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

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 pathogenesis.[1] The mast cell–restricted proteases, that is 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 peptides.[6] Considering that lung mast cells release large amounts of proteolytically active CPA3 upon degranulation, we hypothesized 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 resistance.[7] NvCI is highly specific for the M14A carboxypeptidase subfamily, 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 cross-linking 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 pretreated 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 nebulized 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 normalization of airway resistance and dynamic compliance in these mice (Figure 1D). This strongly supports the notion that CPA activity contributes to the 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, B), 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-33,[10] suggesting an overall detrimental role of these

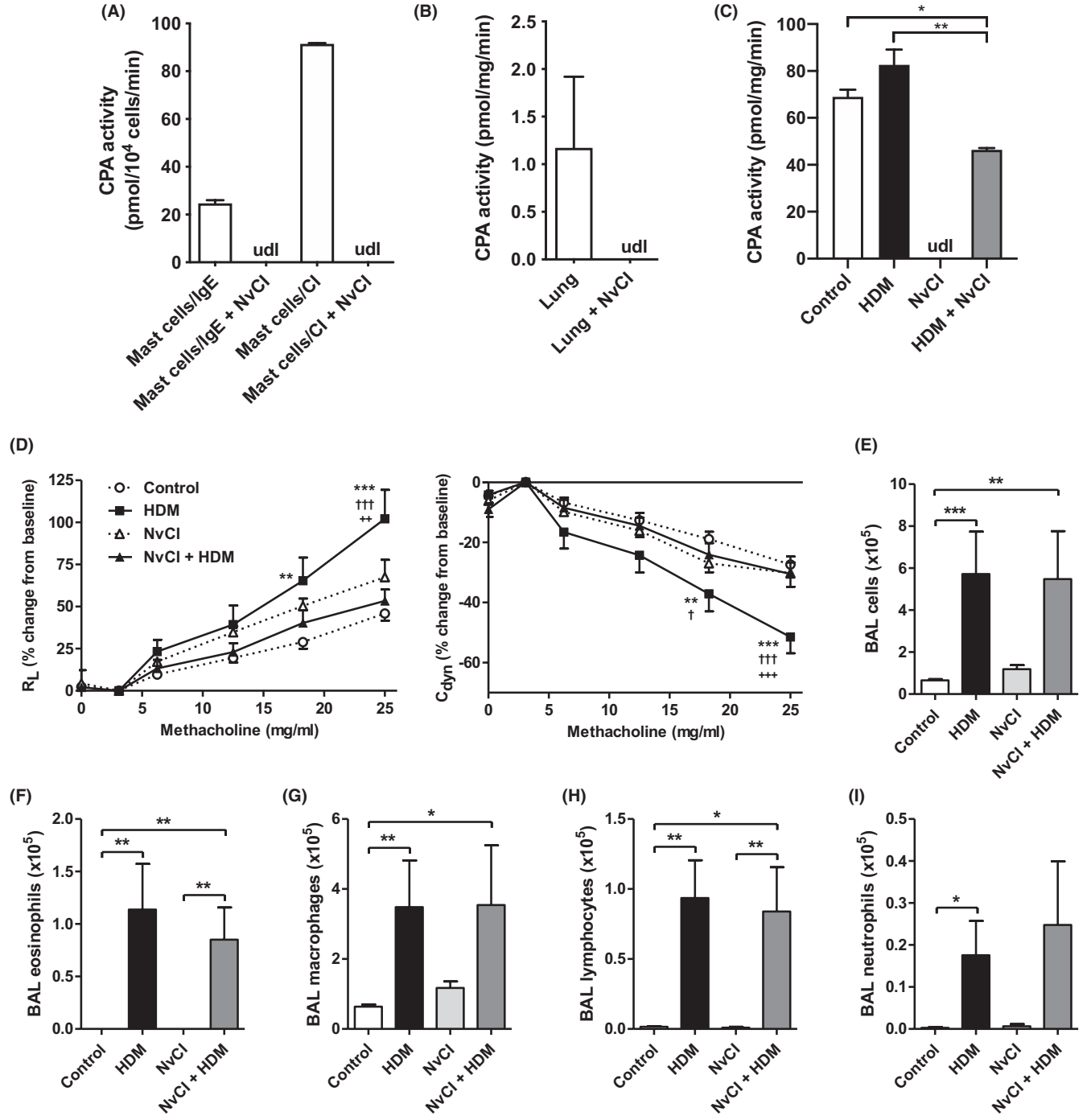

FIGURE 1 Carboxypeptidase inhibitor NvCI inhibits CPA-like activity and reduces AHR, but does not modify BAL leucocyte influx following HDM challenge. A, CPA-like activity in mast cell supernatants, B, in human lung and C, in mouse lung homogenates (udl, under detection level). D, Lung resistance (R_L) and dynamic compliance (C_{dyn}; n = 6–9). HDM versus control (PBS): $^{**}p < 0.01$, $^{***}p < 0.001$; HDM versus NvCI +HDM ††† $p < 0.001$; 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

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 summarize, 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

