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Carboxypeptidase inhibition by NvCI suppresses airway hyperreactivity in a mouse asthma model

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To the Editor,

Mast cells are implicated in asthma and an emerging body of evidence suggests that proteases released by mast cells are crucial players in asthma pathogenesis¹. The mast cell-restricted proteases, i.e. tryptase, chymase and carboxypeptidase A3 (CPA), are stored in large amounts as active enzymes in secretory granules in mast cells. Experimental studies in mouse models suggest that tryptase can contribute to airway hyperreactivity (AHR), a hallmark of asthma, whereas chymase can attenuate several features of asthma including AHR and IL-33 accumulation in lungs^{2,3}. In contrast, there is very limited information on the role of CPA3 in asthma pathogenesis. However, findings from clinical studies suggest a link between Th2-high asthma and expression of CPA3 in the lungs^{4,5}. CPA3 is a proteolytic enzyme with CPA-like activity, which means that it can remove aromatic and aliphatic residues at the C-terminal end of target proteins and peptides⁶. Considering that lung mast cells release large amounts of proteolytically active CPA3 upon degranulation, we hypothesised that CPA activity in lung tissue could have a profound effect on the asthma response.

Here we investigated the contribution of CPA activity to features of asthma using the *Nerita versicolor* carboxypeptidase inhibitor (NvCI) in a mouse model of allergic asthma. NvCI is the strongest natural CPA-inhibitor described so far, with inhibitory constants in the picomolar range, and also displays remarkable bioavailability and resistance⁷. NvCI is highly specific for the M14A carboxypeptidase family, including CPA3. To confirm the inhibitory effect of NvCI on mast cell CPA3, we used a chromogenic CPA substrate (M-2245/AAFP). We found that addition of NvCI completely inhibited CPA activity in supernatants of murine mast cells that were degranulated by IgE crosslinking or calcium ionophore (Figure 1A). Moreover, we detected weak CPA activity in human lung homogenates that was undetectable after adding NvCI (Figure 1B). These findings confirm that NvCI is a potent inhibitor of CPA activity derived from mast cells and located in lung tissue.

We then investigated the effect of CPA inhibition in a house dust mite (HDM)-induced asthma model. Female mice of the BALB/c strain were pre-treated with intraperitoneal injections of NvCI followed by intranasal instillations with HDM extract twice weekly for three weeks (Figure S1). Control groups of mice received HDM, buffer (PBS) or NvCI alone, at the same days. Resident mast cells in mouse lungs contain substantial amounts of CPA^{1,8} and we found increased degranulation of these mast cells upon HDM-exposure (Figure S2), hence indirectly confirming the release of CPA in this model. Moreover, we confirmed the absence of CPA-like activity in the HDM extract (data not shown) and found that NvCI-treatment of mice reduced the total CPA-activity in lung homogenates (Figure 1C). We assessed AHR by measuring the change in lung resistance and dynamic compliance in response to nebulised methacholine compared with baseline values (PBS controls). As expected, mice challenged with HDM exhibited increased lung resistance and decreased dynamic compliance compared with control mice, whereas mice injected with NvCI alone did not differ from controls (Figure 1D). Notably, there was no AHR in the group of NvCI-treated HDM-challenged mice, as demonstrated by normalisation of airway resistance and dynamic compliance in these mice (Figure 1D). This strongly supports the notion that CPA activity contributes to development of AHR in the HDM-induced asthma model. To determine whether the NvCI-mediated attenuation of AHR was accompanied by a decrease in airway inflammation, we analysed HDM-induced accumulation of total cells, eosinophils, macrophages, lymphocytes and neutrophils in bronchoalveolar lavage (BAL). We found that NvCI treatment did not have any statistically significant effect on total or differential cell counts in BAL, although there was a trend for decreased numbers of eosinophils in NvCI-pretreated, HDM-challenged mice (Figure 1E-I).

