific modulator of neutrophil infiltration, balancing chemotactic drive and repulsion. The diminished levels of tumstatin observed in asthmatic individuals may increase susceptibility of asthmatics to aggravated neutrophil inflammation after viral exacerbation, contributing to more severe neutrophilic asthma phenotypes. Furthermore, CP17 may serve as a potential precursor for drug development to combat overwhelming neutrophil inflammation and thus detrimental effects on lung or tissue remodeling in general.

Conflict of Interest

The authors declare no conflict of interest.

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Tables

Donor No.	Sex	Age [years]	Size [cm]	Weight [kg]	BMI [kg/m2]	Medication	Asthma	Allergies	Smoking	Chronic disease	Last respiratory tract infection
C1	M	25	173	65	21.7	No	No	No	No	No	/
C2	F	25	172	62	21.0	No	No	No	No	No	/
C3	F	22	163	60	22.6	No	No	No	No	No	/
C4	M	40	190	125	34.6	Nebivolol	No	No	No	Adipositas, Hypertension	/
D1	F	23	172	65	22.0	No	No	No	No	No	/
D2	M	68	185	96	28.0	Tamsulosin	No	No	No	Prostata hyperplasia	/
D3	M	60	186	118	34.1	Aspirin, Candesartan	No	No	No	Adipositas, Hypertension	/
E1	F	22	168	53	18.8	Maxim	No	No	No	No	/
E2	M	40	182	75	22.6	No	Yes	No	No	No	/
E3	M	29	187	83	23.7	No	No	Yes	No	No	/
E4	F	25	172	67	22.6	No	No	Yes	No	No	/
E5	F	26	157	68	27.6	No	No	No	No	No	/

Tabel 1: Donor population metrics. Donor C1-4 used for migrations with CP17 in fibronectin, Donor D1-3 for migrations with SP17 in fibronectin, donor E1-5 for migrations with CP17 and SP17 with fMLP in fibronectin.

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ID	Predicted class	Confidence
CP17	Antioxidant protein	0.945
SP17	Antioxidant protein	0.935

Table 2: Predicted result from AOP-Pred; Algorithm determines the likelihood that a protein has antioxidant function. A confidence of 1 would imply certainty that a protein has antioxidant capabilities. No information on the antioxidant strength is provided. http://antioxidant.weka.cc/ on 2017/09/13

Figure Legends:

Figure 1: CP17 decreases production rate of ROS. ROS production was measured by preincubating cells with indicated concentration of peptides or vehicle for 1 h. Fifty thousand neutrophils per well were then stimulated with 10nM PMA for 90min and accumulated chemiluminescence was measured every minute with a Lumimeter (BMG Biotech, Germany). A) Accumulated amount of ROS decreases significantly with CP17 but not SP17 preincubation. Accumulated (637 sec, compare supplement figure 1a) relative light units (RLU) are plotted vs. peptide concentrations (µM). ***p<0.001 ****p<0.0001 CP17 vs. SP17 n.s. = not significant. B) Half-maximal production rate was determined from maximal ROS-production rate plateau of each kinetic curve as seen in supplement figure 1a. *p<0.05, ***p<0.001 CP17 vs. vehicle; + p<0.05, ++p<0.01 for SP17 vs. CP17, ### p<0.001 SP17 vs. vehicle, n.s. = not significant. SP17 n=6, CP17 n=14.

Figure 2: CP17 inhibits neutrophil migration in vitro. A) The inhibitory effect of CP17 to the in vitro migration of neutrophil granulocytes was quantified in a microfluidic chemotaxis assay. A directed migration was induced using fMLP and IL-8 gradients (see section methods for details). The effect of the two peptides CP17 (n=4 donors IL-8, n=4 donors fMLP) and SP17 (n=3 donors IL-8, n=4 donors FMLP) were tested in doses of 5µM (CP17 177 /SP17 212 trajectories for migration in IL-8, 395/366 trajectories for migration in fMLP) and 50 µM (446 / 187 trajectories for CP17 / SP17 for migration in IL-8, 573/366 trajectories for migration in fMLP). Blue line corresponds to trajectory from vehicle control run towards IL-8, fast and directed. Green line represents slow and undirected neutrophil migration in the presence of CP17 B) Distribution of directionality and speed in a fMLP gradient (vehicle or 5µM CP17). Each point represents one tracked neutrophil granulocyte and the blue/green lines show the density

