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RESEARCH ARTICLE

Direct vagus nerve stimulation: A new tool to control allergic airway inflammation through α7 nicotinic acetylcholine receptor

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Abstract

Background and Purpose: Asthma is characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness. The use of nicotinic agents to mimic the cholinergic anti-inflammatory pathway (CAP) controls experimental asthma. Yet, the effects of vagus nerve stimulation (VNS)-induced CAP on allergic inflammation remain unknown.

Experimental Approach: BALB/c mice were sensitized and challenged with house dust mite (HDM) extract and treated with active VNS (5 Hz, 0.5 ms, 0.05–1 mA). Bronchoalveolar lavage (BAL) fluid was assessed for total and differential cell counts and cytokine levels. Lungs were examined by histopathology and electron microscopy.

Key Results: In the HDM mouse asthma model, VNS at intensities equal to or above 0.1 mA (VNS 0.1) but not sham VNS reduced BAL fluid differential cell counts and alveolar macrophages expressing α7 nicotinic receptors (α7nAChR), goblet cell hyperplasia, and collagen deposition. Besides, VNS 0.1 also abated HDM-induced elevation of type 2 cytokines IL-4 and IL-5 and was found to block the phosphorylation of transcription factor STAT6 and expression level of IRF4 in total lung lysates. Finally, VNS 0.1 abrogated methacholine-induced hyperresponsiveness in asthma mice. Prior administration of α-bungarotoxin, a specific inhibitor of α7nAChR, but not propranolol, a specific inhibitor of β2-adrenoceptors, abolished the therapeutic effects of VNS 0.1.

Conclusion and Implications: Our data revealed the protective effects of VNS on various clinical features in allergic airway inflammation model. VNS, a clinically

Abbreviations: α-BGTX, α-bungarotoxin; AM, alveolar macrophages; BAL, bronchoalveolar lavage; CAP, cholinergic anti-inflammatory pathway; Cdyn, dynamic compliance; HDM, house dust mite; α7nAChR, α7 nicotinic acetylcholine receptor; NMUR1, neuromedin U receptor 1; R_L, lung resistance; TEM, transmission electron microscopy; TRP, transient receptor potential; VNS, vagus nerve stimulation.

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approved therapy for depression and epilepsy, appears to be a promising new strategy for controlling allergic asthma.

KEYWORDS
asthma, bronchoalveolar inflammation, nicotinic receptors, vagus nerve stimulation

1 | INTRODUCTION

Asthma, one of the most common chronic inflammatory airway diseases, is estimated to affect some 339 million people worldwide (Ellwood et al., 2017). It is characterized by airway inflammation, mucus hypersecretion, collagen production, and airway hyperresponsiveness (AHR) (Page et al., 2016). These pathological features are caused by the infiltration of eosinophils and macrophages, increased production of proinflammatory mediators including Th2 cytokines **IL-4**, **IL-5**, and IL-13, and elevation of serum IgE level (Foster et al., 2017). The mainstay therapy for asthma, consisting of inhaled long-acting β_2-adrenoceptor agonist and inhaled corticosteroid (ICS), is able to control asthma symptoms in the majority of asthmatics (Beasley et al., 2019). However, patients with severe asthma, accounting for approximately 10% of all asthma cases, often respond poorly to ICS and require add-on therapies such as long-acting muscarinic antagonists, leukotriene modifiers, or biologics for full control of asthma symptoms. Unavoidably, patients are subject to increased risk of side effects (Holguin et al., 2020). As such, substantial effort has been made to discover novel anti-inflammatory strategies for asthma, especially for severe asthma.

Acetylcholine (ACh) is synthesized from acetyl coenzyme A and choline by the action of **choline acetyltransferase** (ChAT) (Kistemaker et al., 2014). ACh acts by binding to two classes of cholinergic receptors: metabotropic **muscarinic acetylcholine receptors** (mAChRs) that are G protein-coupled receptors with seven transmembrane domains and ionotropic **nicotinic acetylcholine receptors** (nAChRs) that are cation channels composed of a high diversity of subunits with two ACh binding sites (Wess et al., 2007). Stimulation of mAChRs is involved in airway smooth muscle contraction leading to airway hyperresponsiveness (Cazzola et al., 2021), whereas activation of nAChRs is believed to possess anti-inflammatory actions (Wang et al., 2003). The "Cholinergic Anti-inflammatory Pathway" (CAP) reflexively monitors and adjusts inflammatory responses by inhibiting proinflammatory cytokine synthesis through vagal efferent ACh release and **α7 nicotinic acetylcholine receptor** (α7nAChR) activation (Tracey, 2002). Resembling CAP activation, it has been shown that exogenous ACh or nicotine, by binding to α7nAChRs, inhibited LPS-induced TNF-α production from macrophages (Wang et al., 2003). More recently, α7nAChR agonists like nicotine, PNU-22987, and GTS-21, or **acetylcholinesterase** inhibitors like neostigmine, were found to attenuate allergic airway inflammation (Antunes et al., 2020; Gahring et al., 2020; Mazloomi et al., 2018; Yamamoto et al., 2014; Yuan et al., 2021). However, the role of CAP and the vagus nerve in these protective effects could not be confirmed in these studies (Cremin et al., 2023).

CAP activation can be achieved by pulsed electrical vagus nerve stimulation (VNS) to activate cholinergic efferents (Giebelen et al., 2007). VNS has been demonstrated to be an effective anti-inflammatory therapy in inflammatory disease models such as endotoxin-induced systemic inflammation (Borovikova et al., 2000), inflammatory bowel disease (Bonaz et al., 2016), and rheumatoid arthritis (Koopman et al., 2016). VNS was also found to be effective in alleviating histamine-induced acute bronchoconstriction in animal models (Hoffmann et al., 2009). In clinical studies, VNS has been approved by the FDA as a nondrug therapy for refractory partial-onset epilepsy since 1997 and a therapy for treatment-resistant depression since 2005 (Johnson & Wilson, 2018). Limited clinical data

What is already known

- The "Cholinergic Anti-inflammatory Pathway" (CAP) reflexively monitors inflammatory responses by inhibiting proinflammatory cytokine synthesis.
- Exogenous ACh binds to α7 nicotinic acetylcholine receptor (α7nAChR) to attenuate allergic airway inflammation.

What does this study add

- Vagus nerve stimulation (VNS) reduces inflammation and epithelial cell hyperplasia in a mouse asthma model.
- VNS protective effect on airway inflammation involves α7nAChRs but not β2 adrenergic receptors (β2AR).

What is the clinical significance

- VNS is a novel strategy for asthma management, especially for severe asthma and steroid-resistant asthma.

suggest that noninvasive VNS might provide benefits in lung function and dyspnoea in patients who have respiratory problems, such as acute bronchoconstriction exacerbation due to asthma (Miner et al., 2012; Steyn et al., 2013). More recently, Staats and collaborators showed that noninvasive VNS improved the hospital discharge for two COVID-19 patients with respiratory symptoms (Staats et al., 2020). However, the potential effect of VNS on allergic airway inflammation remains to be evaluated.

The purpose of the present study was to investigate whether CAP activation induced by VNS protects against allergic airway inflammation in a house dust mite (HDM)-induced mouse asthma model. Our findings revealed that VNS equal to or superior to 0.1 mA attenuated allergic airway inflammation in an HDM asthma model via the engagement of macrophage α7nAChR but not β2-adrenoceptors, by suppressing type 2 immune responses and the transcriptional activity of **STAT6** and IRF-4 as well as alveolar ChAT, and may be considered efficient for chronic allergic asthma.

2 | METHODS

2.1 | Animals

Female BALB/c mice of 6 to 8 weeks old were purchased from the InVivos Pte. Ltd. (Singapore) and maintained in a 12-h light–dark cycle with food and water available ad libitum. All experimental protocols were granted approval and performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (Animal protocol number: R20-01056). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2018) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.2 | House dust mite (HDM)-induced allergic airway inflammation in mice

The mouse asthma model was developed as previously described (Chan et al., 2016; Peh et al., 2015). Briefly, mice were anaesthetized with isoflurane (2%–2.5%) and then given 50-μg HDM (*Dermatophagoides pteronyssinus* extract, Greer Laboratories, Lenoir, NC, USA) in 40-μl saline or 40-μl saline alone as a negative control, on days 0, 7, and 14 via the intratracheal (i.t.) route (Figure 1).