To determine the influence of NvCI on tissue inflammation and remodelling in the asthma model, lung sections were stained with periodic acid-Schiff (PAS) or haematoxylin and eosin and analysed for goblet cell accumulation, inflammatory cell infiltrates and thickening of airway smooth muscle layer. The results showed that NvCI significantly decreased accumulation of goblet cells in airway epithelium (Figure 2A, 2B), suggesting that CPA activity contributes to epithelial remodelling induced by HDM exposure. In agreement with the lack of effect on the inflammatory cells of the BAL fluid, NvCI did not influence the HDM-induced infiltration of inflammatory cells around airways or blood vessels (Figure 2C-E). Thickness of the smooth

muscle layer in lung tissue did not differ between groups, and hence no influence of NvCI was detected (Figure 2F).

We have previously shown that extracellular levels of IL-13 and IL-33 can be limited by proteolytic degradation exerted by mast cell serine proteases, including chymase.^{3,9} Notably, intra-epithelial mast cells in asthma exhibit an aberrant protease profile (tryptase⁺, CPA3⁺ and chymase⁻)⁴ and was recently shown to augment epithelial IL-33¹⁰, suggesting an overall detrimental role of these mast cells. To examine the effect of CPA inhibition on cytokine concentrations, we measured a panel of pro-inflammatory cytokines in lung tissue extracts by ELISA. Concentrations of IL-5, IL-33 and CXCL-1 were increased upon HDM-challenge but there was no statistical difference between NvCI-treated and non-treated groups of HDM-challenged mice (Figure 2G). Hence we found no evidence for a regulatory role of CPA activity on levels of cytokines in this model.

To summarise, we demonstrated that NvCI can efficiently inhibit CPA activity derived from murine mast cells or human lungs. In the HDM-induced asthma model, pre-treatment with NvCI reduced the lung CPA-activity and protected against AHR and reduced goblet cell hyperplasia, whereas inflammatory cell-infiltration and cytokine levels were largely unaffected. This suggests that CPA activity is critical for AHR development and contributes to epithelial remodelling in this asthma model. It also suggests that CPA activity has a selective, local effect on airway smooth muscle and epithelium, compatible with the demonstrated accumulation of mast cells in these tissue compartments in asthma^{1,4}. Although inhibition of mast cell CPA3 is a likely explanation for the dampening effect of NvCI in the asthma model, inhibition of other M14A carboxypeptidases cannot be excluded⁷. To discriminate between the role of CPA3 and other carboxypeptidases, future studies including a battery of selective inhibitors of such enzymes and knockout mice are warranted. Our findings provide significant evidence for a detrimental role of carboxypeptidase in experimental asthma and highlight the biomedical potential of targeting carboxypeptidase activity in future asthma therapies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

FIGURE LEGENDS

FIGURE 1 Carboxypeptidase inhibitor NvCI inhibits CPA-like activity and reduces AHR, but does not modify BAL leukocyte influx following HDM challenge. **A**, CPA-like activity in mast cell supernatants, **B**, in human lung, and, **C**, in mouse lung homogenates (udl, under detection level). **D**, Lung resistance (R_L) and dynamic compliance (C_{dyn} ; $n=6-9$). HDM versus control (PBS): $**P<0.01$, $***P<0.001$; HDM versus NvCI + HDM $\dagger\dagger\dagger P<0.001$; HDM versus NvCI $++P<0.01$ (two-way ANOVA). **E-I**, Total and differential counts of cells in BAL ($n=6-9$). $*P<0.05$, $**P<0.01$, $***P<0.001$ (one-way ANOVA). Data were obtained from 4 independent experiments.

FIGURE 2 HDM-induced tissue inflammation and remodelling in NvCI-treated mice. **A**, Periodic acid-Schiff's (PAS) staining (arrows) of lung sections. **B**, Number of PAS⁺ cells per μm airway epithelia ($n=6-9$). **C-E**, Leukocyte infiltrates in the lung tissue ($n=6-9$). **F**, Thickness of airway smooth muscle layer ($n=6-8$). **G** and **H**, Cytokine concentrations in lung homogenates ($n=6-9$). $*P<0.05$, $**P<0.01$, $***P<0.001$ (one-way ANOVA). Data were obtained from 4 independent experiments.

