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RESEARCH PAPER

Targeting the phosphorylation site of myristoylated alanine-rich C kinase substrate alleviates symptoms in a murine model of steroid-resistant asthma

Chien-Neng Wang[1] | Yu-Chao Lin[2] | Bo-Chun Chang[3] | Ching-Hsien Chen[4] | Reen Wu[5] | Chen-Chen Lee[6,7]

[1] Graduate Institute of Basic Medical Science, College of Medicine, China Medical University, Taichung, Taiwan

[2] Division of Pulmonary Medicine, China Medical University Hospital, Taichung, Taiwan

[3] College of Medicine, China Medical University, Taichung, Taiwan

[4] Division of Nephrology, Department of Internal Medicine, University of California at Davis, Davis, California

[5] Center for Comparative Respiratory Biology and Medicine, Internal Medicine, College of Medicine, University of California at Davis, Davis, California

[6] Department of Microbiology and Immunology, School of Medicine, College of Medicine, China Medical University, Taichung, Taiwan

[7] Center of Drug Development, China Medical University, Taichung, Taiwan

Correspondence

Reen Wu, Internal Medicine and VM Anatomy, Physiology and Cell Biology, Center for Comparative Respiratory Biology and Medicine, Genome and Biomedical Science Facility Room 6510/6523, 451 East Health Sciences Drive, University of California at Davis, Davis, CA 95616.
Email: rwu@ucdavis.edu

Chen-Chen Lee, Department of Microbiology and Immunology, School of Medicine, College of Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 40402, Taiwan.
Email: leechenchen@mail.cmu.edu.tw

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Background and Purpose: Myristoylated alanine-rich C kinase substrate (MARCKS), a PKC substrate, facilitates mucus production and neutrophil migration. However, the effects of therapeutic procedures targeting the phosphorylation site of MARCKS on steroid-resistant asthma and the mechanisms underlying such effects have not yet been investigated. We designed a peptide that targets the MARCKS phosphorylation site (MPS peptide) and assessed its therapeutic potential against steroid-resistant asthma.

Experimental Approach: Mice were sensitized with ovalbumin (OVA), alum, and challenged with aerosolized OVA five times a week for 1 month. The mice were intratracheally administered MPS peptides three times a week, 1 hr before OVA challenge. Asthma symptoms and cell profiles in the bronchoalveolar lavage were assessed, and key proteins were analysed using Western blotting.

Key Results: Phosphorylated (p)-MARCKS was highly expressed in inflammatory and bronchial epithelial cells in OVA-immunized mice. MPS peptide reduced eosinophils, neutrophils, mucus production, collagen deposition, and airway hyperresponsiveness. Dexamethasone (Dexa) did not alleviate steroid-resistant asthma symptoms. MPS peptide caused a decrease in p-MARCKS, nitrotyrosine and the expression of oxidative stress enzymes, NADPH oxidase dual oxidase 1 and inducible NOS, in lung tissues. Compared to Dexa, MPS peptides inhibited C5a production and attenuated IL-17A and KC production in the airway more effectively, thus suppressing asthma symptoms.

Conclusions and Implications: Our findings indicate that targeting MARCKS phosphorylation through MPS treatment may inhibit neutrophilic inflammation and relieve asthma symptoms, thereby highlighting its potential as a therapeutic agent for steroid-resistant asthma.

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Abbreviations: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; C5a, complement component 5a; Dexa, dexamethasone; Duox, dual oxidase; FITC, fluorescein isothiocyanate; GATA-3, GATA binding protein 3; HBE, human bronchial epithelial; H&E, haematoxylin&eosin; IHC, immunohistochemistry; IL-1RA, IL-1 receptor antagonist; iNOS, inducible NOS; MARCKS, myristoylated alanine-rich c-kinase substrate; MDR, multiple drug resistance; MPS, MARCKS phosphorylation site; OVA, ovalbumin; PAS, periodic acid–Schiff; PSD, phosphorylation site domain; RORγt, RAR-related orphan receptor γt; T-bet, T-box transcription factor; T_H, T helper cell; TIMP, tissue inhibitors of metalloproteinase

1 | INTRODUCTION

Asthma, which is a chronic inflammatory disease, is considered a health problem worldwide. According to the World Health Organization, 235 million people suffer from asthma globally (http://www.who.int/respiratory/asthma/en/), and its prevalence has increased in the past two decades. Most manage their symptoms with drugs. However, 10–25% of patients require corticosteroids to control asthma symptoms. Even with high-dosage steroid treatments, symptoms may recur frequently (Bel et al., 2011; Chan, 1998; Kupczyk & Wenzel, 2012; Nelson, Leung, & Bloom, 2003; Szefler et al., 2002), indicating the development of steroid-resistant asthma (Chan, 1998; Kupczyk & Wenzel, 2012; Nelson et al., 2003). Therefore, there is a need for a novel therapeutic method to treat this form of asthma.

Myristoylated alanine-rich C kinase substrate (MARCKS), an 87-kDa protein, is the major substrate of **PKC** and is composed of three highly conserved regions: the N-terminal myristoylated domain, the phosphorylation site domain (PSD), and the multiple homology 2 domain. MARCKS is anchored to the cell membrane through an amide bond formed between the amino group of the N-terminal residue and the highly basic PSD of several positively charged lysine residues interacting with negatively charged acidic membrane phospholipids (Harlan, 1991; Sundaram, Cook, & Byers, 2004; Wu, Walaas, Nairn, & Greengard, 1982). The PSD comprises 25 amino acids containing four serine residues that may be phosphorylated by PKC. Phosphorylated MARCKS regulates secretion, migration, and invasion in various cells. Membrane-bound MARCKS is bound to **phosphatidylinositol 4,5-bisphosphate (PIP_2)** and **phosphatidylserine**. Upon phosphorylation by PKC, MARCKS moves from the cell membrane into the cytosol and binds to actin filaments, inducing leukocyte migration (Eckert, Neuder, Park, Adler, & Jones, 2010; Green et al., 2012), lung cancer cell metastasis (Chen et al., 2014), and airway goblet cell mucus secretion (Singer et al., 2004). MARCKS protein is an important molecule that regulates mucin hyper-production and secretion in asthma (Singer et al., 2004).

Mechanisms underlying severe asthma remain unclear. However, activation of $CD4^+$ T helper cells (T_H1, T_H2, and T_H17) and oxidative stress may contribute to severe asthma. MARCKS activation through phosphorylation regulates PIP_2-related pathways, important for lymphocyte activation and inflammatory cell migration. Targeting the phosphorylation site of MARCKS may be a potential therapeutic strategy for steroid-resistant asthma. We designed a peptide targeting the MARCKS phosphorylation site (MPS) and investigated its effects on steroid-resistant asthma in a mouse model and the mechanism underlying these effects. We investigated whether the MPS could be utilized as a novel therapeutic target for steroid-resistant asthma.

What is already known

- Myristoylated alanine-rich C kinase substrate (MARCKS), a PKC substrate, facilitates mucus production and neutrophil migration

What this study adds

- MARCKS phosphorylation highly expressed in airway epithelial cells and inflammatory cells in a murine model of steroid-resisitant-like asthma. A peptide targeting MARCKS phosphorylation site domain showed inhibitory effects of airway dysfunction of allergic asthma.

What is the clinical significance

- There is no effective drug for treatment of steroid-resistance asthma. Targeting MARCKS phosphorylation through MPS treatment may inhibit neutrophilic inflammation and relieve asthma symptoms, thereby highlighting its potential as a therapeutic agent for steroid-resistant asthma.

2 | METHODS

2.1 | Animals

Six-week-old female BALB/cJ mice were purchased from the National Laboratory Animal Centre, National Research Laboratories, Taiwan (RRID:IMSR_JAX:000651). All mice were housed in a pathogen-free environment at the animal centre of China Medical University and were provided with a standard chow diet (carbohydrate: 70%; protein: 20%; fat: 10%) and water ad libitum. The animals were housed in groups of seven per cage under a 12 hr light–dark cycle and fed for 8 weeks. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*. All animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University. Protocols for animal experiments were approved by the Animal Care and Use Committee of China Medical University (Protocol No. 103-128-N).

2.2 | Animal experiments

On Day 0, 6-week-old female BALB/c mice were immunized with 50 μg of ovalbumin (OVA) and 2 mg of alum adjuvant dissolved in 200 μl of PBS through i.p. injection. Boosters composed of 25 μg

OVA and 2 mg alum were administered on Days 4 and 7 (Reddy, Lakshmi, & Reddy, 2012). Sensitized mice were challenged with 4%, 5 ml aerosolized OVA in PBS using a nebulizer (AG-AL1000, Aeroneb[R] Lab Nebulizer Unit, Standard VMD) once a day, 5 days per week, for 1 month as previously described (Lee et al., 2013; Figure 1a). On Days 12, 14, 16, 19, 21, 23, 26, 28, 30, 33, 35, and 37, the mice were administered 0.46 and 2.3 mg·kg^{-1} MPS, 2.3 mg·kg^{-1} A-MPS, and 2.3 mg·kg^{-1} D-MPS peptides (figures represent A-MPS and D-MPS as groups) or 1 mg·kg^{-1} **dexamethasone** (Dexa), intratracheal instillation 1 hr before OVA exposure. Each group was composed of five to seven mice. On Day 38, the mice were killed via CO_2 exposure, and chronic inflammatory responses were noted (Figure 1b).