2.3 | Vagus nerve stimulation (VNS)

VNS was delivered on day 14 under isoflurane anaesthesia with hook-shaped electrodes that were coated with platinum–iridium wires (0.005″ bare, 0.008″ coated), with a distance of about 0.5 mm between hook electrode leads. The electrode was surgically placed around the midsection of the left cervical vagus nerve (Sévoz-Couche et al., 2002) (Figure 1) to minimize undue effects on the heart (Pelleg et al., 1993). An isolated stimulator (Model 4100, A-M Systems, Carlsborg, WA, USA) was used to deliver biphasic rectangular pulses for VNS. As high-frequency (>10 Hz) and/or high-intensity (>0.5 mA) VNS has chronotropic effects on the heart (Lee et al., 2018) and broncho-constrictive effect on the airways (Watson et al., 1992) due to the stimulation of mAChRs (Kawada et al., 2020), we used low frequency (5 Hz) and intensity (0.05 to 1 mA) VNS. These parameters are known to produce anti-inflammatory effects through nAChRs (de Jonge et al., 2005) and prevent bronchoconstriction induced by histamine (Hoffmann et al., 2009). The phase widths of the biphasic pulses were 500 μs, with no interphase delay. A total of 20-min VNS was applied, with 10-min stimulation immediately before and 10-min stimulation immediately after HDM i.t. administration, as performed in other acute inflammation models (Komegae et al., 2018). After

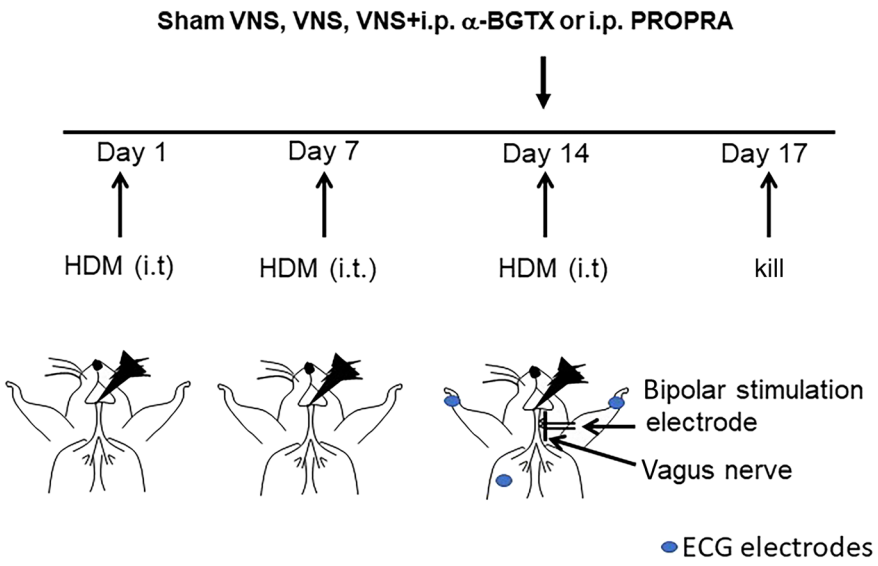

FIGURE 1 A schematic diagram for the development of house dust mite (HDM)-induced airway inflammation model and the vagus nerve stimulation (VNS) treatment protocol. At days 0 and 7, mice were anaesthetised with isoflurane and sensitized via intratracheal (i.t.) administration of either saline or 50-μg HDM extract. On day 14, mice were challenged with the same dose of HDM, with (active VNS) or without (Sham VNS) electrical stimulation of the vagus nerve. Intraperitoneal administration of either α-bungarotoxin (α-BGTX) or propranolol hydrochloride (PROPRA) was given 1 h or 30 min before VNS, respectively. Mice were killed on day 17.

VNS, the wound was closed with permanent interrupted sutures, and the mice were kept under close observation until total recovery from anaesthesia. Sham mice had similar surgery but were not stimulated. Intraperitoneal (i.p.) administration of **α-bungarotoxin** (α-BGTX, 1 μg/kg), a specific α7nACh receptor antagonist (Chen & Patrick, 1997), or propranolol hydrochloride (propranolol, 5 mg·kg^{-1}), a specific β2-adrenoceptor antagonist (Black et al., 1964), was carried out 1 h (Kong et al., 2018) or 30 min (Guyot et al., 2019; Wheeler et al., 2021) before VNS, respectively (Figure 1).

Mouse heart rate was monitored by electrocardiogram (ECG) to make sure it was stable and not modified by VNS (Figure S1).

2.4 | Bronchoalveolar lavage (BAL) fluid, lung tissue and serum collection

Mice were killed on day 17. BAL fluid, blood, and lung tissue samples were collected and processed as previously described (Chan et al., 2016). Briefly, total blood was collected by cardiac puncture, and serum was separated by centrifugation. Tracheotomy was performed, and a cannula was inserted into the trachea. Ice-cold PBS (0.5 ml × 3) was instilled into the lungs, and BAL fluid was collected. BAL fluid levels of IL-4 and IL-5 were measured using ELISA (BD Biosciences, Franklin Lakes, NJ, USA). BAL fluid cells were pelleted, and the number of BAL fluid cells was determined using a haemocytometer. Differential cell types were analysed by flow cytometry. Lung tissue was excised and stored at −80°C for subsequent analysis.

2.5 | Flow cytometry analysis

BAL fluid cells were stained via antibodies targeting surface makers CD45-PE/Cy7 (Thermo Fisher Scientific, Cat# 25-0451-82, RRID:AB_2734986), CD11b-APC (Thermo Fisher Scientific, Waltham, MA, USA, Cat# 17-0112-82, RRID:AB_469343), Ly6G/6C-PerCP/Cy5.5 (Thermo Fisher Scientific, Cat# 45-5931-80, RRID:AB_906247), SiglecF-PE (Thermo Fisher Scientific, Cat# 12-1702-82, RRID:AB_2637129), and CD11c-PB (Thermo Fisher Scientific, Cat# 48-0114-82, RRID:AB_1548654) to differentiate total leukocytes, lymphocytes, alveolar macrophages (AMs), eosinophils, and neutrophils as described previously (Chan et al., 2016). Leukocytes were identified as CD45$^+$, lymphocytes as CD45$^+$SSC$_{low}$, AMs as CD45$^+$CD11c$^+$SiglecF$^+$, eosinophils as CD45$^+$CD11c$^-$SiglecF$^+$, and neutrophils as CD45$^+$Ly6G/6C$^+$CD11b$^+$. The gating strategy for BAL fluid cells is shown in Figure S2. All samples were run on BD LSR Fortessa cell analyser and data were analysed with FlowJo software (BD Biosciences) or Cytobank (https://www.cytobank.org).

2.6 | Histological analysis

Lungs were fixed in 10% neutral formalin, embedded with paraffin, cut into 5-μM sections, and stained with haematoxylin and eosin (H&E) for examining cell infiltration and with periodic acid-fluorescence Schiff (PAFS) for mucus production. Images were captured by Thunder Imaging System (Leica, Wetzlar, Germany). Scoring of H&E and PAFS staining was performed as described previously (Peh et al., 2015).

2.7 | Transmission electron microscopy (TEM) analysis

Lung tissues were fixed in 2.5% glutaraldehyde in PBS, washed three times in 0.1-M cacodylate buffer, and postfixed in 1% buffered osmium for 1 h at room temperature. Lung samples were then dehydrated in ethanol and embedded in Araldite medium. Ultrathin sections were cut using an ultracut microtome (Leica) and stained with uranyl acetate and lead citrate. The stained lung sections were examined in a JEM 1400 transmission electron microscope operated at 100 kV and equipped with a Matataki Flash sCMOS camera (JEOL, Peabody, MA, USA, RRID:SCR_020179).

2.8 | Immunofluorescence staining

Lung tissues were fixed in 2% paraformaldehyde (PFA) + 30% sucrose fixative, and frozen in Tissue-Tek Optimum Cutting Temperature compound (Tissue-Tek OCT, Sakura Finetek, Torrance, CA, USA) for 10-μm cryo-sectioning and subsequent immunofluorescence staining. Briefly, tissue sections were incubated in 0.2% bovine serum albumin (BSA) blocking solution, followed by incubation overnight at 4°C of (i) anti-ChAT rabbit monoclonal primary antibody (EPR 16590, dilution 1:300; Abcam, Cambridge, UK, Cat# ab178850, RRID:AB_2721842) and Cy3-conjugated antirabbit secondary antibody (dilution 1:300, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, Cat# 611-164-215, RRID:AB_2922862) to detect ChAT, (ii) anti-α7nAChR rabbit monoclonal primary antibody (dilution 1:200; Alomone Labs, Jerusalem, Israel, Cat# ANC-007-FR, RRID:AB_2756676) and Cy3-conjugated antirabbit secondary antibody (dilution 1:300, Jackson ImmunoResearch Laboratories, Cat# 611-164-215, RRID:AB_2922862) to detect α7nAChR, (iii) anti-CD68 rat monoclonal primary antibody (dilution 1:300; BIORAD, Hercules, CA, Cat# MCA1957, RRID:AB_322219) and Cy3-conjugated antirat secondary antibody (dilution 1:300, Jackson ImmunoResearch Laboratories, Cat# 611-164-215, RRID:AB_2922862) to detect macrophages.

FITC/AF488-conjugated antismooth muscle actin antibody (SMA, mouse clone 1A4; Sigma-Aldrich, St. Louis, MI, USA, Cat# F3777, RRID:AB_476977) was used to identify airway smooth muscle cells. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) for cell nuclei visualization for histological analysis. A widefield fluorescence microscope (Axio Imager.Z1, Carl Zeiss MicroImaging, Inc., Jena, Germany) was used to view the specimens. Fiji (ImageJ) was used to analyse and quantify the fluorescence in the images obtained. Density was calculated as the mean of fluorescence in

bronchial and peribronchial zones relative to total density, in four fields per lung section.