Figure 1

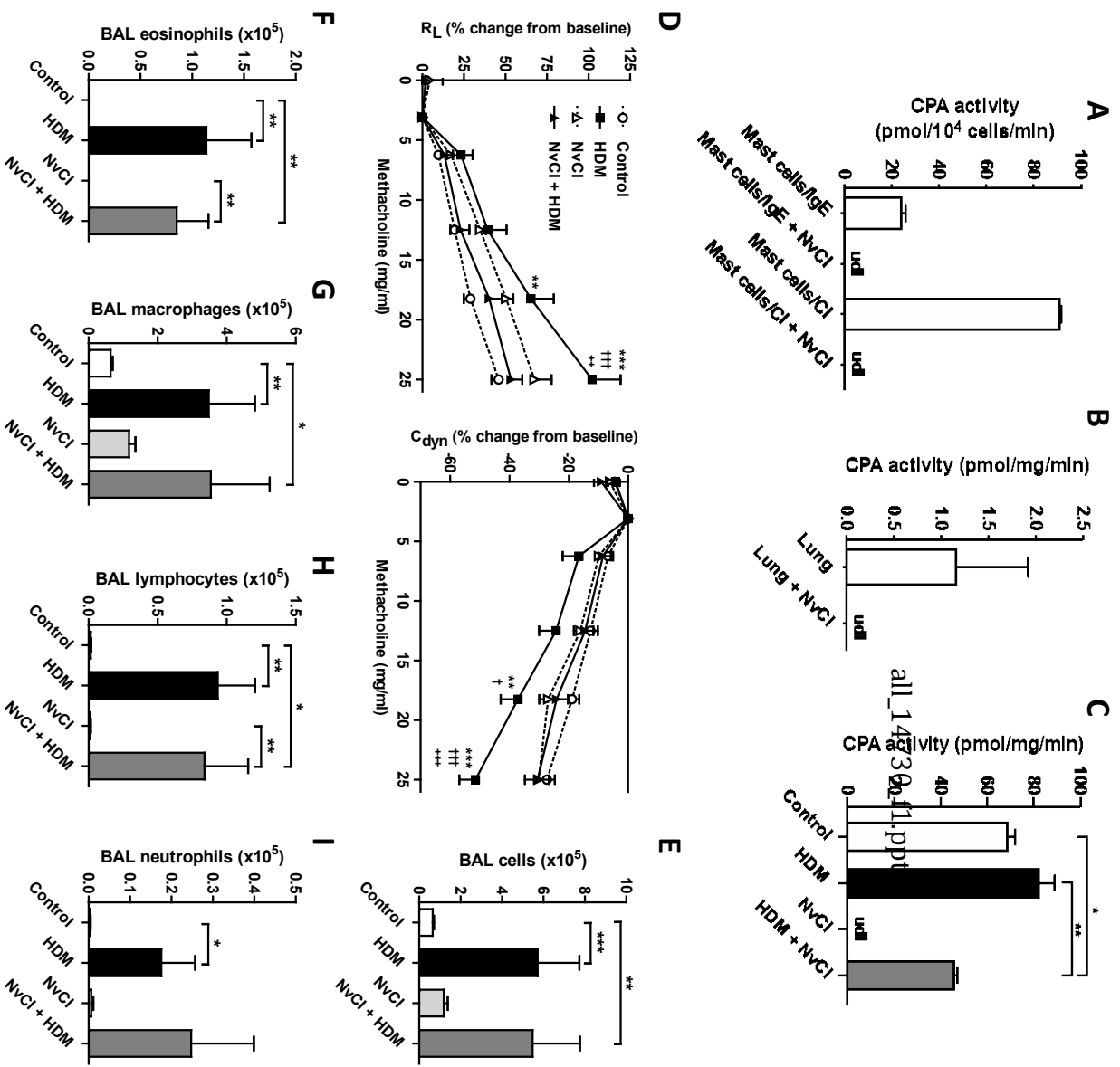