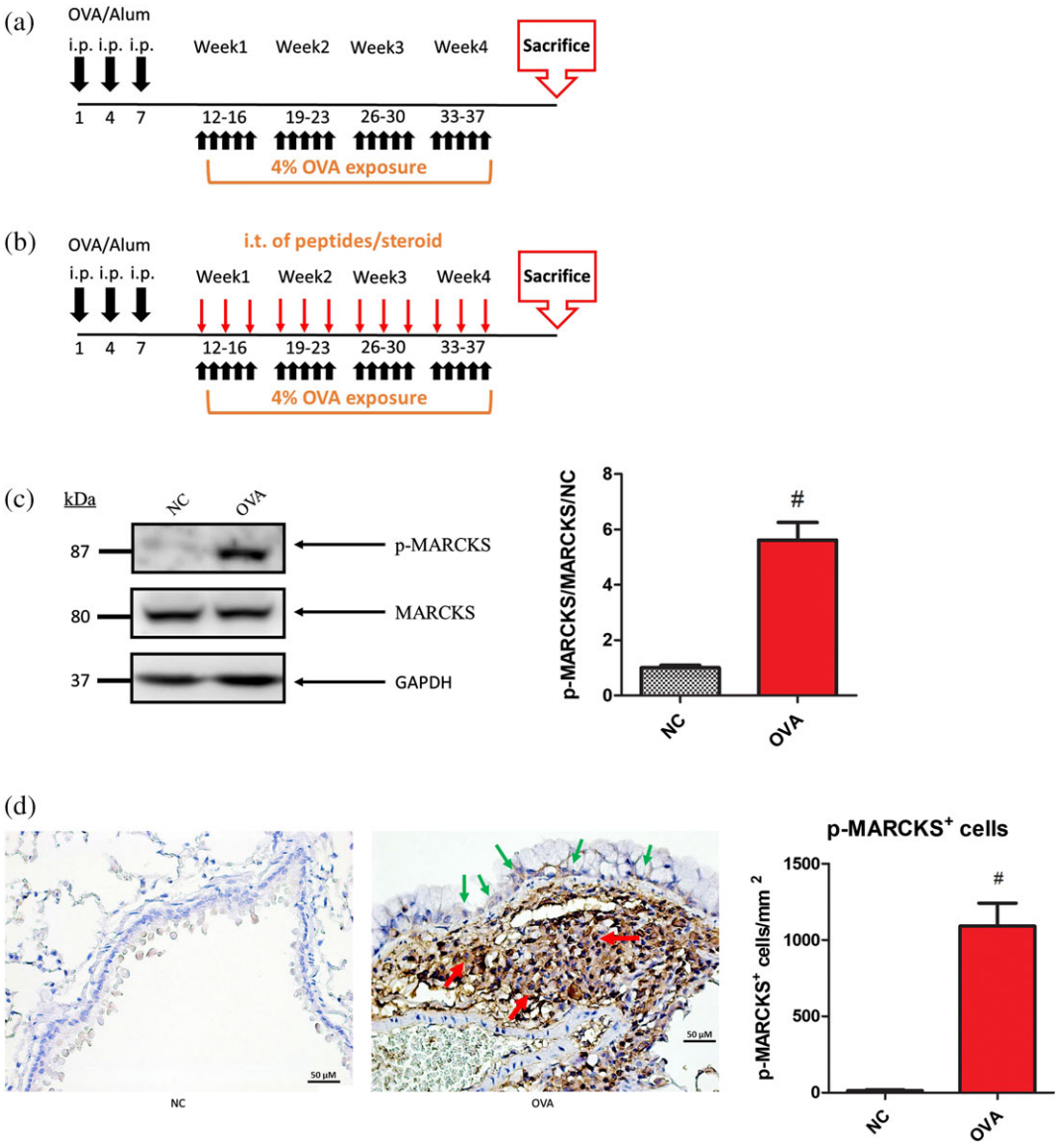

FIGURE 1 Elevation of phosphorylated (p)-MARCKS levels in the airways of OVA-immunized mice. (a) OVA-induced steroid-resistant-like asthma model. Six-week-old mice were sensitized with OVA/alum, as described in 2. On Day 12, mice were exposed to PBS or OVA (4% weight per volume diluted in 0.9% saline; 5 ml per inhalation) 5 days per week for 4 weeks. On Day 38, mice were killed; the lungs were embedded in paraffin and analysed via Western blot and immunohistochemical (IHC) staining. (b) Brief scheme of the generation of the mouse steroid-resistant model. Mice were sensitized with aerosolized OVA 5 times·week^{-1} for 1 month (black arrow). On Days 12, 14, 16, 19, 21, 23, 26, 28, 30, 33, 35, and 37, mice were administered various MPS peptides/steroids which were intratracheally instilled 1 hr before OVA challenge (red arrow). On Day 38, mice were killed, and the lungs were collected for further experimentation. (c) Proteins were extracted from homogenized lungs and subjected to Western blot to detect p-MARCKS. p-MARCKS was quantified using ImageJ. (d) Paraffin-embedded sections of mouse airway tissues were de-paraffinized, IHC stained with anti-Ser$^{152/164}$ p-MARCKS antibody (green arrows represent epithelial cells and red arrows represent inflammatory cells). The scale bar is 50 μm. Data are expressed as means and SEM for NC and OVA groups (n > 11). $^{\#}P < 0.05$, as compared to the NC group

2.3 | Bronchoalveolar lavage and lung histology

Bronchoalveolar lavage (BAL) was performed by injecting 1 ml of HBSS and gently aspirating 1 ml of BAL. The procedure was performed three times. BAL samples were centrifuged at 200× *g* and 4°C for 5 min. Supernatant from the first BAL sample was stored at −20°C for cytokine analysis by ELISA. BAL cells were counted using a haematocytometer. Cytospin slides of BAL cells were stained with May–Giemsa stain. The cell profile of 300 cells in at least four fields of view was determined under a light microscope (Lee et al., 2010; Lee et al., 2013). The lungs were fixed with 10% formalin and sectioned for haematoxylin & eosin (H&E) staining, periodic acid–Schiff (PAS) staining, and Masson's trichrome staining, which were performed to quantify the number of infiltrating cells, mucus production, and collagen deposition. Quantification of inflammatory cells, PAS-positive bronchial epithelial cells, and the collagen deposition area was performed according to a previous study (Lee et al., 2010).

2.4 | Immunohistochemistry

Lung sections were de-paraffinized and hydrated, and epitope retrieval was performed according to manufacturer's protocol (Thermo Quanto Detection system, CA). Briefly, the lung sections were stained with hydrogen peroxide and protein block and treated with rabbit polyclonal anti-Ser$^{152/156}$ phosphorylated MARCKS (cat#07-1238, Millipore, Bedford, MA) at 1:100 dilution rate and PIP_2 antibody (ab811039, Abcam, Cambridge, MA) afterwards at a 3 μg·ml^{-1} dilution rate. Sections were then stained with secondary HRP-labelled polymer and DAB (Antibody amplifier + System-HRP, Thermo) and counter-stained with haematoxylin. Immunostained sections were viewed under a Zeiss microscope (Stuttgart, Germany). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander, Roberts et al., 2018).

2.5 | Cytokine assay

BAL supernatants were stored at −20°C prior to analysis via ELISA, performed according to the manufacturer's instructions. Standards were prepared from recombinant mouse **IFN-γ**, **IL-4**, **IL-13**, **IL-17**, **IL-33**, **eotaxin** **(CCL11)**, and **KC** **(CXCL1)** (R&D systems, Minneapolis, MN).