The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.9 | Immunoblotting

Total proteins from lung tissues were extracted in M-PER® Mammalian Protein Extraction Reagent containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Total lung lysates were separated in 10% SDS-PAGE and immunoblots probed with anti-p-STAT6 (Cell Signalling Technologies, Danvers, MA, USA, Cat# 56554, RRID:AB_2799514), anti-STAT6 (Cell Signalling Technologies, Cat# 9362, RRID:AB_2271211), and anti-IRF4 (Cell Signalling Technologies, Cat# 15106, RRID:AB_2798709) antibodies. β-actin (Proteintech, Rosemont, IL, USA, Cat# CL594-66009, RRID:AB_2883475) was used as a loading control. Immunoblots were visualized and documented using ChemiDoc™ Touch Gel Imaging System (Bio-Rad Laboratories, RRID:SCR_021693). Band intensity was quantitated using ImageJ software (NIH).

2.10 | Measurements of airway hyperresponsiveness

Tracheotomy and intubation were performed as described previously (Peh et al., 2015). The trachea was intubated with a cannula that was connected to the Buxco Resistance and Compliance System (Data Sciences International, St. Paul, MN, USA). Lung resistance (R_L) and dynamic compliance (Cdyn) in response to either saline or nebulized methacholine (2.5–20 mg·ml^{-1}) were recorded and analysed using FinePointe software (Data Sciences International). Results are expressed as a percentage of the respective basal values in response to PBS.

2.11 | Statistical analysis

All data are expressed as mean ± SEM. One-way ANOVA of raw data without prior normalization followed by Dunnett's test was used to determine the significant differences between treatment groups with GraphPad PRISM (8.0.1). Two-way repeated ANOVA was performed for airway hyperresponsiveness analysis (factors: groups and methacholine doses). Comparisons between groups and HDM were performed (*), and a specific comparison between HDM + VNS and either HDM + VNS + α-BGTX or HDM + VNS + propranolol was also conducted (#). Post-hoc tests were run only if F achieved P<0.05 and there was no significant variance inhomogeneity. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Beuve, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Annett, et al., 2023; Alexander, Kelly, et al., 2023; Alexander, Mathie, et al., 2023).

3 | RESULTS

3.1 | Vagus nerve stimulation (VNS) attenuates house dust mite (HDM)-induced airway inflammation through α7nAChR activation

Clusters of different immune cells including alveolar macrophages, eosinophils, lymphocytes, and neutrophils from mouse BAL fluid were plotted by high-dimensional flow cytometry analysis based on surface markers (Figure S2). HDM challenge markedly increased the number of total inflammatory cells in BAL fluid, especially eosinophils and macrophages, as compared with saline (Figure 2a). Sham VNS had no effect on HDM-induced inflammatory cell infiltration (Figure 2a). VNS applied at 0.1, 0.5, or 1 mA, but not 0.05 mA, prevented against HDM increases in inflammatory cells (Figure 2a). There was no statistical difference between data obtained with VNS equal to or superior to 0.1 mA, and the subsequent experiments were performed with the lower effective intensity, that is, 0.1 mA, indicated throughout the manuscript as VNS 0.1.

Pretreatment with α-BGTX, a specific α7nAChR antagonist, prevented the anti-inflammatory effects of VNS 0.1 on total inflammatory cells, macrophages, and eosinophils (Figure 2b). Because β2-adrenoceptors may also participate in an anti-inflammatory effect through catecholamine release by sympathetic nerves, which are influenced by vagal afferents (Wolterink et al., 2022), we administered propranolol, a specific antagonist, before VNS 0.1. Unlike α-BGTX, propranolol pretreatment failed to reverse the preventive effect of VNS 0.1 on BAL fluid cells (Figure 2c).

Type 2 cytokines IL-4 and IL-5 in the BAL fluid were markedly elevated by HDM challenge. Active VNS 0.1 drastically suppressed BAL fluid IL-4 and IL-5 levels, which were reversed by prior treatment with α-BGTX (Figure 3a).

HDM challenge induced bronchial epithelial hypertrophy as well as marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues (Figure 3b). HDM challenge also promoted mucus hypersecretion in goblet cells, and VNS 0.1 markedly mitigated HDM-induced goblet cell mucus hypersecretion (Figure 3c). That beneficial effect of VNS 0.1 was abolished by pretreatment of α-BGTX.

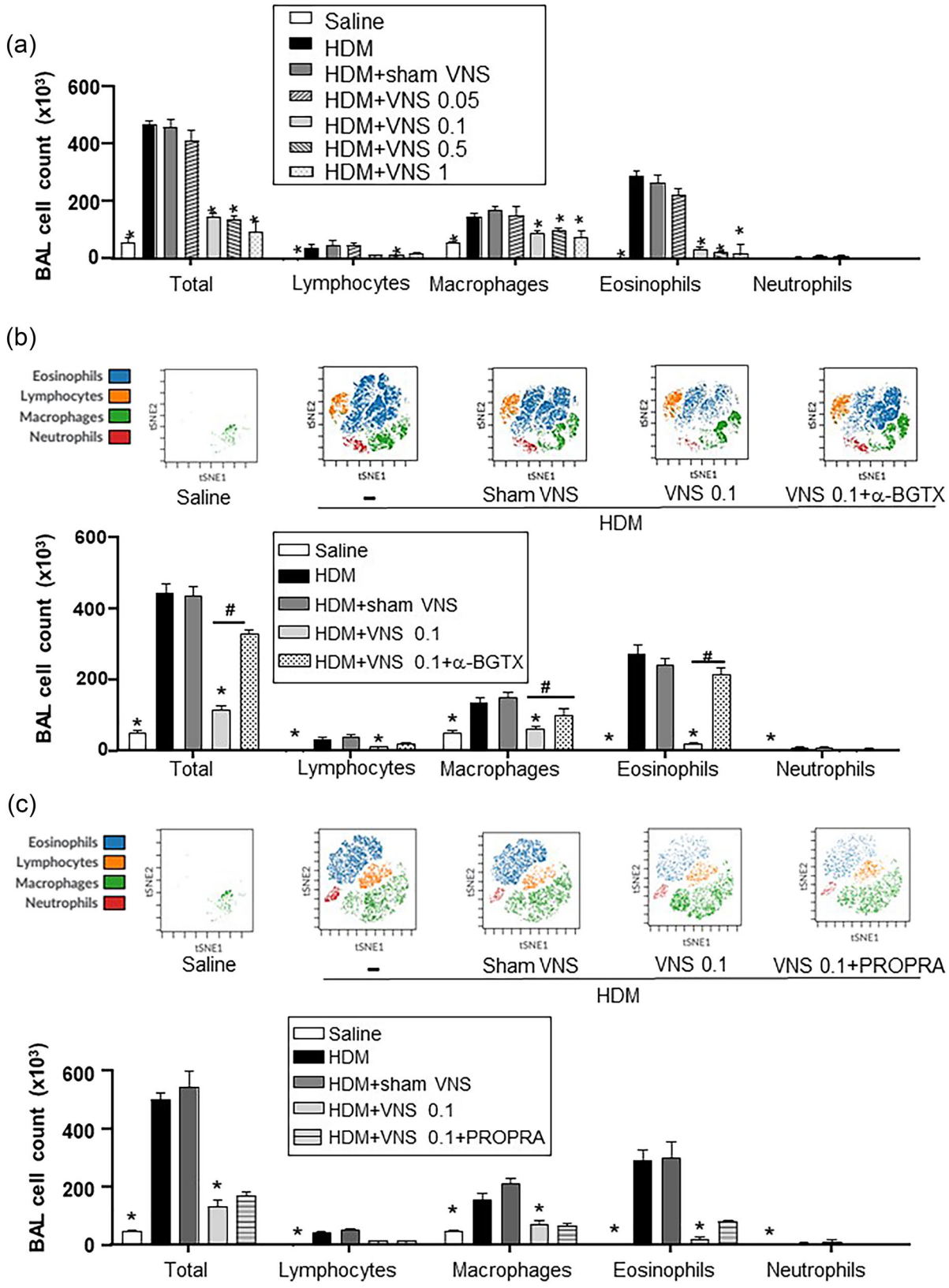

FIGURE 2 Legend on next page.

We have analysed α7nAChR expression and observed that upon HDM challenge, the fluorescent density of these receptors was markedly elevated in the alveoli and in the perivascular area. They were expressed mostly on macrophages, but they were also observed on some goblet cells (Figure 4a), without overlapping smooth muscle cells (Figure S3). VNS 0.1 abolished HDM-induced α7nAChR expression, and prior administration of α-BGTX reversed this effect (Figure 4b).