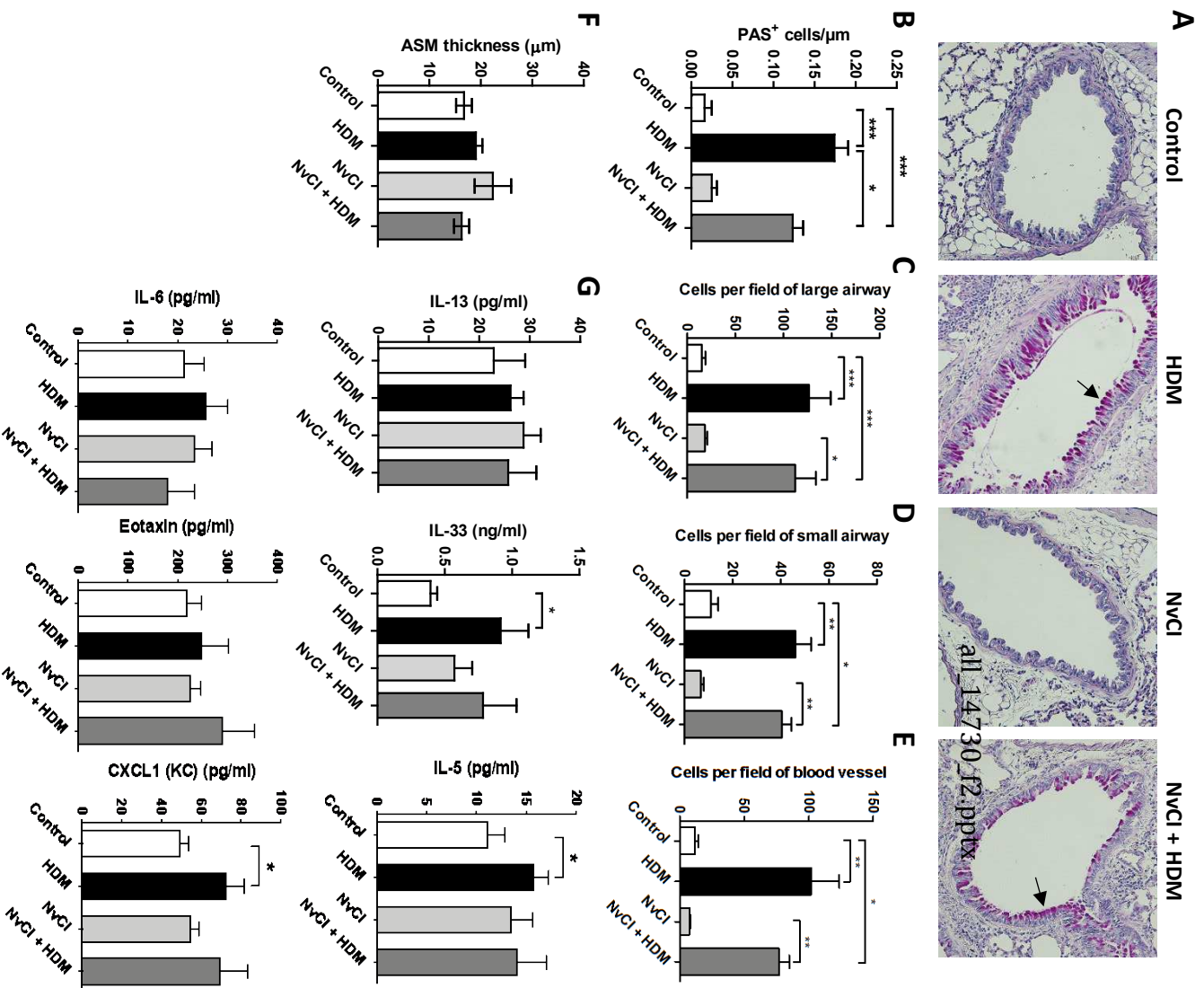