2.6 | Western blot analysis

Lung tissues were homogenized via a lysis buffer (25 mM Tris–HCl, 2 mM EDTA, 1 mM benzamidine, 1 mM PMSF; pH 7.4) containing protease and phosphatase inhibitors (Thermo Fisher Scientific). Appropriate homogenate volumes, containing 50 μg of proteins per well, were quantified using a Bradford Assay Kit (cat#500-0006, Bio-Rad, Hercules, CA) and separated via 10% SDS-PAGE. The proteins were then transferred to PVDF membranes and probed with anti-Ser$^{159/163}$ phosphorylated MARCKS antibody (ab81295, RRID: AB_2773726; Abcam, Cambridge, MA) at a 1:2,000 dilution for Western blotting. Anti-FOXP3 at a concentration of 4 μg·ml^{-1} (ab20034, RRID:AB_445284; Abcam), anti-MARCKS at 1:500 dilution, anti-**DUOX-1 (dual oxidase 1)** at 1:500 dilution, anti-**DUOX-2** at 1:2,000 dilution, anti-total **PKC-β**II at 1:500 dilution, anti-GATA binding protein 3 (GATA-3) at 1:500 dilution, anti-**RAR-related orphan receptor γt (RORγt)** at 1:500 dilution, anti-T-box transcription factor (T-bet) at 1:500 dilution (SC-100777, RRID:AB_1125958; SC-48858, RRID:AB_2094184; SC-49939, RRID:AB_2094195; SC-210, RRID:AB_2252825; SC-268, RRID:AB_2108591; SC-28559, RRID:AB_2285218; SC-21749, RRID:AB_2094195; Santa Cruz, CA, USA), anti-3-nitrotyrosine at 1:1500 dilution (ab61392, RRID: AB_942087; Abcam), anti-complement component 5a (**C5a**; Genetax 11625, RRID:AB_1949789), anti-Ser^{660} phosphorylated PKC-βII at 1:1,000 dilution (Cell Signaling #9371, RRID:AB_2168219), anti-**inducible NOS (iNOS)** at 1:500 dilution, and anti-GAPDH at 1:1000 dilution (#NB300-605, RRID:AB_10002794; #NB300-221, RRID:AB_10077627, mouse monoclonal, Novus Biologicals, Littleton, CO; Chan, Lien, Lee, & Huang, 2016) were used. Following incubation with HRP-conjugated secondary antibodies, signals were visualized using a Millipore ECL Kit (Millipore, Bedford, MA) according to the manufacturer's instructions.

2.7 | Measurement of airway hyperresponsiveness by non-invasive whole body plethysmography

Mice were placed in whole body plethysmography (BUXCO, USA) chambers for 15 min. Baseline readings of airway resistance were collected every 2 s for 3 min. The baseline for resistance index was determined using PBS as a control, and 500 μl each of varying **methacholine** concentrations (0, 3.125, 6.25, and 12.5 mg·ml^{-1}) was used for repeat nebulization for 2 min and an additional 6-min cycle. Airway resistance was determined by calculating the dimensionless parameter enhanced pause (Reddy et al., 2012).

2.8 | In vivo fluorescence imaging with the IVIS spectrum system

Retention time of MPS in the lung tissues was determined via in vivo fluorescence imaging using the IVIS spectrum imaging system (Caliper Life Sciences) 0 min, 3 min, 6 hr, 24 hr, and 48 hr following intratracheal instillation of FITC-conjugated MPS peptide (2.3 mg·kg^{-1}) into BALB/c mice. At specified time periods following intratracheal instillation of MPS peptide, the mice were killed and the lungs isolated for fluorescence imaging. Fluorescence intensities of MPS peptides were analysed using accessory software (Living Image Version 4.0; Caliper Life Sciences). Ratios of fluorescence intensities in the MPS peptide-treated group and those in the control group were calculated using ImageJ software. (ImageJ, RRID:SCR_003070).

2.9 | Statistical analysis

Results are presented as mean ± SD for $n \geq 5$. The programme Instat (GraphPad software) was used for statistical analysis. Group comparisons using one-way ANOVA followed by Newman–Keuls post hoc test were conducted for cell counts in BAL fluid, H&E, PAS, Masson's trichrome pathology index, airway hyperresponsiveness (AHR), Western blotting, and cytokine levels. In Figure 1c,d, data were analysed using Student's paired *t*-test. Statistical significance was set at $P < 0.05$. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology.

2.10 | Reagents and chemicals

Ovalbumin (OVA; grade V), methacholine, and dexamethasone (Dexa) were purchased from Sigma Chemical Co. (St. Louis, MO). HBSS was obtained from Invitrogen (Carlsbad, CA). Alum adjuvant was purchased from Thermo Fisher Scientific (AlumImuject; Pierce Chemical, Rockford, IL). The MPS peptide (N-KKKKRFSFKKSFKLSGFSFKKNKK-C), A-MPS peptide; (N-KKKKRFAFKKAFKLAGFAFKKNKK-C), D-MPS peptide (N-KKKKRFDFKKDFKLSGFSFKKNKK-C), and MPS, A-MPS, and D-MPS peptides conjugated to fluorescein isothiocyanate (FITC) at the C-terminal were purchased from KareBay™ Biochem's (Monmouth Junction, NJ).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Cidlowski et al., 2017; Alexander, Fabbro et al., 2017).

3 | RESULTS

3.1 | Elevation of MARCKS phosphorylation in the lungs in a murine model of steroid-resistant asthma

To create a murine steroid-resistant asthma model, mice were sensitized with OVA combined with alum and exposed to OVA for 4 weeks (Figure 1a,b). MARCKS was highly expressed in the lungs of OVA-immunized mice (Figure 1c). It was also expressed in bronchial epithelial cells (green arrows) and inflammatory cells (red arrows; Figure 1d). Steroid-resistant-like mice exhibited severe airway inflammation (Figures 2c,d), mucus production (Figure 2e), collagen deposition (Figure 2f), and AHR (Figure 2g). The mice were resistant to Dexa treatment.

3.2 | MPS peptide inhibits asthma symptoms by interfering with MARCKS phosphorylation in a murine steroid-resistant-like asthma model

We designed a peptide that targets the MARCKS phosphorylation to investigate potential therapeutic effects of MPS peptides on steroid-resistant asthma (Figure 2a). To determine the retention time of MPS peptides, MPS peptides conjugated to FITC (2.3 $mg{\cdot}kg^{-1}$) were intratracheally instilled in mice and MPS peptide levels measured at different time points. The fluorescence of MPS peptide reached its peak at 3 min (Figure 2b), declined sharply, sustained at 48 hr, and disappeared at 72 hr (Figure S4). The fluorescence of low dose MPS peptide (0.46 $mg{\cdot}kg^{-1}$) reached its peak at 3 min but disappeared at 24 hr (left panel; Figure S4). We investigated the ability of three designated MPS peptides in human bronchial epithelial (HBE) cell line to enter cells and localize intracellularly. HBE cells were treated with FITC-labelled MPS, A-MPS, and D-MPS peptides. MPS, A-MPS, and D-MPS peptides anchor in the cell membrane and enter HBE cells as a complete cytosolic green fluorescence, extending into the nuclei (Figure S1). Peptide fluorescence was sustained for 48 hr in HBE cells (data not shown). Based on these results, the time interval between intratracheal instillation of MPS peptide and the relevant assays was set as 48 hr. Following intratracheal instillation of MPS peptide at 0.46 and 2.3 $mg{\cdot}kg^{-1}$, OVA-induced airway inflammation, mucus production, collagen deposition, and AHR were inhibited in a dose-dependent manner (Figure 2c–g). Analysis of inflammatory cell profiles of BAL showed that MPS peptide inhibited infiltration of neutrophils, eosinophils, and lymphocytes in steroid-resistant-like mice. Dexa was able to suppress the infiltration of eosinophils and lymphocytes but not that of neutrophils (Figure 2c). To determine whether suppression of asthma symptoms following MPS peptide treatment was associated with MARCKS phosphorylation, we assessed p-MARCKS expression in the lungs. The expression of p-MARCKS was higher in the lungs of OVA-immunized mice. It decreased upon treatment with MPS peptide but was unaffected by Dexa treatment (Figure 2h). Thus, targeting p-MARCKS with MPS peptides may block asthma symptoms.

3.3 | MPS peptide reduces IL-17A, C5a expression, and oxidative stress production and contributes to the inhibition of asthma symptoms in a murine steroid-resistant asthma model

T helper (T_H) cells, T_H1, T_H2, and T_H17, are involved in airway inflammation in steroid-resistant asthma (Hansbro et al., 2017; Linden, 2006; Zijlstra, Ten Hacken, Hoffmann, van Oosterhout, & Heijink, 2012). We observed a similar phenomenon. In steroid-resistant mice, T_H1-specific cytokine IFN-γ, T_H2-related cytokines, IL-4, IL-13, and eotaxin, and T_H17-related cytokines, IL-17 and KC, were all increased in BAL or in the lungs (Figure 3a). Following treatment with 2.3 $mg{\cdot}kg^{-1}$ MPS peptide, all these cytokines were suppressed. In contrast, Dexa inhibited IFN-γ, IL-4, and eotaxin and slightly attenuated IL-13 and