3.2 | Vagus nerve stimulation (VNS) ameliorates house dust mite (HDM)-induced airway remodelling through α7nAChR activation

In contrast to the predominant ciliated epithelial cells identified in saline-challenged mice, pseudostratified epithelial cells, hyperplasia of goblet cells with vacuoles containing electron lucent substance, and ciliated cells with dense vacuoles (mucus granules), were shown in the epithelial lumen from HDM-exposed mice using TEM (Figure 5a). VNS 0.1, but not sham VNS, reduced goblet cell hyperplasia induced by HDM, and this protective effect of VNS 0.1 was abolished by prior administration of α-BGTX (Figure 5a). In addition, TEM also uncovered an increase in collagen bundles in lung tissues from mice challenged with HDM (Figure 5b). VNS 0.1, but not sham VNS, was able to decrease HDM-induced collagen deposition, and prior administration of α-BGTX reversed that beneficial effect of VNS 0.1 (Figure 5b).

3.3 | Vagus nerve stimulation (VNS) down-regulates house dust mite (HDM)-induced increases in ChAT in airway epithelial cells

Small airways expressing muscarinic receptors are involved in all stages of the pathophysiological manifestation of asthma, including airway hyperresponsiveness (Cazzola et al., 2021). Both neuronal and nonneuronal cells such as bronchial epithelial cells can express ChAT to produce and release ACh (Proskocil et al., 2004). In response to inflammatory stimuli, epithelial cell-derived ACh (considering that the small airways are not innervated by extrinsic or intrinsic cholinergic neurons: Koarai & Ichinose, 2018), can bind to muscarinic receptors to promote airway hyperresponsiveness. ChAT expression has been shown to be up-regulated in an allergic asthma model (Liu et al., 2018). Upon HDM challenge, the fluorescent density of ChAT in bronchial epithelium was markedly elevated (apical level) (Figure 6a). VNS 0.1 abolished HDM-induced ChAT expression, but that effect was reversed by prior administration of α-BGTX (Figure 6b).

3.4 | Vagus nerve stimulation (VNS) inhibits the type 2 transcription factors lung STAT6 and IRF-4

The classical type 2 cytokines IL-4 and IL-13 have been shown to orchestrate allergic airway inflammation and remodelling by promoting JAK1/STAT6 and interferon regulatory factor 4 (IRF-4) signalling pathways (Gao et al., 2013). HDM challenge phosphorylated and activated transcription factor STAT6 and increased IRF4 protein levels in total lung lysates. VNS 0.1, but not sham VNS, suppressed HDM-induced phosphorylation of STAT6 and up-regulation of IRF-4 protein expression in total lung lysates (Figure 7). The inhibitory effects of VNS 0.1 on p-STAT6 and IRF-4 were abrogated by the pretreatment with α-BGTX (Figure 7).

3.5 | Vagus nerve stimulation (VNS) blocks house dust mite (HDM)-induced airway hyperresponsiveness

Lung resistance (R_L) and dynamic compliance (C_{dyn}) are the two parameters used to evaluate airway hyperresponsiveness in asthma in response to bronchoconstrictors like methacholine, a cholinergic agent acting on the muscarinic receptors present in bronchial smooth muscle (Kim et al., 2014). HDM-challenged mice developed airway hyperresponsiveness characterized by high R_L and low C_{dyn}. VNS 0.1, but not sham VNS, effectively blocked the increase in R_L and enhanced the decrease in C_{dyn} upon HDM challenge (Figure 8). The beneficial effects of VNS 0.1 were eradicated by prior administration of α-BGTX (Figure 8).

4 | DISCUSSION

The use of nicotine or specific agonists for α7nACh receptors has been shown to protect against airway inflammation in allergic asthma models (Antunes et al., 2020; Gahring et al., 2020; Mazloomi et al., 2018; Yuan et al., 2021). Here, we show for the first time that active VNS can also attenuate HDM-induced allergic airway inflammation through CAP activation. In mice sensitized and challenged with the aeroallergen HDM, VNS 0.1 reduced BAL fluid cell counts, the

FIGURE 2 Vagus nerve stimulation (VNS) attenuates house dust mite (HDM)-induced recruitment of inflammatory cells in bronchoalveolar lavage (BAL) fluid. (a) Total and differential cell counts in mouse BAL fluid in mice challenged by HDM, without or with stimulation of the vagus nerve at 0.05 (VNS 0.05), 0.1 (VNS 0.1), 0.5 (VNS 0.5), or 1 (VNS 1) mA. Clusters were identified based on cell surface marker expressions as described in Section 2. Values are shown as means ± SEM of five animals. *Significant difference from HDM: $^*P < 0.05$. (b,c) Total and differential cell counts in mouse BAL fluid, with flow cytometry viSNE, showing BAL fluid macrophage, eosinophil, neutrophil, and lymphocyte populations in HDM challenged animals with or without α-bungarotoxin (αBGTX) 1 h prior to VNS 0.1 (b), or propranolol hydrochloride (PROPRA) 30 min prior to VNS 0.1 (c). Values are shown as means ± SEM of five different animals in b and c. *Significant difference from HDM: $^*P < 0.05$. #Significant difference between VNS 0.1 and VNS 0.1 + α-BGTX or VNS 0.1 + PROPRA: $^{\#}P < 0.05$.

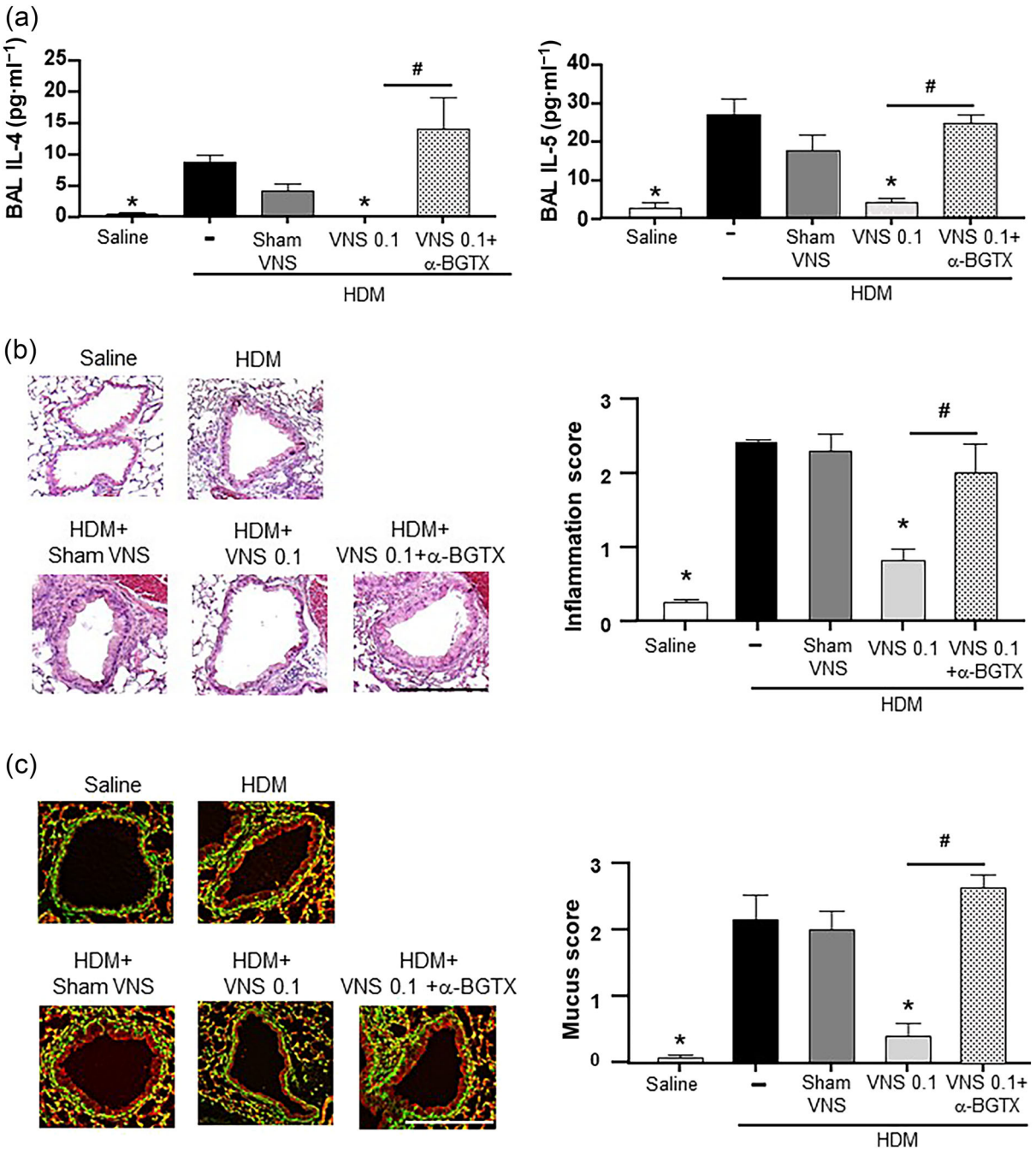

FIGURE 3 Vagus nerve stimulation (VNS) 0.1 attenuates house dust mite (HDM)-induced allergic airway inflammation. (a) Levels of cytokines IL-4 and IL-5 in BAL fluid were determined by ELISA. (b) Lung sections stained with haematoxylin and eosin (H&E) with corresponding quantitative analysis. Scale: 100 μm. (c) Lung sections for mucus staining with periodic acid-fluorescence Schiff (PAFS) solution with corresponding quantitative analysis. Scale: 100 μm. Values are shown as means ± SEM of five mice. *Significant difference from HDM: $^{*}P < 0.05$. #Significant difference between 0.1 VNS and 0.1 VNS + α-BGTX: $^{\#}P < 0.05$.