Figure 2



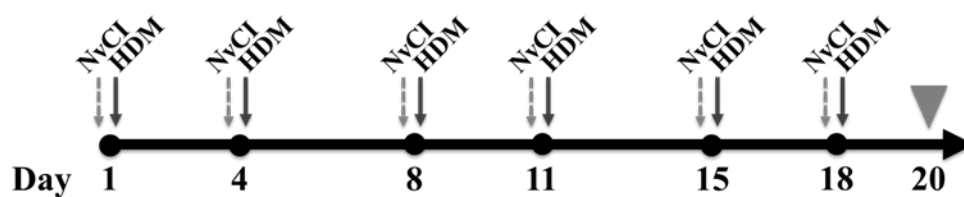


FIGURE S1 Protocol for treatment with NvCI in a mouse model of asthma. Mice were given six repeated intranasal instillations during three weeks with HDM extract (3 μ g protein in 30 μ l PBS) or PBS alone. Groups of mice were treated with 20 mg/kg NvCI by intraperitoneal injections 30 minutes before every intranasal HDM-challenge. Control mice received NvCI alone. On day 20, AHR to methacholine was assessed and BAL fluid and lung tissue were collected.

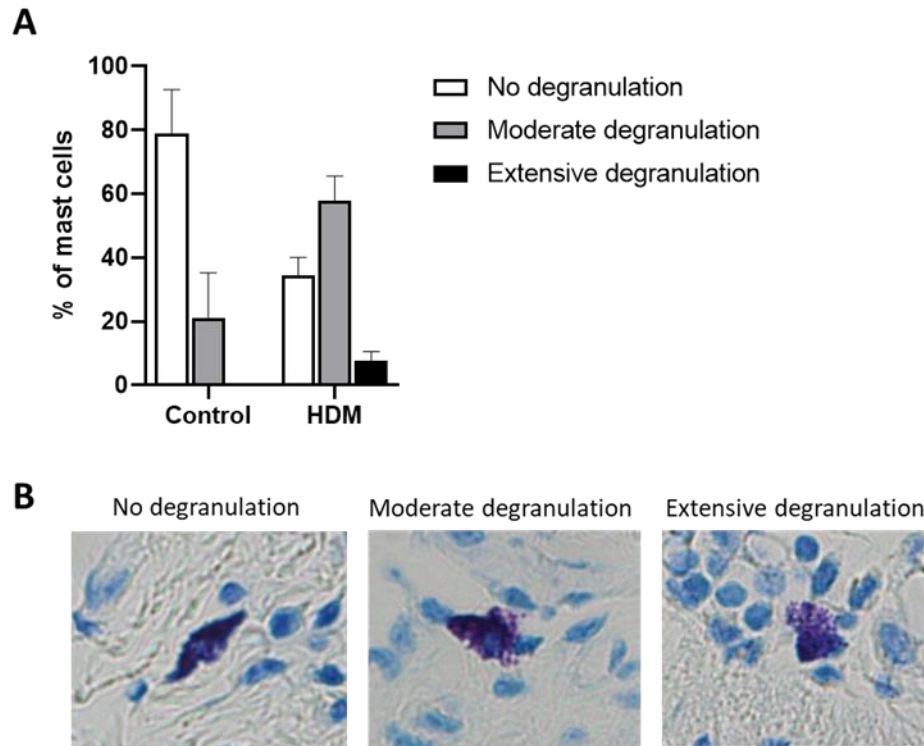


FIGURE S2 Increased mast cell degranulation upon HDM-exposure. Lung tissue sections from mice given six repeated intranasal instillations with either 3 μ g HDM extract (HDM; n=6) or PBS alone (control; n=5) were stained with Toluidine blue. Mast cells around larger airways were categorized into three groups depending on the degree of mast cell degranulation (no, moderate or extensive). A, Data is presented as % of mast cells in each category (mean + SEM). A multinomial logistic mixed model demonstrated a significant effect ($\text{Pr} > F$ 0.01) of HDM-treatment on the degree of degranulation. B, Representative pictures of mast cells.