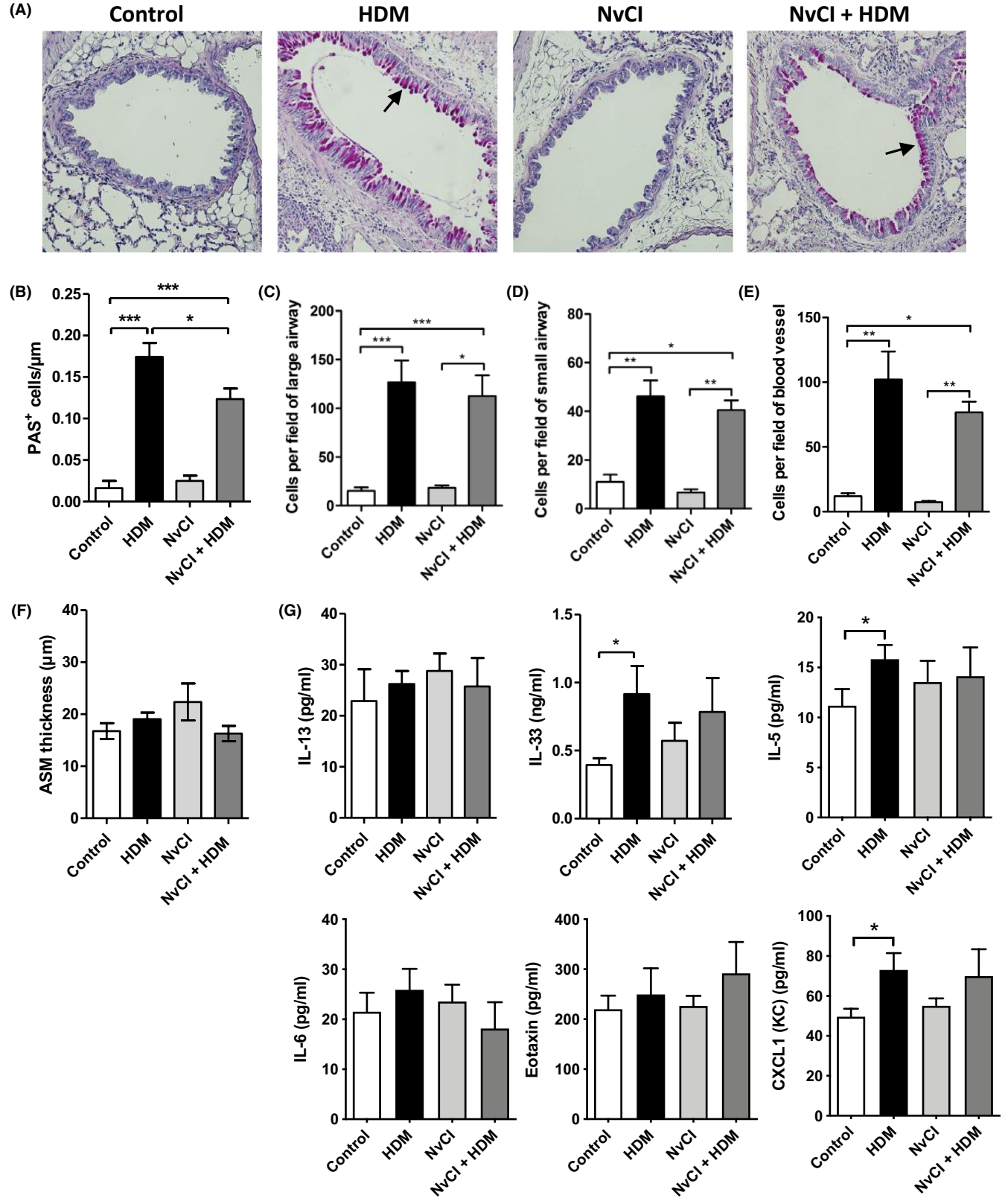

FIGURE 2 HDM-induced tissue inflammation and remodelling in NvCl-treated mice. A, Periodic acid-Schiff's (PAS) staining (arrows) of lung sections. B, Number of PAS^+ cells per µm airway epithelia (n = 6–9). C-E, Leucocyte infiltrates in the lung tissue (n = 6–9). F, Thickness of airway smooth muscle layer (n = 6–8). G, 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

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 excluded.[7] 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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KEYWORDS

asthma, carboxypeptidase activity, house dust mite, mast cell, NvCI carboxypeptidase inhibitor

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

1. Pejler G. The emerging role of mast cell proteases in asthma. *Eur Respir J* 2019;54(4):1900685.
2. Cui Y, Dahlin JS, Feinstein R, et al. Mouse mast cell protease-6 and MHC are involved in the development of experimental asthma. *J Immunol* 2014;193(10):4783-4789.
3. Waern I, Lundequist A, Pejler G, Wernersson S. Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Mucosal Immunol* 2013;6(5):911-920.
4. Dougherty RH, Sidhu SS, Raman K, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in TH2-high asthma. *J Allergy Clin Immunol* 2010;125(5):1046-1053 e8.
5. Fricker M, Gibson PG, Powell H, et al. A sputum 6-gene signature predicts future exacerbations of poorly controlled asthma. *J Allergy Clin Immunol* 2019;144(1):51-60 e11.
6. Tanco S, Lorenzo J, Garcia-Pardo J, et al. Proteome-derived peptide libraries to study the substrate specificity profiles of carboxypeptidases. *Mol Cell Proteomics*. 2013;12(8):2096-2110.
7. Covaleda-Cortes G, Hernandez M, Trejo SA, et al. Characterization, recombinant production and structure-function analysis of NvCI, a picomolar metallocarboxypeptidase inhibitor from the marine snail nerita versicolor. *Mar Drugs* 2019;17(9):511.
8. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proc Natl Acad Sci USA* 2011;108(34):14210-14215.
9. Waern I, Karlsson I, Thorpe M, et al. Mast cells limit extracellular levels of IL-13 via a serglycin proteoglycan-serine protease axis. *Biol Chem* 2012;393(12):1555-1567.
10. Altman MC, Lai Y, Nolin JD, et al. Airway epithelium-shifted mast cell infiltration regulates asthmatic inflammation via IL-33 signaling. *J Clin Invest* 2019;129(11):4979-4991.

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