recruitment of alveolar macrophages expressing α7nAChR, pulmonary cell infiltration and mucus hypersecretion, and ameliorated airway hyperresponsiveness. Histological and TEM analyses revealed that VNS 0.1 also inhibited eosinophil degranulation, goblet cell hyperplasia, and collagen deposition. The mechanisms underlying its anti-inflammatory effects may be attributable to the blockade of type 2 cytokines IL-4 and IL-5, activated STAT6 and IRF-4 signalling pathways, and the ACh biosynthesis enzyme ChAT expression in the allergic airways. The beneficial effects of VNS 0.1 could be abrogated by prior administration of α-BGTX but not propranolol, highlighting an essential role of parasympathetic efferents and α7nAChR activation in mediating antiasthma actions of VNS.

In addition to controlling the classic physiological functions (e.g. heart rate, gastric motility, pupil constriction) (Beaumont

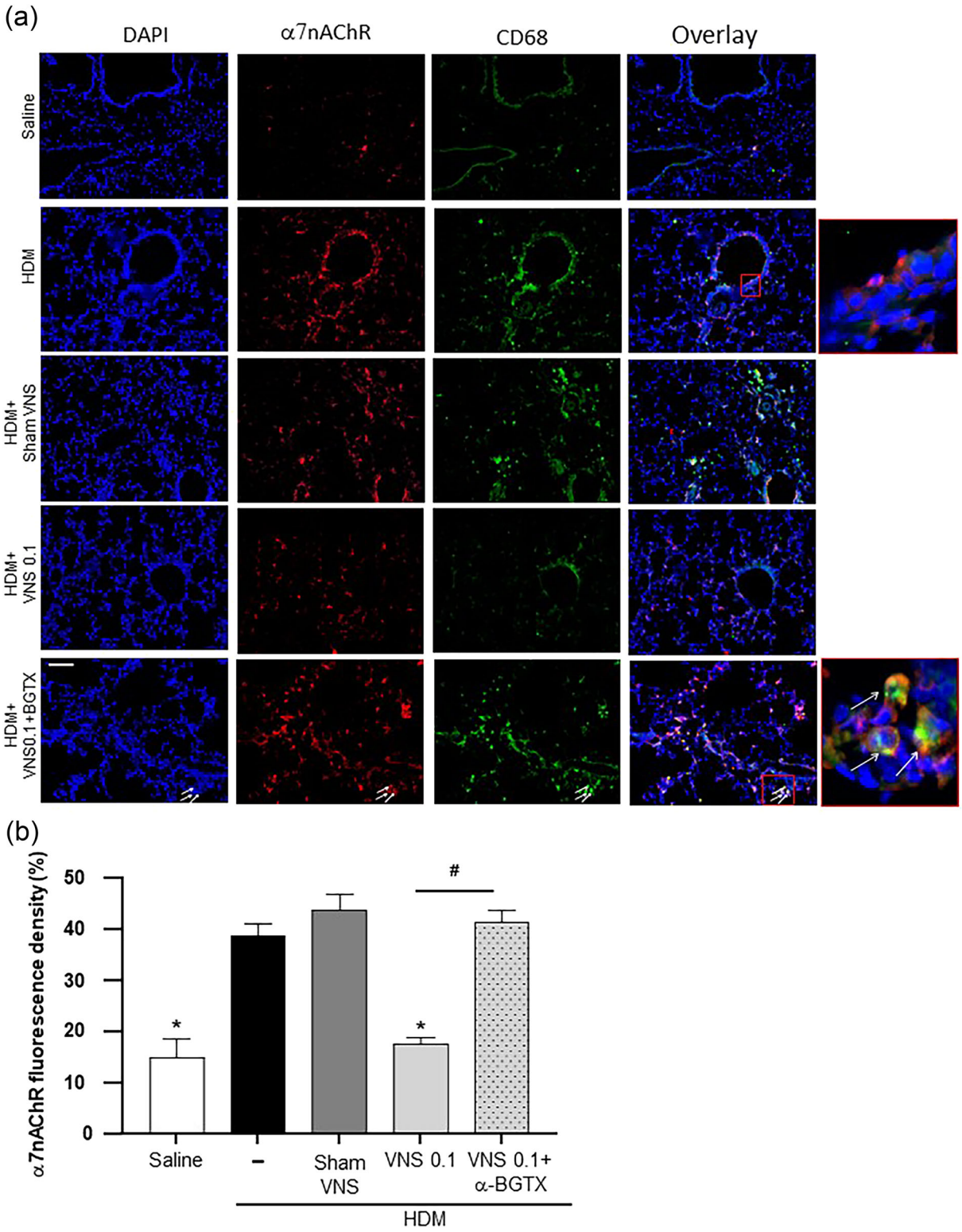

FIGURE 4 Vagus nerve stimulation (VNS) 0.1 reduces the level of macrophages and goblet cells expressing α7nAChR. (a) Lung sections were stained for α7nAChR (red), CD68 (macrophages, green), and DNA nuclear stain (DAPI, blue). Scale: 50 μm. Macrophages and goblet cells are shown with higher magnification. Examples of double-staining are identified with white arrows. (b) Quantification of fluorescent intensity of α7nAChR in alveolar and peribronchial area. Values are shown as means ± SEM of five animals. *Significant difference from house dust mite (HDM): $^{*}P < 0.05$. #Significant difference between VNS 0.1 and VNS 0.1 + α-bungarotoxin (α-BGTX): $^{\#}P < 0.05$.

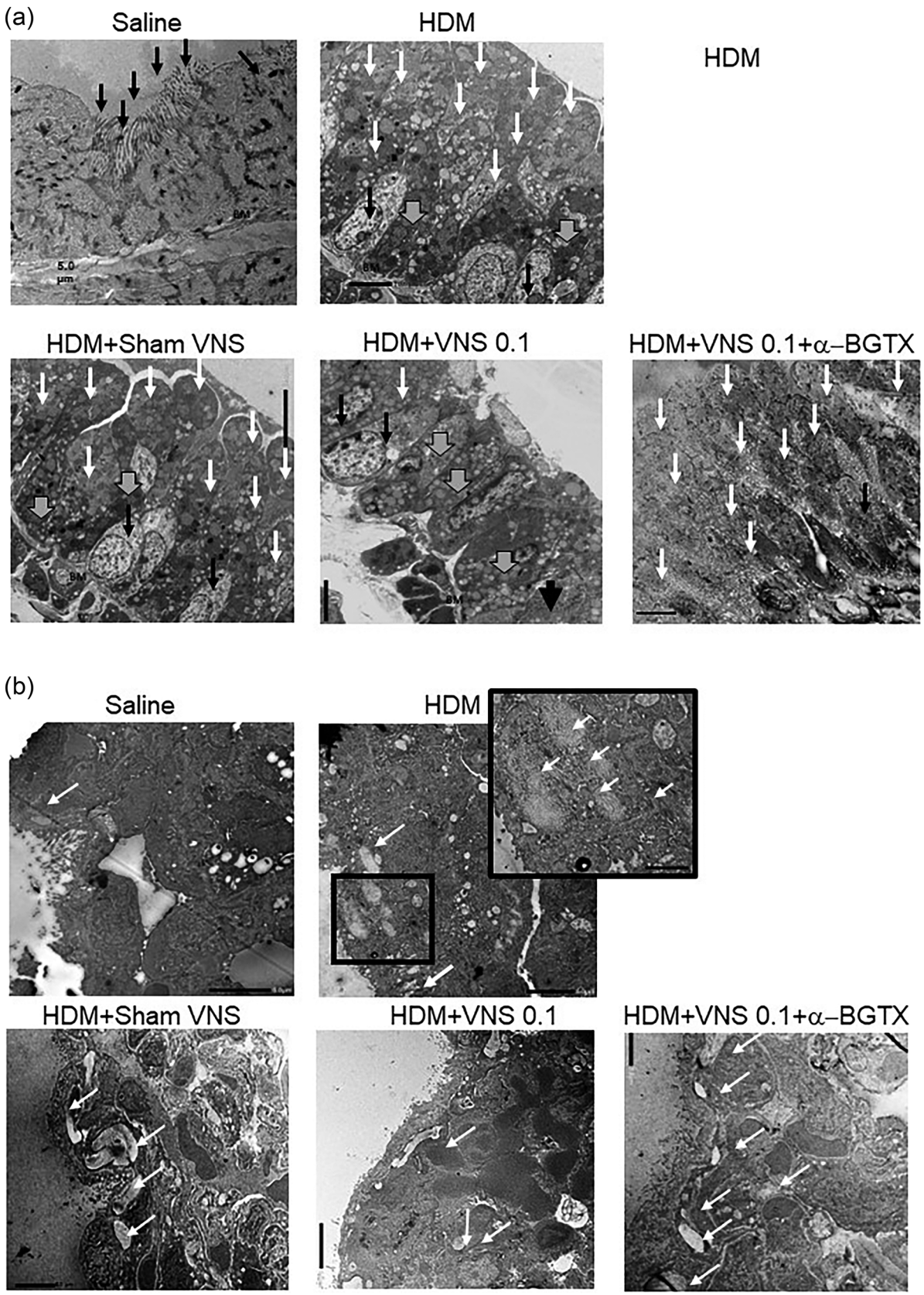

FIGURE 5 Legend on next page.