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distribution. In this representation, the inhibitory effect results in a shifted centroid of the CP17 distribution (green) compared to the vehicle distribution (blue) towards a slower and more undirected migration pattern. C) Change of directionality after CP17 treatment. Higher dose of peptides (50µM) reverses effects. D) fMLP results in stronger decrease of directionality in the low concentration but no difference in the high concentration. E) CP17 compared to SP17 reduces the speed of neutrophils in an IL-8 gradient. F) In fMLP all doses of either CP17 or SP17 result in a strong reduction of speed. CP17 significantly reduces (compared to SP17) migration speed. Blue arrow indicates increase of speed or directionality, red error denotes a reduction. Green labeled bars show CP17, red filled bars SP17. *$p<0.05$, ** < 0.001, ****$p<0.0001$.

Figure 3: CP17 selectively decreases neutrophil infiltration in a mouse model of asthma exacerbation. Animals were prepared as described in the methods sections. Cytospins were analysed and total cell counts were used to assess the effects of peptide treatment. A) BAL Neutrophil cell counts are significantly enhanced after PolyI:C instillation (Exa=exacerbation) and is unaffected by SP17 treatment. CP17 significantly ameliorates this increase. B) Eosinophil granulocytes in BAL are increased after OVA challenge but remain unaltered after peptide treatment. No effects are observed for lymphocytes or macrophages in BAL. C) Histological analysis shows reduced mucus production in CP17 treated cells (upper panel, PAS). Hematoxylin and Eosin staining reflects the reduction of neutrophil infiltration with reduced interstitial accumulation of cells (black arrows). D) Assessment of mucus producing cells using a newCAST system. Number of mucus producing cells are significantly reduced with CP17 treatment. E) Comparison of lung resistance of animals, which received polyI:C, polyI:C and SP17 or CP17 respectively. No significant difference was observed. F) Comparison of dynamic compliance of animals, which received polyI:C, polyI:C and SP17 or CP17 respectively. No significant difference was observed. *$p<0.05$, ***$p<0.001$, n.s. = not significant.

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Exa SP17 CP17
0
10
20
30
40
50
surface covered by
goblet cells (%)
*
Figure 3
A)
PBS SP17 CP17 OVA SP17 CP17 Exa SP17 CP17
0.0

5.0Å~104
1.0Å~105
1.5Å~105
2.0Å~105
2.5Å~105
BAL Neutrophils (cells/ml)
*** ***
OVA OVA+ polyI:C
B)
PBS SP17 CP17 OVA SP17 CP17 Exa SP17 CP17
0.0
5.0Å~104
1.0Å~105
1.5Å~105
2.0Å~105
2.5Å~105
BAL cells (cells/ml)
macrophages lymphocytes eosinophils
OVA OVA+ polyI:C
***
n.s.
n.s.
C)
H&E PAS
Exacerbation SP-17 CP-17
D)
1 10 100
2
3
4
Methacholine (mg/mL) RI (
cmH2O/ml/s)
OVA EX CP17
OVA EX
OVA EX SCP
E)
1 10 100
0.00
0.02
0.04
0.06
Methacholine (mg/mL)
C_{dyn} (mL/cmH2O)
OVA EX CP17
OVA EX
OVA EX SCP
F)
n.s.
n.s.
Supplement Figure 1
ROS (relative chemiluminescence units)
0
1
2
3
20 40 60
0
100%
100%
SP17
CP17
50%
50%
$A_{SP17} = A_{CP17}$
$m_{SP17} > m_{CP17}$
Time (min)
A)
0.0
2.0
4.0
6.0
8.0
10.0

IL-8 release (ng/ml)
CP17 SP17
control 0.5 5.0 50.0
10 100 1000 10000
0
100
200
300
time (sec)
RLU
Vehicle Medium
10 100 1000 10000
0
100
200
300
time (sec)
RLU
CP17 SP17
10 100 1000 10000
0
100
200
300
time (sec)
RLU
CP17 SP17
10 100 1000 10000
0
100
200
300
time (sec)
RLU
CP17 SP17
50µM 5.0µM 0.5µM control
B)
C) R848
0
20
40
60
Neutrophil Elastase (ng/mL)
50.0 5.0 0.5 Control
CP17 SP17
D) R848 E) PMA Stimulation
0
20
40
60
80
peptide concentration (µM)
Number of NETs (·10^3)
0.0 0.5 5.0 50.0
CP17 SP17
PBS SP17 CP17
0
10
20
30
40
50
surface covered
by goblet cells (%)
OVA groups
n.s.
Macrophages Lymphocytes Eosinophils
0
1Å~10^5
2Å~10^5
Cell number
BAL Cells
PBS, SP17 PBS, CP17
LPS, SP17 LPS, CP17
**
n.s.
**