MATERIALS AND METHODS

Mice and house dust mite-induced allergic airway inflammation

Female BALB/c mice at 9-12 weeks of age (Taconic Biosciences, Lille Skensved, Denmark) were anaesthetised with isoflurane and instilled intranasally on days 1, 4, 8, 11, 15 and 18 with 3 µg house dust mite (HDM) extract from the species *Dermatophagoides farinae* (Greer Laboratories, Lenoir, NC), dissolved in 30 µL PBS, as previously described¹. Non-inhibitor treated mice received HDM alone (HDM group, n = 9), and inhibitor-treated mice were injected intraperitoneally with 20 mg/kg *Nerita versicolor* carboxypeptidase inhibitor (NvCI)² 30 minutes before each intranasal challenge with allergen (NvCI + HDM group, n = 8). Groups of mice were injected intraperitoneally with NvCI alone (NvCI group, n = 6) or were instilled intranasally with PBS alone (Control group, n = 8) on days 1, 4, 8, 11, 15 and 18. All mouse experiments were approved by the local ethics committee (Dnr. C162/14) and were conducted in accordance with the EU Directive 2010/63/EU for animal experiments.

Measurement of airway hyperreactivity

Mice were anaesthetised with pentobarbital sodium (50 mg/kg) (Sigma-Aldrich, St Louis, MO), tracheostomised, intubated to a small animal ventilator (FinePointe, Buxco, Winchester, UK) and ventilated at 160 breaths/min with a tidal volume of 0.25 mL. Prior to measurements, mice were briefly ventilated to reach an acclimatisation baseline. Lung function was assessed with a dose-response curve to increasing doses of aerosolised methacholine (Sigma-Aldrich) via the tracheal cannula. Lung resistance (R_L) and dynamic compliance (C_{dyn}) are shown as percentage change from baseline.

BAL and lung tissue sampling and homogenisation

Bronchoalveolar lavage (BAL) was performed, directly after lung function assessment, with 2 x 1 mL Hank's balanced salt solution (HBSS, Sigma Aldrich). Cytospin slides were prepared and percentage of leukocyte populations was determined after conventional May Grünwald/Giemsa staining. For

cytokine measurements, right lung lobes were frozen on dry ice and stored at -70°C. Lung tissue was homogenised in PBS containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) at a concentration of 50 mg lung tissue/mL buffer. Homogenates were centrifuged at 800 x g at 4°C for 8 min, and supernatants were collected for cytokine measurements using ELISA.

Cytokine ELISA

Cytokine levels in mouse lung homogenates was determined with ELISA kits for IL-5, IL-6, IL-33 (eBioscience, San Diego, CA, USA), CCL11/eotaxin (R&D Systems, Minneapolis, MN, USA) and CXCL1/KC (Thermo Scientific Inc, Waltham, MA, USA) according to the manufacturer's instructions.