et al., 2017; Lu et al., 2020), the vagus nerve is also implicated in immune modulation, and its stimulation leads to effective anti-inflammatory actions through a mechanism classically called CAP (Tracey, 2002). In lymph tissues, CAP involves vagal motor cholinergic neurons which target the celiac/superior mesenteric ganglia; their stimulation induces sympathetic nerve activation and noradrenaline (NA) release in the spleen and mesenteric nodes (Murray et al., 2021; Tracey, 2002). NA in these secondary lymphoid organs elicits ACh release from ChAT+ T-cells in a β2-adrenoceptor dependent manner, to inhibit TNFα production by macrophages that express α7nAChR (Murray et al., 2021). At the level of the lung, there is no ChAT+ T cells and ACh from parasympathetic efferents directly targets α7nAChR macrophages in the alveoli (McGovern & Mazzone, 2010). It is important to note that the action of efferent cholinergic neurons in the lung in the context of inflammation is dual (Cremin et al., 2023; Wolterink et al., 2022). On the one hand, ACh dampens type 2 innate lymphoid cell (ILC2) function (Galle-Treger et al., 2016), via the stimulation of α7nAChR (de Jonge et al., 2005). Also, residential alveolar macrophages and other infiltrated immune cells express various levels of α7nAChR (Blanchet et al., 2007), and their activation by ACh contributes to the anti-inflammatory effects of CAP. Conversely, cholinergic descending fibres also mediate type 2 innate lymphoid cell (ILC2)-mediated allergic responses (production of IL-5 and IL-13) via the secretion of the neuropeptide NMU, which acts on the neuromedin U receptor 1 (NMUR1) (Blake et al., 2019).

We first explored the role of the vagus nerve and α7nAChR in mice challenged by HDM, which is well known to produce a good model for the exploration of asthma (Piyadasa et al., 2016). We applied VNS at different intensities (0.05 to 1 mA). VNS is effective at reducing BAL fluid cell count when the intensity is equal to or above 0.1 mA. We have used the lower effective level of intensity (0.1 mA) throughout the experiments (indicated as VNS 0.1) to preserve the nerve function. The administration of αBGTX drastically prevents the protective effect of VNS 0.1 on HDM-induced inflammatory cells. In addition, in HDM-challenged mice, the level of macrophages expressing α7nAChR increases, and this effect is prevented by VNS 0.1 alone, but not when α-BGTX was administered before VNS, suggesting a key role of cholinergic parasympathetic efferents in this effect.

We also explored the possibility that VNS 0.1 could involve the participation of vagal afferents and β2-adrenoceptors. The vagus nerve is composed of three fibre groups whose diameters, conduction speeds, and thresholds vary for activation: large-diameter fast-conducting myelinated fibres with an amplitude duration threshold ranging between 0.02 and 0.2 mA (A afferent fibres); small- and medium-diameter myelinated fibres with a higher excitation threshold between 0.04 and 0.6 mA (B efferent fibres); and very small unmyelinated fibres that require stimulation currents higher than 2.0 mA (C afferent fibres) (Groves & Brown, 2005). Therefore, VNS applied with intensities lower than 2 mA as performed in our study here is likely to activate efferent B and afferent A (rather than C) fibres. Thus, the beneficial effects of VNS 0.1 involving α7nAChR can be attributed to B efferent activation but may also, at least in part, involve the activation of abdominal A afferents. A vago-vagal (A afferent–B efferent) circuit participates in the classic CAP-induced anti-inflammatory effects in the gastro-intestinal tract (Liu et al., 2007) and may also participate here. However, there is a linear relationship between A afferent fibre activation and activity, while this relationship is exponential (with a fast maximal plateau) for B efferents (Chang et al., 2020). Our data show that VNS was not effective at 0.05 mA, but there was a maximal plateau from 0.1 mA and above, independent of the level of stimulation. Therefore, it is likely that abdominal A afferents may only play a minor role, if any, in the cholinergic protective effects on inflammatory cells induced by VNS induced by B motor neurons.

Also, although VNS 0.1 is presumably too low to activate C-afferent fibres (Groves & Brown, 2005), we could not rule out that these neurons may have also been involved, at least in part, in VNS effects. The activation of vagal abdominal C-fibres are known to produce anti-inflammatory effects through NA and β2-adrenoceptor signalling on macrophages and/or type 2 innate lymphoid (ILC2) cells, after the stimulation of sympathetic neurons projecting to the spleen (Komegae et al., 2018), but also to the lung (Kummer et al., 1992). However, this possibility seems unlikely because the use of propranolol, a specific inhibitor of β2-adrenoceptors, was not able to prevent the beneficial effect of VNS 0.1. To note, there is also a cross-talk between C-fibres originating from the lung and the immune system (Blake et al., 2019; Wolterink et al., 2022). Lung C-fibres can be stimulated by activating nociceptive transient receptor potential (**TRP**) cation channel proteins (e.g., TRPV1, TRPA1) and have been found to play a critical role in driving immune cell cytokine production and airway hyperreactivity in a model of asthma induced by ovalbumin (OVA) in mice through TRPA1 (Caceres et al., 2009). However, as described above, lung C-fibres are not likely to have been stimulated in our study; moreover, we found that airway hyperresponsiveness is not increased but reduced by VNS 0.1.

FIGURE 5 Vagus nerve stimulation (VNS) 0.1 inhibits goblet cell hyperplasia and collagen deposition in house dust mite (HDM)-induced allergic airway. (a) Representative TEM images of bronchial epithelium. Ciliated cells (Ci) are recognized by its columnar shape, large basal nucleus, electron lucent cytoplasm, and the presence of unaltered cilia. Nonciliated Clara cells (C) are columnar-shaped with a large amount of cytoplasmic electron dense secretory granules beside the cell organelles. Goblet cells (G) are dome-shaped and identified by membrane-bound mucous granules containing electron lucent substance. In saline control mice, bronchial epithelium is regular, and mainly populated with Ci (black arrows). In HDM-challenged mice, numerous G (white arrows) and C appear, causing pseudostratification and hyperplasia, and Ci are compressed towards the side. In VNS- but not sham VNS-treated HDM mice, the number of secretory cells (G and C) was reduced, and that effect was reversed by i.p. administration of α-BGTX. (b) Representative TEM images of lung subepithelial area. Large collagen fibres (white arrows) are found in the lung subepithelial area of HDM-exposed mice. Collagen bundles are shown with higher magnification. In VNS- but not sham VNS-treated asthma mice, the apparition of collagen deposition was reduced. Prior administration of α-bungarotoxin (α-BGTX) reversed the antifibrotic effect of VNS.