n.s.
n.s.
BAL Neutrophils
0.0
5.0Å~104
1.0Å~105
1.5Å~105
2.0Å~105
2.5Å~105
Cell number
##
*
PBS LPS
SP17 CP17
Exacerbation SP17 CP17
0
5
10
15
ΔCT (vs. ACT)
CXCL1
n.s. n.s.
Exacerbation SP17 CP17
0
5
10
15
ΔCT (vs. ACT)
CCL20
*
n.s.
n.s.
0
2
4
6
8
10
Methacholine (100mg/mL)
RI (cmH2O/ml/s)
PBS SCP CP17 PBS SCP CP17 PBS SCP CP17
OVA OVA + PolyI:C
****
****
****
0.00
0.02
0.04
0.06
0.08
0.10
Methacholine (100mg/mL)

C_{dyn} (mL/cmH_2O)
PBS SCP CP17 PBS SCP CP17 PBS SCP CP17
OVA OVA + PolyI:C
****
*
n.s.
Supplement Figure 2
A) B)
C) D)
E) F)
PAS+ Cells
PBS PBS LPS LPS
0
50
100
150
MPC (per HPF)
**
SP17 CP17
###
n.s.
G) H)

Supplement

Legends:

Supplement Figure 1: Effects of CP17 on ROS kinetics, degranulation, secretion and NETosis. A) Production rate after CP17 exposure shows repressed kinetics. Representative line-graphs of ROS production with and without CP17 and SP17 treatment. Relative light units (RLU) are plotted vs. time in seconds. Logarithmic x-axis for improved display of ROS production ramp-up phase. B) Schematic drawing of ROS production: Treatment with SP17 results in an unaltered rate of production (red curve) of ROS over time. At the half-maximal level of ROS production, the rate of ROS production (slope m) is determined. Green curve shows ROS production with CP17 treatment. Both areas (red, green) under the curve are equal and an end-point measurement of accumulated ROS would detect no difference. C) IL-8 secretion after R848 stimulation is not altered with CP17 or SP17 preincubation. D) Degranulation (neutrophil elastase) is not significantly altered after CP17 or SP17 treatment after R848 stimulation. E) NETosis is not altered with CP17 or SP17 treatment. Neutrophils were stimulated with 10nM PMA and fluorescent signal of secreted DNA was measured after 2h and referenced to standard curve. Plot displays number of NETs vs. peptide concentration. Dashed line CP17, straight line SP17, x-axis at logarithmic scale.

Supplement Figure 2: CP17 has no effect on lung function and chemokine production in exacerbating animals. Animals were prepared as described in the methods sections. A) Comparison of lung resistance at 100mg/mL methacholine in animals, which received PBS, SP17, CP17 only, OVA alone or with respective peptides and polyI:C together SP17 or CP17. Significant increases in airway resistance were observed OVA vs. PBS and PolyI:C vs. OVA. ****$p<0.0001$ B) Comparison of lung compliance at 100mg/mL methacholine in animals, which received PBS, SP17, CP17 only, OVA alone or with respective peptides and polyI:C together SP17 or CP17. *$p<0.05$, ****$p<0.0001$, n.s. = not significant C) Neither CP17 nor SP17 shows an impact on total lung mRNA expression of CXCL1 (KC). n.s. = not significant D) CCL20 mRNA expression in total lung homogenates is not significantly altered after CP17 treatment. *$p<0.05$, n.s. = not significant E) CP17 reduces bronchoalveolar lavage neutrophil cell counts in a mouse model of neutrophil asthma when compared to SP17. # $p<0.05$ OVA+LPS vs. PBS, ## $p<0.01$ OVA+LPS vs. PBS, * $p<0.05$ CP17 vs. SP17 in OVA+LPS F) Macrophages and lymphocytes are increased in bronchoalveolar lavage after OVA and LPS challenge (neutrophilic asthma mouse model) but remain unchanged after peptide treatment. No effects observed for eosinophil granulocytes in BAL. **$p<0.01$, PBS vs. OVA+LPS G) Assessment of mucus producing cells in OVA only groups using a

newCAST system. Number of mucus producing cells are not altered with CP17 treatment. H) Mucus producing cells (MPC) are significantly reduced after CP17 (vs. SP17) treatment in animals with OVA+LPS challenge. PBS vs. OVA+LPS CP17 n.s. = not significant, PBS vs. OVA+LPS SP17 ### $p<0.001$, ** $p<0.01$