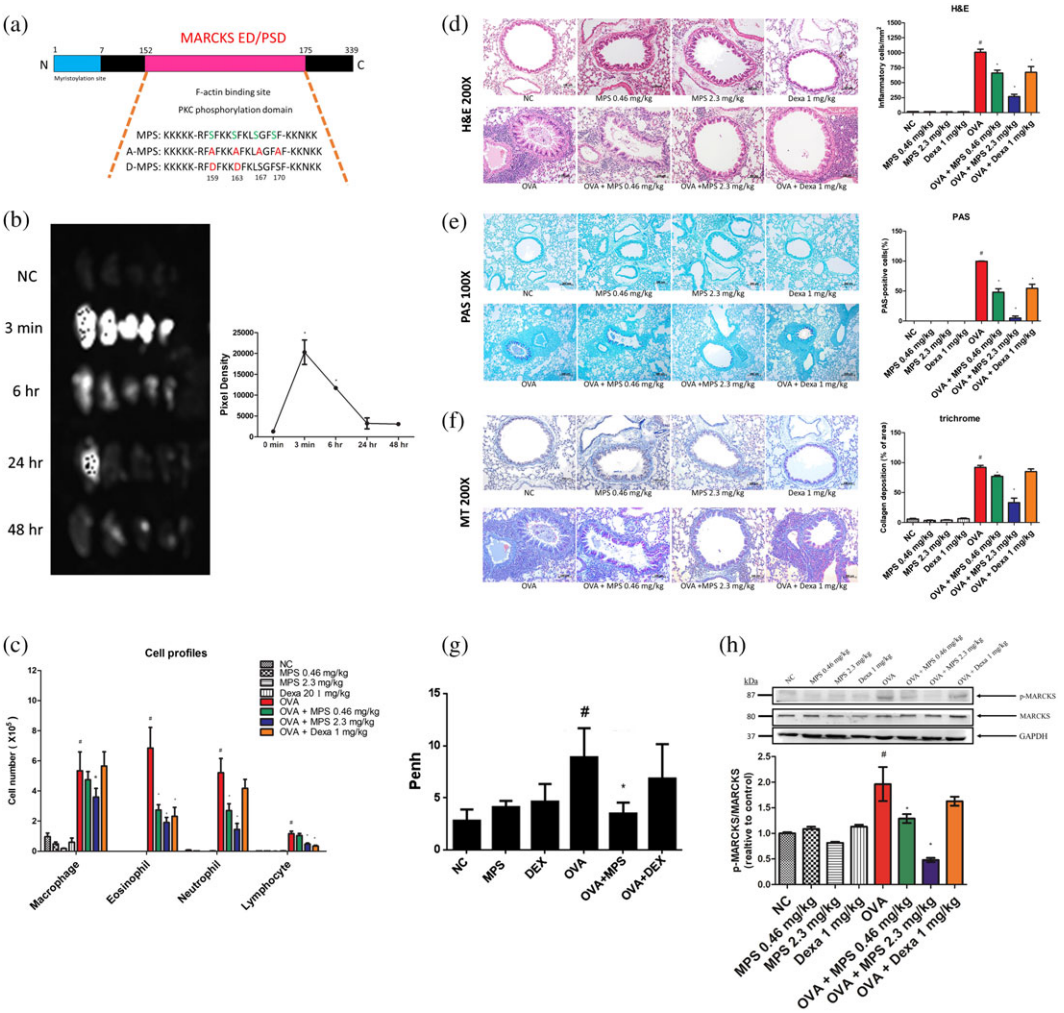

FIGURE 2 Asthma symptoms were alleviated by a peptide targeting the MARCKS phosphorylation site. (a) Amino acid sequences of MPS, A-MPS, and D-MPS peptides. (b) Retention time of MPS peptides in the airway. MPS-FITC peptides at concentrations of 0.46 and 2.3 mg·kg^{-1} were intratracheally instilled in mice at different time points (0 min, 3 min, 24 hr, 48 hr, and 72 hr), and the lungs were dissected, and fluorescence was measured under an IVIS system. (c) As described in 2, mice were killed on Day 38, and differential cell counts of BAL were assessed via May–Giemsa staining. (d–f) The lungs were stained using H&E, periodic acid–Schiff (PAS), and Masson's trichrome (MT) staining. Bar represents 100 μm for H&E and MT; 200 μm for PAS. (g) Airway resistance was determined via whole body plethysmography. (h) Lung proteins were subjected to Western blot to assess Ser$^{159/163}$ p-MARCKS, MARCKS, and GAPDH expression. Proteins were quantified using ImageJ. Data are expressed as mean ± SD ($n \geqq 5$). $^{\#}P < 0.05$; $^{*}P < 0.05$, compared to NC and OVA groups

KC production but did not inhibit IL-17. MPS peptide also inhibited expression of T_H-specific transcription factors (T-bet T_H1, GATA-3 T_H2, and RORγt T_H17 in lung tissues). By contrast, Dexa inhibited GATA-3 expression and slightly suppressed T-bet expression but not RORγt (Figure 3b). This trend was similar to that for T_H-specific cytokines. In a previous study it was found that mice subjected to prolonged 3% OVA exposure (three times a week for a total period of 6 weeks) displayed airway tolerance with a reduction in total BAL cells, eosinophils, neutrophils, and lymphocytes; however, exposure to different concentrations of OVA for 6 weeks caused airway inflammation (Sethi & Naura, 2018). To confirm effects of immune tolerance in our mouse model, we investigated regulatory T cell specific transcription factor, FOXP3, expression in the lungs. FOXP3 expression in lung tissue was not increased in our steroid-resistant-like asthma model (Figure 3b). Therefore, airways exhibiting severe allergic asthma symptoms did not induce naturally occurring Foxp3$^+$CD4$^+$CD25$^+$ T cell (nTreg) activation in our mouse model.

Next, cytokine/chemokine arrays were utilized to characterize the cytokine/chemokine profiles following MPS peptide and Dexa treatment. Of the 40 cytokines, chemokines and acute-phase proteins that were screened, C5a and tissue inhibitors of metalloproteinase (**TIMP-1**), which were markedly overexpressed in steroid-resistant mice, were down-regulated by MPS peptide but not by Dexa (Figure 3c). C5a is important for the recruitment and activation of granulocytes (Ehrengruber, Geiser, & Deranleau, 1994; Zeck-Kapp, Kroegel, Riede, & Kapp, 1995). In addition, **IL-1 receptor antagonist, IL-1Ra** (receptor antagonist for IL-1β), levels in BAL were lower in Dexa-treated steroid-resistant mice than in OVA- or MPS peptide-treated mice (Figure 3c). Other chemokines **CXCL-13**, soluble **intercellular adhesion molecule-1** (sICAM-1), KC and the stromal