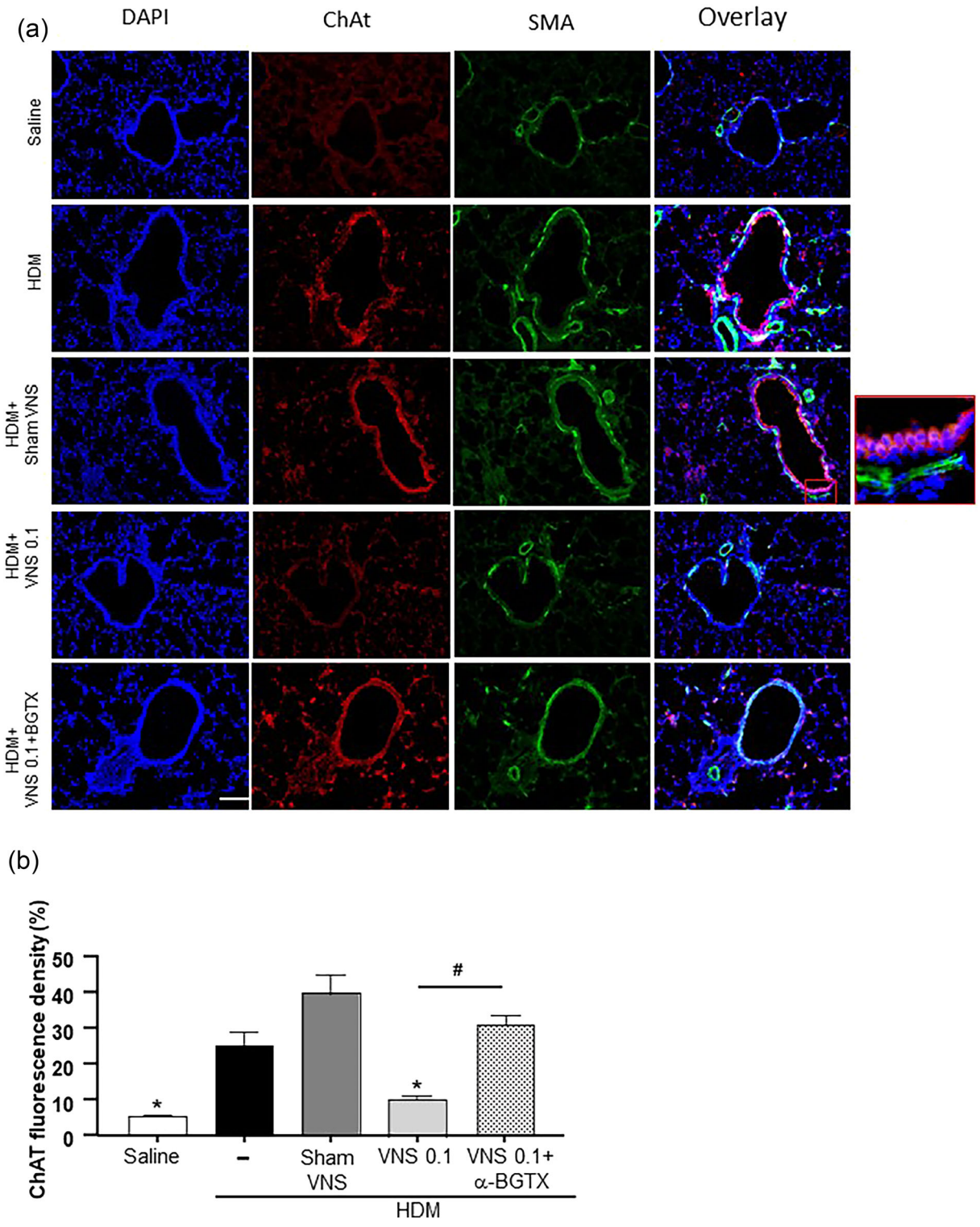

FIGURE 6 Vagus nerve stimulation (VNS) 0.1 down-regulates house dust mite (HDM)-induced cholinergic acetyl transferase (ChAT) expression in peribronchiolar areas of the allergic airway. (a) Lung sections were stained for ChAT (red), smooth muscle actin (SMA, green), and DNA nuclear stain (DAPI, blue). Scale: 50 μm. (b) Quantification of fluorescent intensity of ChAT in bronchial epithelium. Values are shown as means ± SEM of five animals. *Significant difference from HDM: $^{*}P < 0.05$. #Significant difference between VNS 0.1 and VNS 0.1 + α-bungarotoxin (α-BGTX): $^{\#}P < 0.05$.

Altogether, our data give evidence for the key role of vagal efferents and α7nAChR in the reduction of cell infiltration in HDM challenged animals. Using immunofluorescent techniques, we observed that α7nAChRs are present in all groups we have studied, but their expression is increased in HDM and HDM + Sham animals. This is due to a recruitment of macrophages and the appearance of mucus-

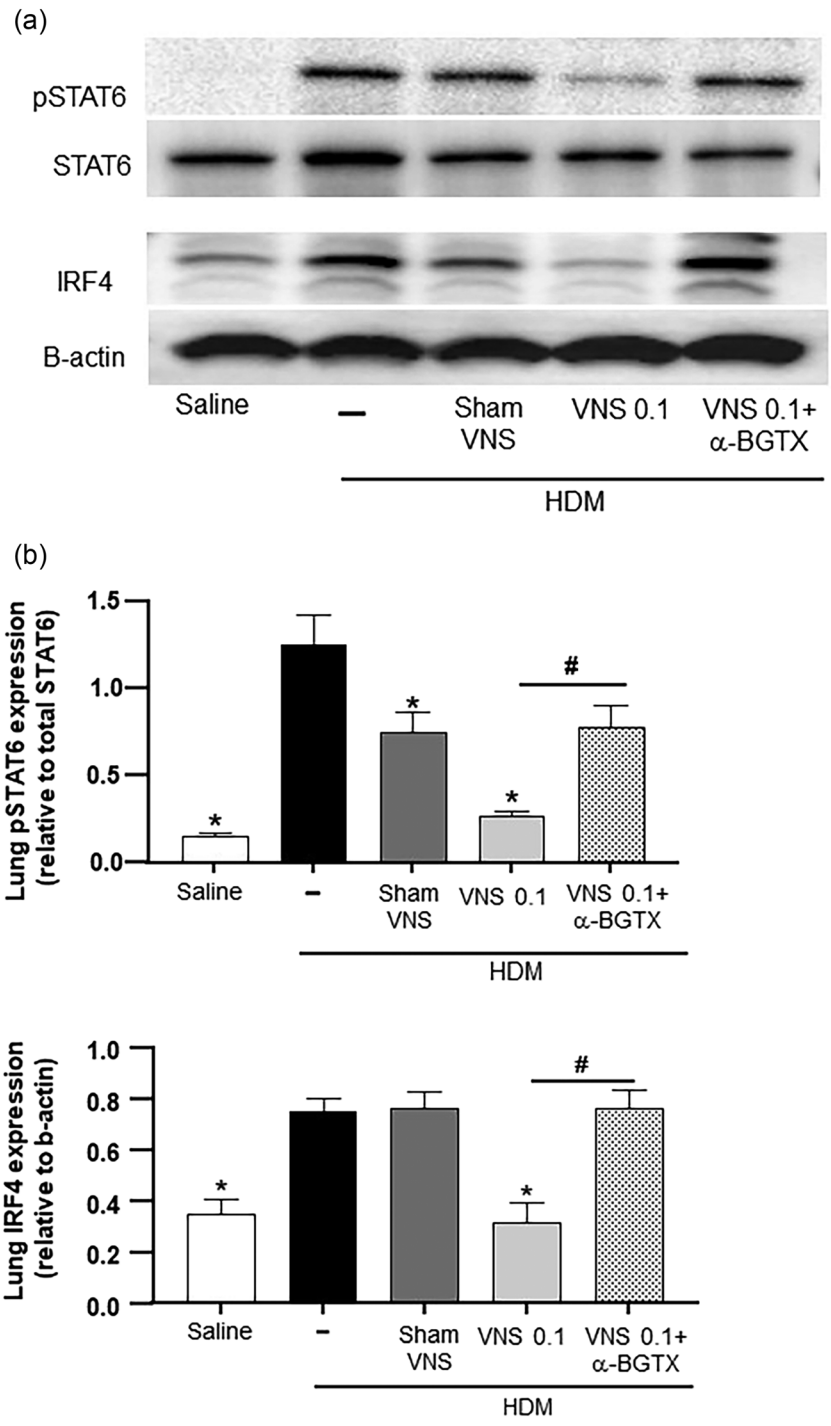

FIGURE 7 Vagus nerve stimulation (VNS) 0.1 modulates house dust mite (HDM)-induced transcription factors in allergic airway. (a) Representative immunoblots of phospho-STAT6 and total STAT6 in mouse total lung lysates with corresponding quantitative analysis. (b) Representative immunoblots of IRF-4 in mouse total lung lysate with corresponding quantitative analysis. Values are shown as mean ± SEM of five mice. *Significant difference from HDM: $^{*}P < 0.05$. #Significant difference between VNS 0.1 and VNS 0.1 + α-bungarotoxin (α-BGTX): $^{\#}P < 0.05$.

producing goblet cells expressing these receptors, showing that these cells can be a target for ACh to reduce cytokine production and mucus secretion. Indeed, macrophages, goblet cells, and therefore α7nAChR expression are reduced when VNS 0.1 is performed in HDM animals. The increase in macrophage and goblet cells expression of α7nAChR is not affected when prior administration of αBGTX is performed. In line with these data, we show ultrastructural changes in HDM-exposed airways. VNS 0.1 suppresses mucus hypersecretion as shown by PAFS staining, as well as reduced goblet cell hyperplasia and mucus granules inside the cells as visualized by TEM. We have also observed the presence of disjointed cells and collagen bundles, as reported in the lungs of asthmatic patients (Shahana et al., 2005). Altogether, these data indicate that VNS modulates both macroscopic and microscopic hallmarks of allergic airways, through α7nAChR signalling.

Allergic asthma is characterized by elevation of type 2 cytokines including IL-4, IL-5, and IL-13. IL-5 is a key regulator of eosinophil migration, proliferation, maturation, and survival (Garcia et al., 2013).