Lung histology

Left lung lobes from mice were preserved in 4% paraformaldehyde, paraffin-embedded, sectioned and stained with conventional haematoxylin and eosin staining. Tissue inflammation was evaluated by blinded counting of infiltrated cells per defined field of lung sections measuring 0.06 mm². The mean number of cells from at least three randomly selected regions around small airways, large airways and blood vessels were used for statistical calculations. Thickness of the airway smooth muscle (ASM) layer around similar-size bronchi was measured with a Nikon Microphot-FXA microscope using a 10x objective lens (Bergström Instrument AB, Stockholm, Sweden) and Eclipse Net software (version 1.20, Developed by Laboratory Imaging, Prague, Czech Republic) on haematoxylin and eosin-stained sections. ASM layer thickness was measured every 30 µm around the bronchi and average thickness was calculated. The effect of HDM-exposure on mast cell degranulation was evaluated by examining Toluidine blue stained lung tissue sections. Mast cells around larger airways were categorized into three groups depending on the degree of mast cell degranulation (no, moderate or extensive). A multinomial logistic mixed model demonstrated a significant effect ($P < 0.01$) of HDM-treatment on the degree of degranulation. Mast cells from the HDM-treated group had a higher predicted probability of moderate (0.6673) or extensive (0.0701) degranulation than mast cells from the control group (0.2673 for moderate and 0.0135 for extensive degranulation). For periodic acid-Schiff's (PAS)

staining, slides were deparaffinised, hydrated, oxidised in 0.05% periodic acid for 5 min, washed in H₂O, stained with Schiff's reagent (Sigma-Aldrich) for 30 min, washed in H₂O for 3 min and counterstained with Mayer's haematoxylin for 30 s. Number of goblet cells per μ m basement membrane (airway epithelia) was counted with a Nikon Microphot-FXA microscope using a 40x objective lens and Eclipse Net software. All histological assessments were performed by trained investigators blinded to experimental information.

Carboxypeptidase A activity and inhibition

Bone marrow-derived mast cells (BMMCs) were cultured as previously described³. IgE anti-TNP (BD Biosciences Pharmingen, Stockholm, Sweden) was added to 1×10^6 BMMCs at 1 μ g/mL, followed by incubation overnight at 37°C. Cells were washed three times and resuspended in Tyrode's buffer. OVA-TNP was added (0.4 μ g/mL final concentration) and supernatant was collected after 1 h. For calcium ionophore activation of BMMCs, A23187 was used at a final concentration of 2 μ M. Tumor-free human lung tissue was obtained from lung cancer patients as previously described⁴ with approval by the local ethics committee (Dnr 2013/223). Lung tissue was homogenised in PBS containing EDTA-free protease inhibitor cocktail (Roche Diagnostics) at a concentration of 50 mg lung tissue/mL buffer. Homogenates were centrifuged at 800 x g at 4°C for 8 min, and supernatants were collected for further analysis. Carboxypeptidase A (CPA) activity in HDM extract (200 mg/mL), lung tissue and BMMC supernatants, with or without the addition of 10 μ g/mL NvCI,² was measured using the chromogenic CPA substrate M2245/AAFP, constituted by the N-(4-Methoxyphenylazoformyl)-L-phenylalanine derivative⁵, according to the manufacturer's instructions (Bachem, Bubendorf, Switzerland)^{6,7}. The reaction was allowed to proceed for 2 h at room temperature and absorbance was measured at 405 nm every 10 min using a FARCyte microplate reader (Amersham Biosciences, Uppsala, Sweden). In separate experiments, lungs were collected from NvCI-treated HDM-exposed mice and from control mice given either HDM, PBS or NvCI alone. Lung tissue was homogenised at a concentration of 50 mg lung tissue/mL in lysis buffer (10 mM Tris-HCl pH 7.4, 4 M NaCl, 0.1% w/v PEG 3350) containing EDTA-free protease inhibitor cocktail, mixed

under vortex agitation for 15 min and then centrifuged at 16 000 x g at 4°C for 20 min. Measurement of CPA activity in supernatants was performed as previously described⁸. Enzymatic activity, expressed as pmol/mg/min or pmol/cell/min, was calculated from the change of absorbance over time and from the initial substrate concentration.

Statistical analysis

Statistical significances were calculated by two-way ANOVA for airway reactivity measurements and one-way ANOVA for differential counting, ELISA and histology. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used for statistical analyses. All values are displayed as mean $\pm$ SEM and $P < 0.05$ was considered significant. A multinomial logistic mixed model was used to model the probabilities for different degranulation degrees.

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