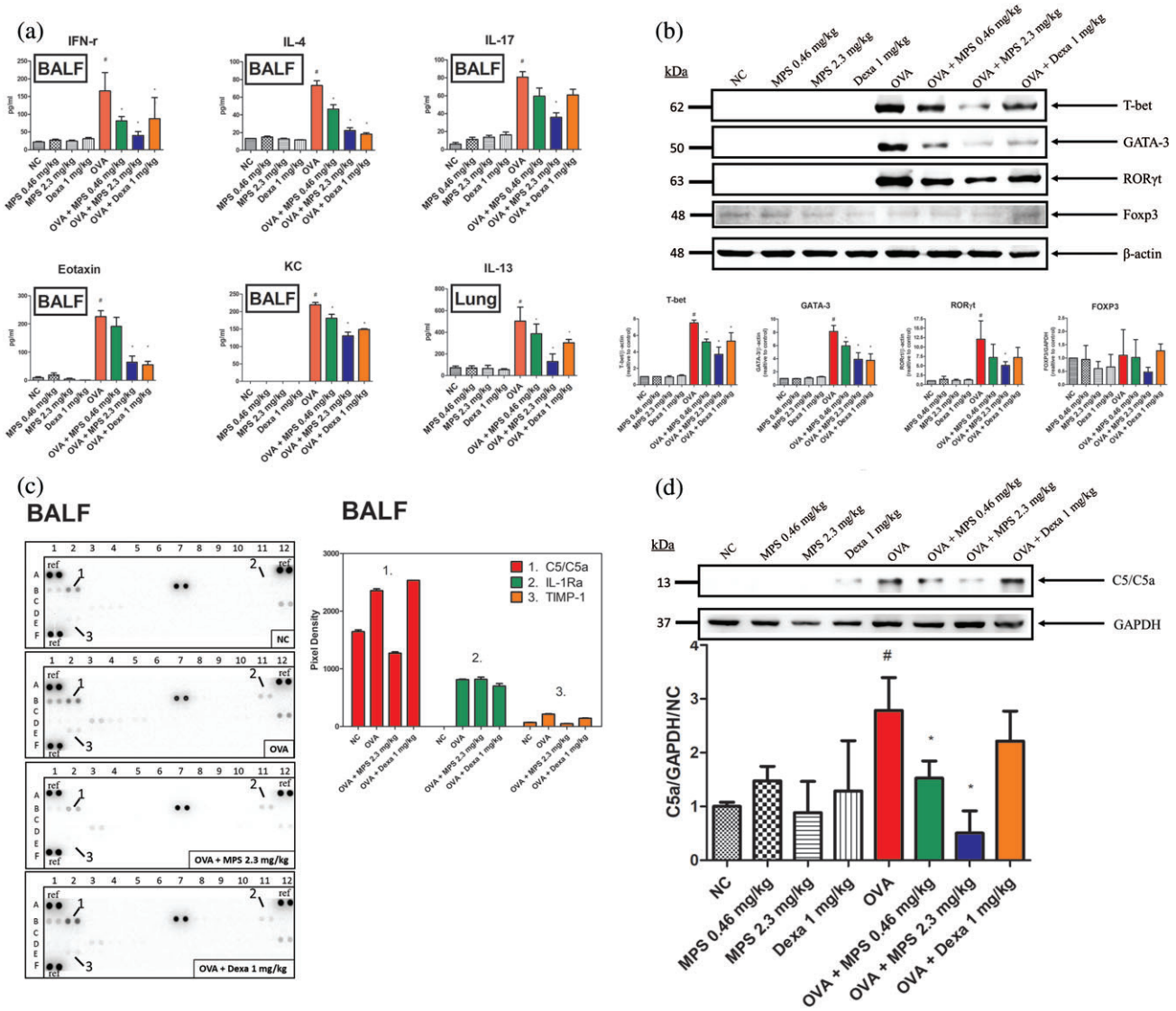

FIGURE 3 MPS peptides decreased T_H1-, T_H2-, and T_H17-related cytokines and specific-transcriptional factors as well as C5a expression in the lungs. (a) On Day 38, mice were killed, BAL fluids collected, and lung proteins analysed via ELISA. (b) Proteins were extracted from homogenized lungs and subjected to Western blotting to assess T-bet, GATA-3, RORγt, FOXP3, and β-actin expressions. (c) BAL fluids were analysed using a mouse cytokine array to screen 40 cytokines, chemokines, and complement and acute-phase proteins in the BAL. Pixel density plots of C5/C5a, IL-1Ra, and TIMP-1 were analysed using ImageJ. (d) Proteins were extracted from homogenized lungs and subjected to Western blotting to assess C5a and GAPDH expressions. These proteins were quantified using ImageJ. Data are expressed as mean ± SD ($n \geqq 5$). $^{\#}P < 0.05$, compared to the NC group; $^{*}P < 0.05$, compared to the OVA group

cell-derived factor-1 (SDF-1), were also increased in the OVA treatment group (Figure S5). MPS peptide and Dexa both reduced CXCL-13, KC, and SDF-1 levels but not that of sICAM-1.

In steroid-resistant asthma, airway inflammatory cell activation causes oxidative stress in the lungs, suppressing lung function, increasing mucus production, and reducing corticosteroid responsiveness in asthma (Saleh, Ernst, Lim, Barnes, & Giaid, 1998). Thus, oxidative stress is a pathological mechanism, which induces steroid-resistant responses in asthma. Therefore, we examined oxidative stress in lung tissues. We found that numerous proteins had been nitrosylated. Also, levels of the oxidative indicator, 3-nitrotyrosine (3-NT), and related enzymes including dual oxidase-1 (Duox-1), Duox-2, and iNOS in the lung tissues of the OVA group were increased (Figure 4). The 2.3 mg·kg^{-1} MPS peptide treatment group, but not Dexa treatment, significantly reduced 3-NT, Duox-1, and iNOS expressions in the lungs.

3.4 | MPS peptide interferes with p65 phosphorylation but does not affect the reduction of HDAC-2 in the lungs in a murine steroid-resistant-like asthma model

To clarify mechanisms underlying the effect of MPS peptide on steroid-resistant signalling, histone deacetylase-2 (HDAC-2) protein

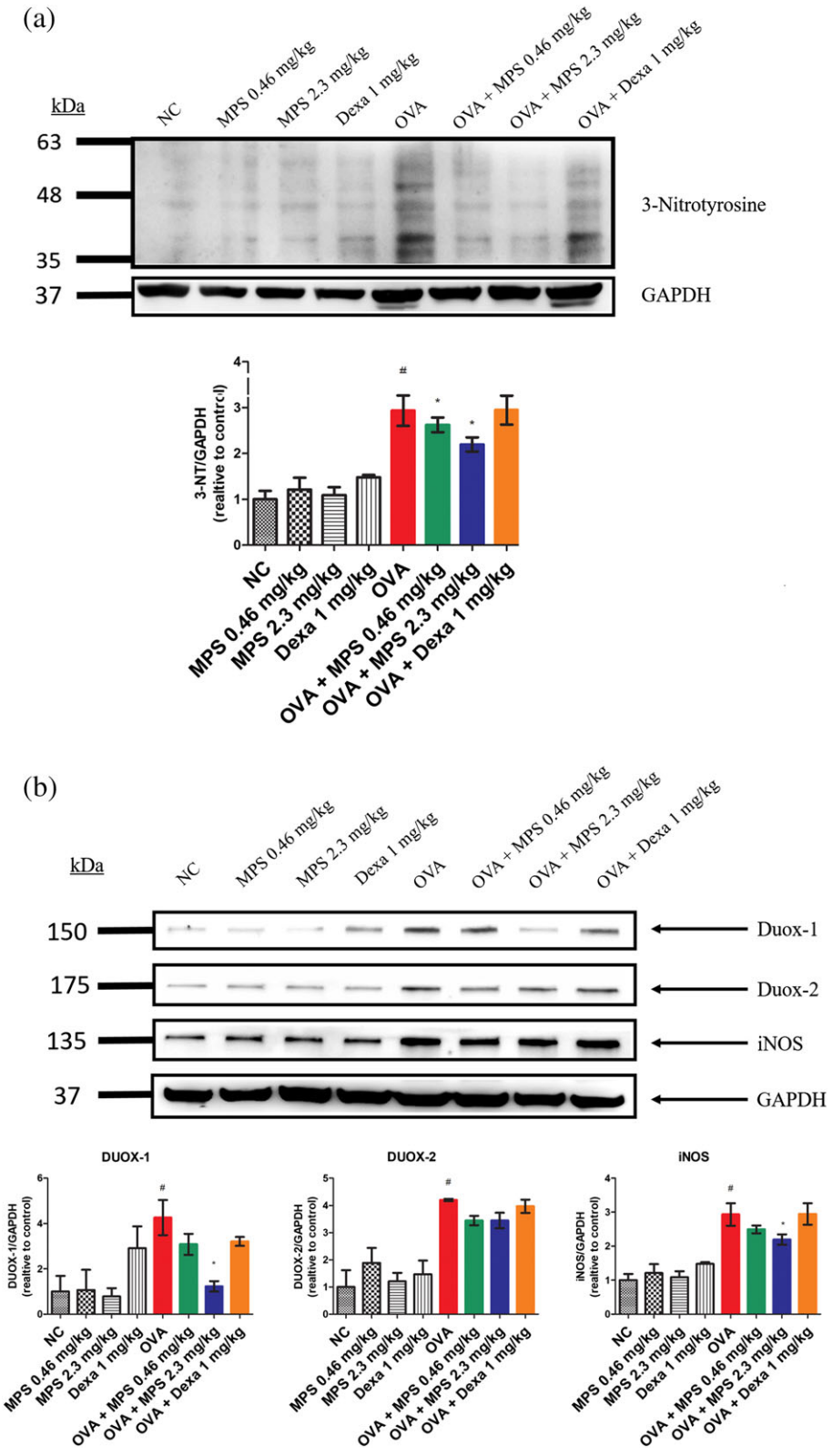

FIGURE 4 Suppression of oxidative stress in lung tissues from steroid-resistant mice. Proteins were extracted from homogenized lungs and subjected to Western blotting to assess the expression of (a) 3-nitrotyrosine (3-NT) and (b) dual oxidase-1 (Duox-1) and Duox-2, inducible NOS (iNOS), and GAPDH. These proteins were quantified using ImageJ. Data are expressed as mean ± SD (n = 5). $^{\#}P < 0.05$, as compared with the NC group; $^{*}P < 0.05$, as compared with the OVA group

expression and NF-κB activation, involved in steroid-resistant signalling (Barnes, 2011; Ito et al., 2008), were investigated in lung tissue. HDAC-2 protein expression was significantly decreased in the OVA-immunized group that showed steroid-resistant features, whereas Dexa treatment did not prevent this HDAC-2 reduction (Figure S2). However, MPS peptide was also unable to reverse HDAC decline in the OVA-immunized group. As NF-κB, which is a key transcription factor activating inflammatory cytokines, is induced by high oxidative stress in the OVA-immunized group in the steroid-resistant-like model, we investigated whether MPS peptide inhibited asthma symptoms by activating NF-κB. A high dose of MPS peptide treatment suppressed high pp65 expression in the OVA-immunized group whereas Dexa treatment did not change pp65 expression (Figure S6).

3.5 | Mutation of Ser residues in MPS to Asp or Ala blocks MPS peptide-induced anti-asthma effects in a murine steroid-resistant asthma model

We investigated the molecular mechanisms underlying inhibition of asthma symptoms by MPS peptide treatment. First, two different mutant MPS peptides were designed (Figure 2a): A-MPS (four Ser residues mutated to Ala which cannot be phosphorylated) and D-MPS (two Ser residues mutated to Asp to mimic protein structure of phosphorylated serine residue). Intratracheal instillation of A-MPS or D-MPS (2.3 $mg \cdot kg^{-1}$) blocked MPS peptide-induced inhibition of asthma symptoms, including airway inflammation (Figures 5a,b), mucus production (Figure 5c), collagen deposition (Figure 5d), and AHR (Figure 5e–g). Furthermore, A-MPS and D-MPS did not inhibit OVA-induced MARCKS phosphorylation in the lungs (Figure 6). Finally, unlike MPS peptide, A-MPS and D-MPS peptide were unable to suppress an increase in phosphorylated PKC expression in the lungs of murine steroid resistant-like-asthma models (Figure 6).