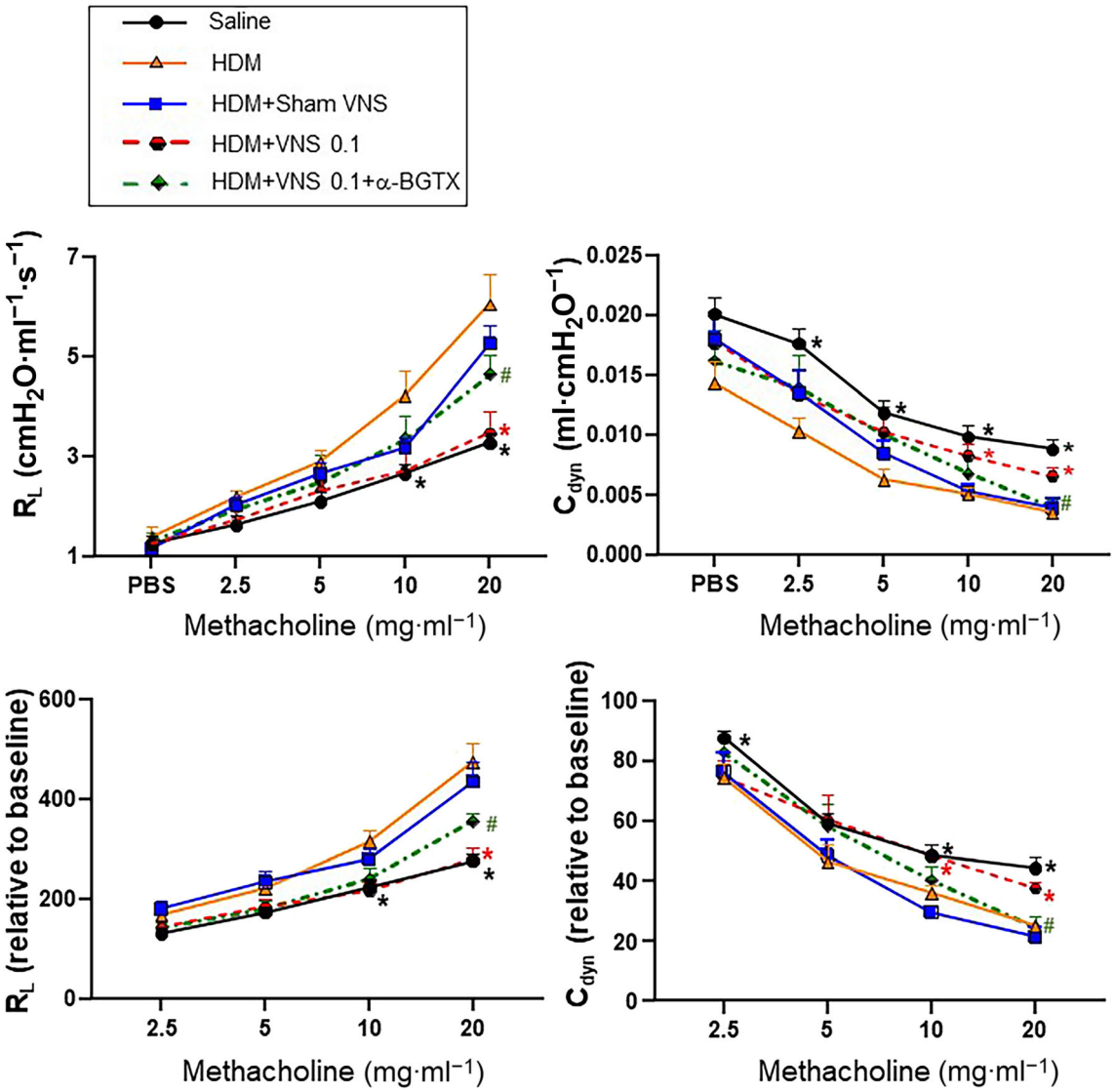

FIGURE 8 Vagus nerve stimulation (VNS) 0.1 blocks house dust mite (HDM)-induced airway hyperresponsiveness (AHR). AHR in response to increasing concentrations of aerosolized methacholine was measured on day 17. AHR is expressed as absolute values (A) or percentage change compared with the baseline (PBS) level of (B) lung resistance (R_L) and (B) dynamic compliance (C_{dyn}). Values are shown as means ± SEM of five mice. *Significant difference from HDM: *$P < 0.05$. #Significant difference between VNS 0.1 and VNS 0.1 + α-bungarotoxin (α-BGTX): #$P < 0.05$.

IL-4 is crucially involved in Th2 cell differentiation, immunoglobulin (Ig) class switching to IgE and eosinophil trafficking (Pelaia et al., 2022). Binding of IL-4 to the **IL-4 receptor** results in JAK1 phosphorylation, which in turn recruits and phosphorylates transcription factor STAT6 for subsequent nuclear translocation and activation of target genes including GATA-3, CRTH2, arginase 1, and Fizz1, leading to allergic airway inflammation and remodelling (Oh et al., 2010). IRF4 is also one of the target genes controlled by STAT6, and functions as another important transcription factor that orchestrates type 2 immune responses in both M2 and Th2 cell polarization (Gao et al., 2013; Satoh et al., 2010). IRF4-deficient mice produced drastically less IL-4 and IL-5 (Flutter & Nestle, 2013). In the present study, VNS 0.1 blocked BAL fluid levels of IL-4 and IL-5, as well as STAT6 phosphorylation in airway inflammation through α7nAChR activation. Though the effects of HDM in the lymph tissues (i.e., the spleen or lymphatic nodes) are limited, in vitro studies showed that there is an increased production of HDM-specific IL-5 and IL-13 by splenocytes (Cates et al., 2004; Johnson et al., 2004). Low-frequency VNS has been shown to reduce IL-4 levels in septic rats; as such, it improves multiple organ dysfunction and reverses immunosuppression of T lymphocytes (Ren et al., 2018). VNS 0.1 stimulates motor vagal fibres to produce an anti-inflammatory effect in the lung through α7nAChR activation. VNS 0.1, through abdominal A afferent stimulation, may reduce the level of cytokines in the spleen through β2-adrenoceptor signalling on ChAT+ T cells (see above). However, the fact that propranolol has no effect on the anti-inflammatory

effects of VNS 0.1 in BAL fluid suggests that β2-adrenoceptor activation in the spleen, if present, would not influence the effects of VNS 0.1 in the lung.

ChAT, the enzyme responsible for the biosynthesis of ACh (Kistemaker et al., 2014), has been shown to localize at the apical end of the bronchial epithelium (Kummer et al., 2006; Wessler & Kirkpatrick, 2008). The basal level of ChAT is low in these cells from healthy subjects (Kawashima et al., 2007), but it is markedly increased in patients with inflammatory conditions such as chronic obstructive bronchitis (Reinheimer et al., 1998) and asthma (Liu et al., 2018). In the present study, we observed a higher expression of ChAT in bronchial epithelial cells in HDM-exposed mice. Epithelial cell-derived ACh may contribute to goblet cell hyperplasia, mucus hypersecretion, and even airway hyperresponsiveness (Gosens & Gross, 2018; Kistemaker et al., 2014) in HDM asthma mice. VNS 0.1 attenuated the HDM-induced increase in epithelial ChAT and methacholine-induced airway hyperresponsiveness through α7nAChR activation.

5 | CONCLUSION

Taken altogether, our data have demonstrated for the first time that VNS, as a nondrug therapy, is equally effective in treating the hallmarks of allergic asthma as compared with pharmacological intervention with nicotine agonists. The fact that the administration of the specific α7nACh receptor antagonist α-BGTX (and not propranolol) blocked all effects induced by VNS strongly implicates that the activation of cholinergic vagal efferents is imperative not only for anti-inflammatory but also for anticonstrictive effects in the lung. Although ACh is the primary neurotransmitter contained in vagal postganglionic parasympathetic fibres in the lung (Hummel et al., 2019), we cannot rule out that some of the beneficial effects obtained with VNS may involve the activation of noncholinergic nonadrenergic (NANC) fibres, especially in airway hyperresponsiveness as they are known to reduce bronchoconstriction (Hoffmann et al., 2012; Krishnakumar et al., 2002). Noninvasive VNS has already been approved by the FDA for the treatment of depression and epilepsy, and has also been proven to be effective in a clinical study to reduce asthma exacerbation (Miner et al., 2012). Thus, our findings support VNS as a novel and safe strategy for asthma management, especially for severe asthma and steroid-resistant asthma.

AUTHOR CONTRIBUTIONS

C. Sévoz-Couche: Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **W. Liao:** Formal analysis (equal); methodology (equal); supervision (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **H. Y. C. Foo:** Formal analysis (supporting). **I. Bonne:** Data curation (lead); formal analysis (supporting); software (equal). **T. B. Lu:** Software (supporting). **C. Tan Qi Hui:** Formal analysis (equal); software (equal). **W. Y. X. Peh:** Methodology (supporting). **S.-C. Yen:** Methodology (supporting). **W. S. F. Wong:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); supervision (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

We confirm there is Data availability in this submission.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Natural Products Research, Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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