Other Supporting Information

The R code for the migration analysis is publicly available here:
https://github.com/cells2numbers/2017_migration_analysis_tumstatin

Supplement Methods:

SiMA Tracking quality measures

To further assess the quality of the tracking results we followed the scheme presented in [27] and calculated the valid observation time (VOT) which is defined as the length of all valid trajectories divided by the sum of the length of all valid and invalid trajectories. The VOTs for all analysed experiments are summarised in Suppl. Table ST1 and reveal a high tracking quality with an average VOT of 0.84.

NETosis Screening Assay

Assay was performed as previously described [59], Briefly, neutrophil concentration was set to $1x10_6$/mL in medium (0.5% BSA in RPMI) including a standard curve in 5-plets. Final number of cells were (multiplied by 10_3) 75, 50, 25, 12.5, 6.25 neutrophils per well (steril 96-well plate, clear bottom, black rims, Corning, Simga-Aldrich, Germany). For stimulation, each well contained 35.000 neutrophils in 100µL 0.5% BSA/RPMI1640 (Biochrom AG, Berlin, Germany) and 10nM PMA (in DMSO vehicle) was used for stimulation. Neutrophils were pre-incubated with SP17 or CP17 at 0.5, 5.0 and 50.0 µM for 1h at 37ÅãC, 5%CO_2 before 10nM PMA was added and final incubation for 2.5h. The 96-well plate was then centrifuged for 5min at 500rcf, 2µg/mL propidium-iodide was added to each well (incubated for 15min) and plate was measured (ex544nm, em590nm) with spectrometer (FLUOStar Omega, BMG, Germany). From the standard curve the fluorescence signal was used to calculate NETs numbers. All measurements were performed as replicates of 5.

Stimulation for secretion and degranulation

Neutrophil concentration adjusted to $1x10_6$/mL in medium (0.5% BSA in RPMI). Cells were preincubated with SP17 or CP17 at 0.5, 5.0 and 50.0 µM for 1h at 37ÅãC, 5%CO_2 and subsequently stimulated (vehicle control for peptides, H_20) with 1.5 µg/mL R848 (Invivogen). Cells were incubated at 37ÅãC with 5%CO_2 for 1 h NE and MMP-9 measurements, or 24 h for CXCL8 measurements. Cell-free supernatant and neutrophil cell pellets were collected and stored at −80ÅãC for analysis.

IL-8 enzyme-linked immunosorbent assay (ELISA)

CXCL8 production was measured using a sandwich ELISA in duplicate. Specific ELISA kits from R&D Systems (Minneapolis, MN, USA) were used according to the manufacturer's instructions. Detection limit was 15.6 pg/mL. The plate was then measured with a spectrometer (FLUOStar Omega, BMG, Germany).

NE activity assay

NE activity was measured in triplicates using a fluorescent assay from Cayman Chemicals (Ann Arbor, MI, USA) according to the manufacturer's instructions. Fluorescence readings from samples were compared with a standard curve of known concentrations of NE to determine the concentration. Detection limit was 3.1 ng/mL.

Mouse Model with LPS-OVA

Exacerbation model was established as previously described [30]. Briefly, mice were sensitized to OVA/LPS by inhalation (OVA grade VI, Sigma, Deisenhofen, Germany) on days 1 and 7. Mice were exposed three times to an OVA (OVA grade V, Sigma) aerosol (1% wt/vol in PBS) on days 14 and15 in order to induce acute allergic airway inflammation. An amount of 1nmole CP17, vehicle (PBS) or scrambled peptide (SP17) was co-instilled after the OVA exposure and on day 14 and 15. All animals were sacrificed by cervical dislocation under deep anesthesia on day 16. Control animals were sham sensitized to PBS, received treatment and were subsequently challenged with PBS aerosol (PBS group). Bronchoalveolar lavage, lung tissue samples and serum were collected.