4 | DISCUSSION

The protein, MARCKS, induces vesicle release during exocytosis and cell migration via actin cytoskeleton remodelling. It plays an important role in the pathophysiology of several pulmonary diseases such as allergic asthma (Singer et al., 2004) and in the development of lung cancer (Chen et al., 2014), breast cancer (Browne et al., 2013; Chen et al., 2015), renal cell carcinoma (Chen et al., 2017), hepatocellular carcinoma (Naboulsi et al., 2016), and pancreatic cancer (Brandi et al., 2016). However, the role of MARCKS phosphorylation in airway inflammatory diseases such as severe, steroid-resistant asthma remains unclear. We detected an elevation in p-MARCKS expression in the lungs of murine steroid-resistant asthma models. We showed that MPS peptide, which targets the MPS, alleviated asthma symptoms including airway inflammation, mucus production, airway remodelling, and airway hyper-responsiveness in steroid-resistant models. This suggested that MPS is a potential target for the treatment of steroid-resistant asthma. These results suggested that MPS peptide inhibited asthma symptoms by interfering with MARCKS phosphorylation. The phosphorylation of MARCKS by PKC causes translocation of MARCKS into the cytosol and the release of PIP_2 (Glaser & Wanaski, 1996; Morton et al., 2012). PIP_2 is then activated by PLC-γ1 and phosphatidylinositol-4, 5-bisphosphate 3-kinase (PtdIns-3-OH kinase) allowing it to activate downstream signalling pathways involving immune cell activation. We also found that MPS peptide attenuated PKC phosphorylation which may reduce MARCKS phosphorylation. However, PKC is activated by calcium ions (Ca^{2+}) and DAG, released following MARCKS phosphorylation. Therefore, inhibition of PKC phosphorylation may occur after MPS peptide interferes with MARCKS phosphorylation. This may require further investigation. We also investigated PIP_2 expression in the lungs using immunohistochemical. PIP_2, which was detected in both bronchial and alveolar epithelial cells of normal lungs, was highly expressed in epithelial cells and inflammatory cells of inflamed lungs (Figure S7). Following MPS peptide treatment, the PIP_2 level was reduced through attenuation of inflammatory cell infiltration in the lungs. The effect of MPS peptide on PIP_2 downstream signalling pathways needs further investigation.

To understand the mechanisms by which MPS peptide inhibits MARCKS phosphorylation, two MPS mutant peptides, A-MPS and D-MPS, were used. Both peptides did not alleviate asthma symptoms in a murine steroid-resistant-like asthma model. This indicates that Ser residues in MPS peptide are important for relieving asthma symptoms. D-MPS did not inhibit MARCKS phosphorylation because D-MPS mimics phosphorylated MPS in that it possesses excess negative charge, thereby reducing its electrostatic interaction with acidic lipids such as phosphatidylserine (McLaughlin & Aderem, 1995; Seykora, Myat, Allen, Ravetch, & Aderem, 1996). Therefore, D-MPS was unable to bind to the cell membrane. However, A-MPS did not suppress MARCKS phosphorylation and asthma symptoms, although no excess negative charge was introduced. Such inability of A-MPS to reduce asthma symptoms and MARCKS phosphorylation will be investigated.

The cytokine profile in BAL was analysed. MPS peptide inhibited expression of T helper cell (T_H) 1-, T_H2-, T_H17-specific cytokines (IFN-γ, IL-4, and IL-17) and transcription factors (T-bet, GATA-3, and RORγt) in the lungs. This may be due to inhibition of MARCKS phosphorylation, which attenuated activation of lymphocytes, including T_H cells, by down-regulating PIP_2/PLC/inositol 1,4,5-triphosphate, DAG/NF-κB, activator protein 1, and nuclear factor of activated T-cell signalling pathways (Foster, De Hoog, & Mann, 2003; Saito et al., 2003; Strzelecka-Kiliszek, Korzeniowski, Kwiatkowska, Mrozinska, & Sobota, 2004). In contrast, Dexa inhibited the production of T_H1- and T_H2-specific cytokines but not that of the T_H17-specific cytokine IL-17. T_H17 causes steroid resistance in murine asthma models (Ano et al., 2013), patients with inflammatory diseases, and bronchial epithelial cells (Ramesh et al., 2014; Schewitz-Bowers et al., 2015; Vazquez-Tello et al., 2010). The mechanisms underlying T_H17-induced steroid resistance are still unclear. Ramesh et al. (2014) demonstrated the expression of the protein multi-drug resistance type 1 (MDR1) by T_H17 cells, which is refractory to glucocorticoid-mediated T cell

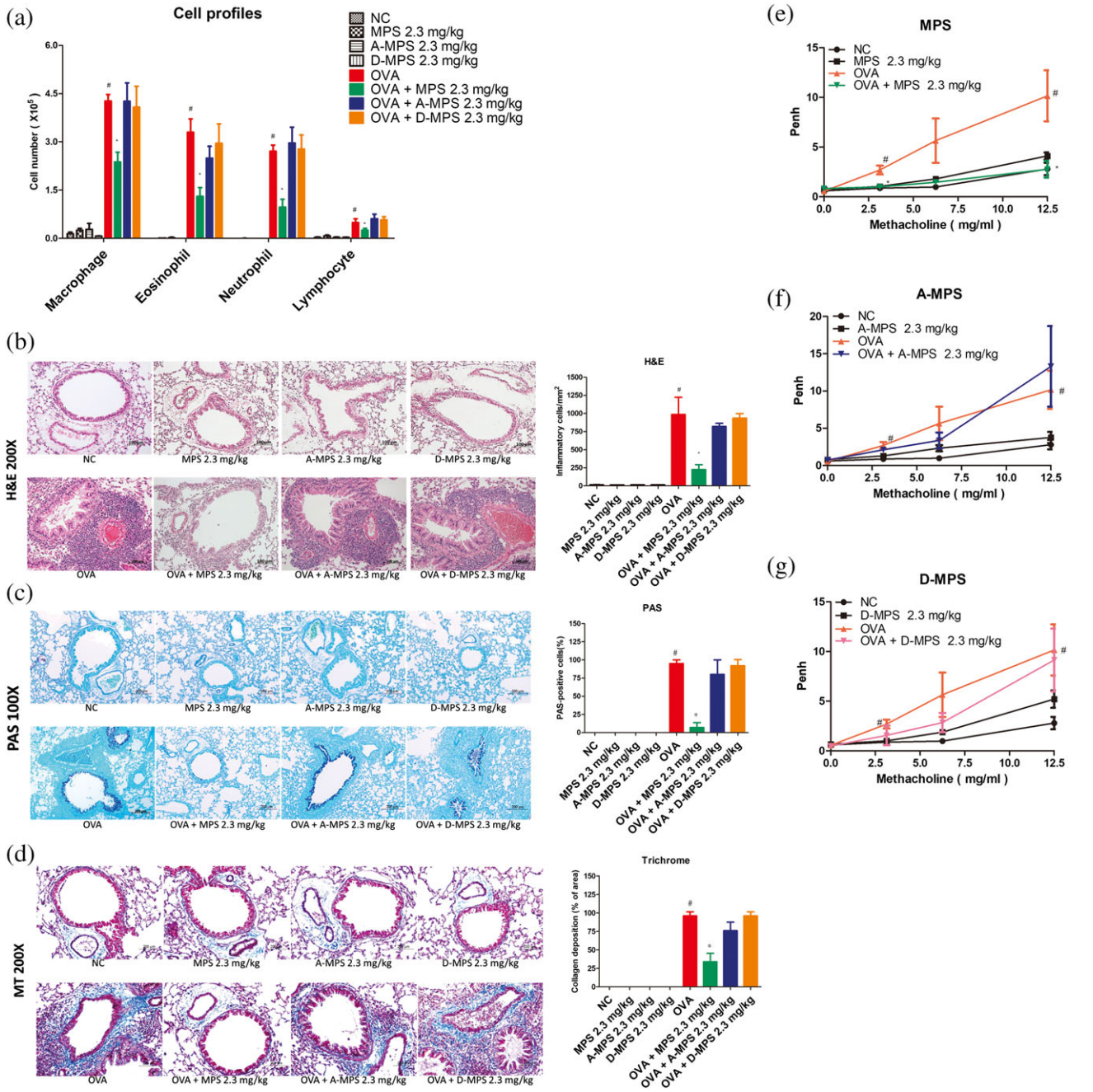

FIGURE 5 The effects of MPS peptide, A-MPS, and D-MPS on asthma symptoms in steroid-resistant mice. (a) BAL differential cell counts were assessed via May–Giemsa staining. (b–d) Lung sections were stained using H&E, PAS, and Masson's trichrome (MT) to assess inflammatory cell infiltration, mucus production, and collagen deposition around the airways, respectively. High magnification bar represents 100 μm for H&E and MT and 200 μm for PAS. (e–g) Airway resistance was determined through whole body plethysmography. Data are expressed as mean ± SD of pulmonary resistance values (RL) ($n \geqq 5$). $^{\#}P < 0.05$, compared to the NC group; $^{*}P < 0.05$, compared to the OVA group

suppression in Crohn's disease. The role of MDR1^{+} T_H17 cells in the pathological mechanism of steroid-resistant asthma and inhibition of T_H17 cell activation by MPS peptide in our model still needs investigation. In addition to T_H17 cells, neutrophils released IL-17A and RORγt in mouse models and human in vitro studies (Taylor et al., 2014). Therefore, attenuation of IL-17A-dependent asthma symptoms by MPS peptide may contribute to inhibition of neutrophil infiltration in the airways.

The complement system is an evolutionarily component of the innate immune system. C5a, a powerful chemoattractant, regulates inflammatory responses and function and recruits granulocytes and monocytes (Guo & Ward, 2005). In our study, the C5a increase in the lungs, and BAL observed in a murine steroid-resistant-like asthma model was inhibited by MPS peptide. However, the levels of C5a, reportedly a steroid-resistant inflammatory mediator, which were shown to be elevated in the BAL of asthmatic patients (Krug, Tschernig,

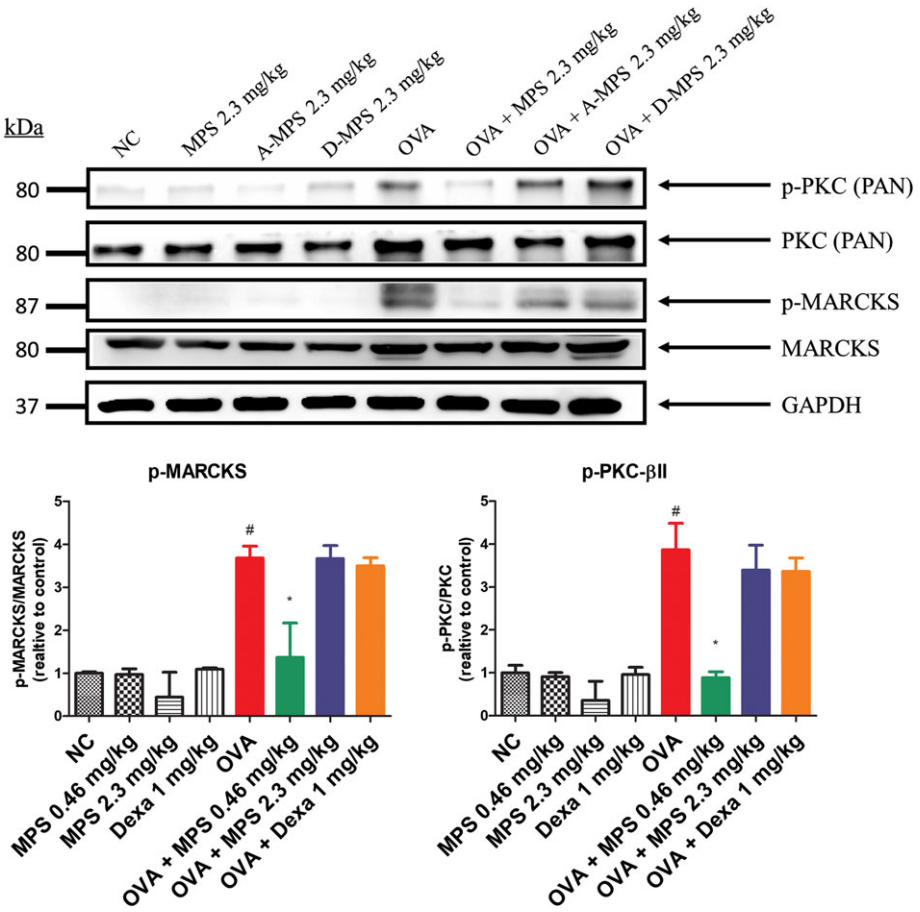

FIGURE 6 Suppression of p-MARCKS expression by MPS peptides in steroid-resistant mice. Lung proteins were subjected to Western blot to assess the expression of phospho-PKC (p-PKC), total PKC-βII, p-MARCKS, MARCKS, and GAPDH. Proteins were quantified using ImageJ. Data are expressed as mean ± SD (n = 5). $^{\#}P < 0.05$, compared to NC group; $^{*}P < 0.05$, compared to the OVA group

Erpenbeck, Hohlfeld, & Köhl, 2001), were shown to be inhibited by steroid treatment in cardiopulmonary bypass patients (Engelman et al., 1995). Therefore, the mechanism underlying the loss of response to steroids in steroid-resistant asthma needs further investigation.

In conclusion, the MARCKS phosphorylation site may serve as a potential therapeutic target in the treatment of steroid-resistant asthma. Additionally, MPS peptide shows potential as a steroid-resistant asthma drug.

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AUTHOR CONTRIBUTIONS

This study was designed, directed, and coordinated by R.W., and C.-C.L. C.-C.L., as the corresponding author, provided conceptual and manuscript editing. C.-N.W. performed the experiments, modulated the animal models, analysed the data, and wrote and edited the manuscript. Y.-C.L. and B.-C.C. assisted C.-N.W. to carry out the experiment and data analysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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 Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

ORCID

Chen-Chen Lee https://orcid.org/0000-0003-1271-5591

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table S1. Quantification of mouse cytokines, chemokines, and complement and acute-phase proteins in bronchoalveolar lavage (BAL) fluids by cytokine arrays.

Mean of Pixel Density					
Location	Cytokines	NC	OVA	OVA + MPS 2.3 mg/kg	OVA + Dexa 1 mg/kg
B1	CXCL13 (A)	422	1493	313	746
B3	C5a	1645	2356	1272	2534
B5	G-CSF	-	-	-	-
B7	G-MCSF	-	-	-	-
B9	CCL1/TCA-3	-	-	-	-
B11	Eotaxin	-	-	-	-
B13	sICAM-1 (B)	10257	9034	8977	9938
B15	IFN-γ	-	-	-	-
B17	IL-1α	-	-	-	-
B19	IL-1β	-	-	-	-
B21	IL-1ra	-	813	817	702
B23	IL-2	-	-	-	-
C1	IL-3	-	-	-	-
C3	IL-4	-	-	-	-
C5	IL-5	-	-	-	-
C7	IL-6	-	-	-	-
C9	IL-7	-	-	-	-
C11	IL-10	-	-	-	-
C13	IL-13	-	-	-	-
C15	IL-12 p70	-	-	-	-
C17	IL-16	-	-	-	-
C19	IL-17	-	-	-	-
C21	IL-23	-	-	-	-
C23	IL-27	-	-	-	-
D1	IP-10	-	-	-	-
D3	I-TAC/CXCL11	-	-	-	-

D5	KC (C)	80	637	390	450
D7	M-CSF	79	92	19	38
D9	CCL2	-	90	-	42
D11	CCL12	-	-	-	-
D13	CXCL9	-	-	-	-
D15	MIP-1a	-	-	-	-
D17	MIP-1B	-	-	-	-
D19	MIP-2	-	22	10	-
D21	RANTES	-	-	-	-
D23	SDF-1/CXCL12 (D)	768	1636	308	751
E1	TARC/CCL17	-	-	-	-
E3	TIMP-1	70	214	48	142
E5	TNF-a	-	-	-	-
E7	TREM-1	-	-	-	-

The level of chemokines (chemokine (C-X-C motif) ligand (CXCL)-13, complement 5a (C5a), Soluble intercellular adhesion molecule-1 (sICAM-1), KC, chemokine (C-C motif) ligand (CCL)-2, CXCL2 (MIP-2) and stromal cell-derived factor (SDF-1), cytokine (IL-1ra, macrophage colony-stimulating factor (M-CSF)) and enzyme (metallopeptidase inhibitor 1 (TIMP-1)) were measured using mouse cytokines assay. “-“ indicated below detection limit.

UC Supporting information

Methods

Peptide treatment in HBE cells and confocal microscope

A human bronchial epithelial (HBE) cell line was obtained from Dr. Reen Wu, University of California, Davis. HBE cells were cultivated in BEGM media (BEBM media supplemented with 0.5 ng/mL hEGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.5 μg/mL epinephrine, 50 μg/mL gentamycin, 50 μg/mL amphotericin B, 6.5 ng/mL triiodothyronine, 10 μg/mL transferrin, 0.13 mg/mL bovine pituitary extract and 100 μg/mL penicillin, streptomycin, and fungizone (all supplied by Lonza). HBE cells (2×10^5 cells/mL) were seeded on a 4-well chamber slide (Thermo Fisher Scientific™ Nunc™) for 24 h. Cell were then washed with HBSS and incubated with 100 nmol MPS-FITC, A-MPS-FITC, and D-MPS-FITC at 37°C for 5 min. After peptide incubation, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and subjected to confocal microscopy. NHBE cells were visualized and images were captured using the Leica TCS SP2/SP8X confocal microscope (Heidelberg, Germany).

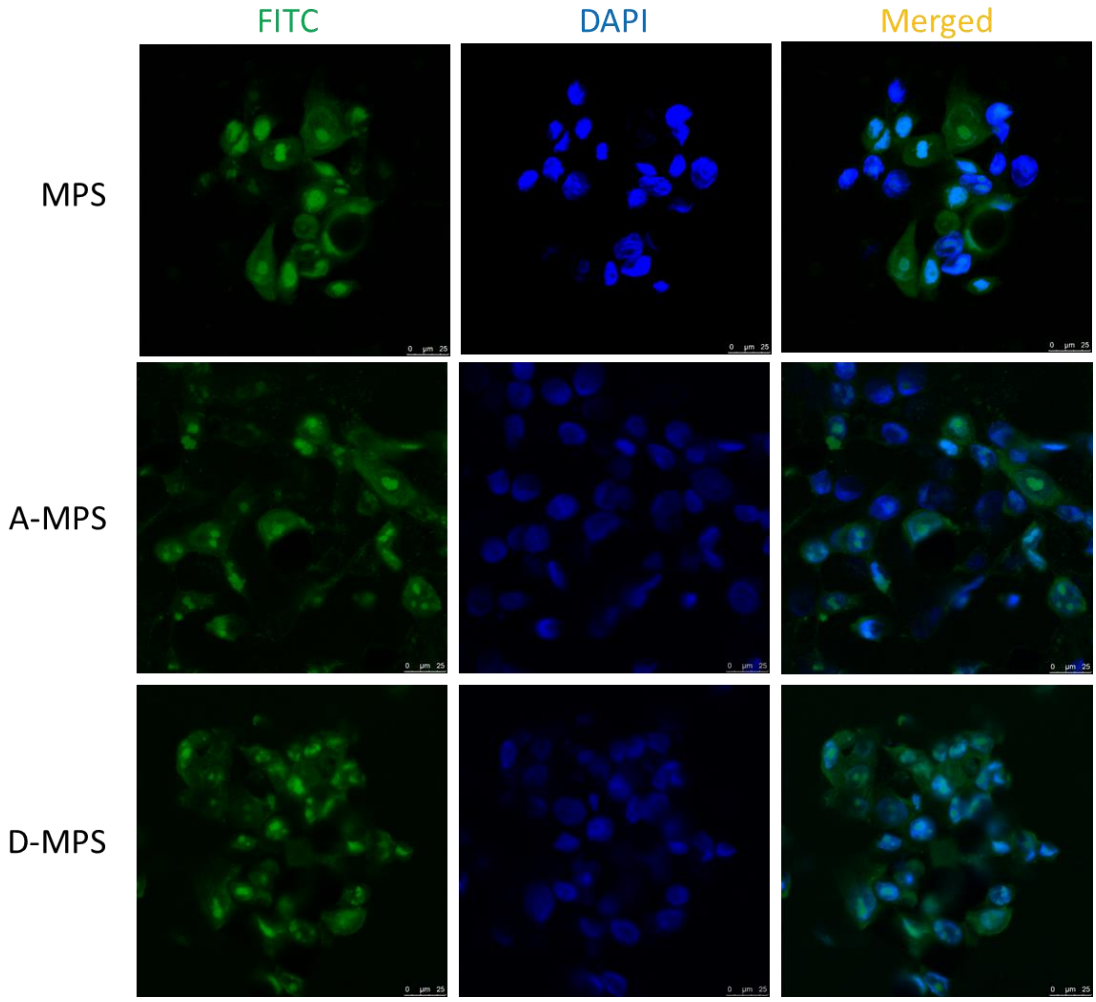


Figure S1. Intracellular localization of MPS, A-MPS, and D-MPS peptides in NHBE cells. NHBE cells were seeded in a chamber slide for 24 h and then incubated with 100 nmol MPS-FITC, A-MPS-FITC, and D-MPS-FITC at 37°C for 5 min. The cells were washed thrice with 1 ml HBSS and then subjected to confocal microscopy. Nuclei were stained with DAPI which showed blue fluorescence. Green fluorescence indicates different MPS peptides. Scale bar: 25 μm, N = 5.

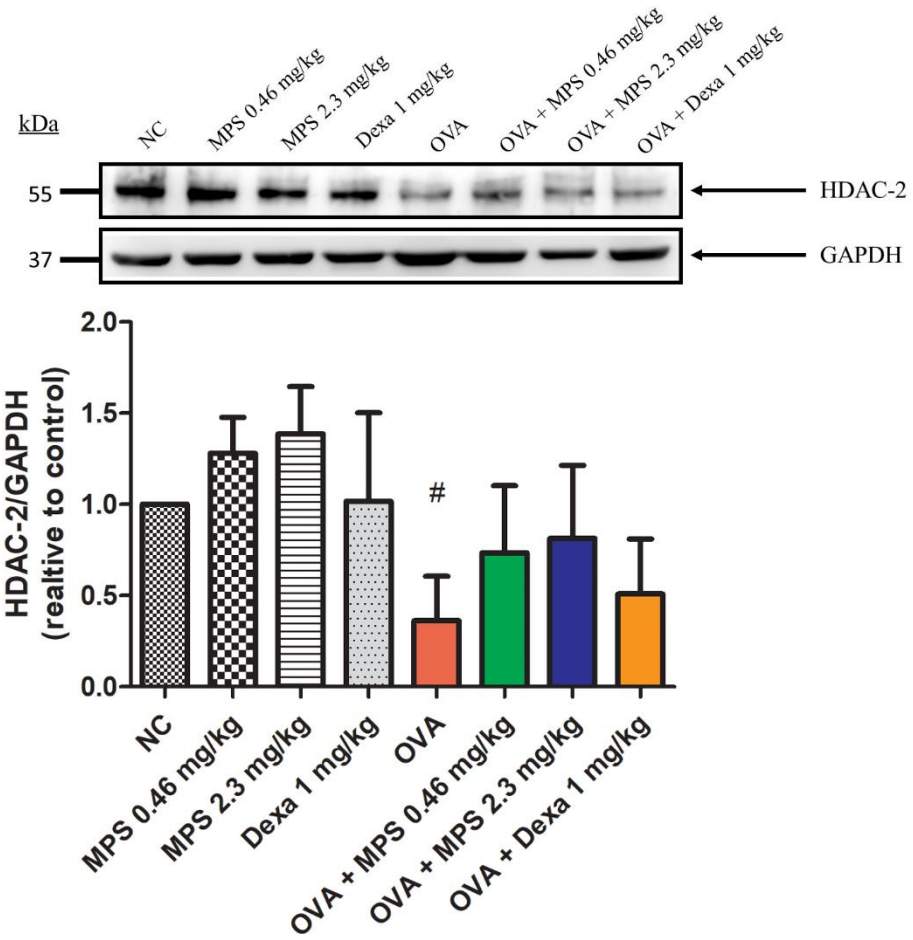
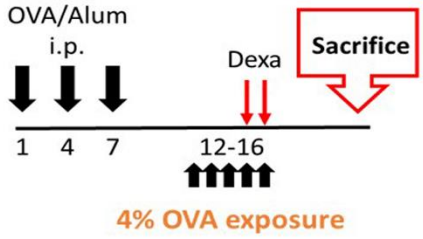
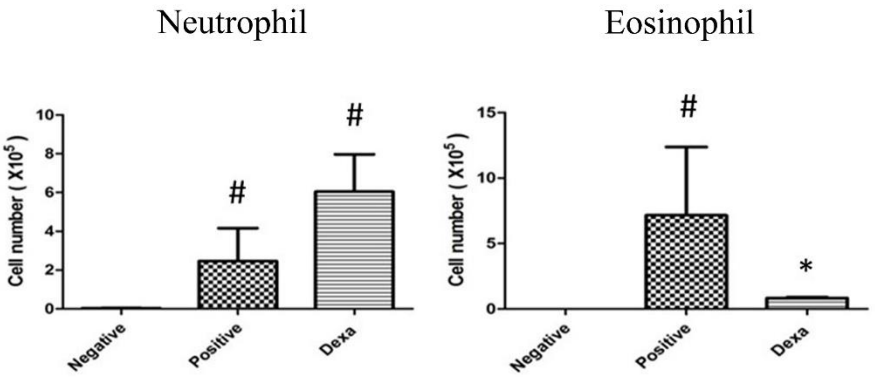


Figure S2. The effect of HDAC-2 protein expression in a murine model of steroid-resistant like asthma. The lung proteins were subjected to western blot analysis to assess the expression of HDAC-2 and GAPDH. The proteins were quantified using Image J. Data are expressed as the mean ± S.D. (n = 5). $^{\#}P < 0.05$, as compared with the NC group.

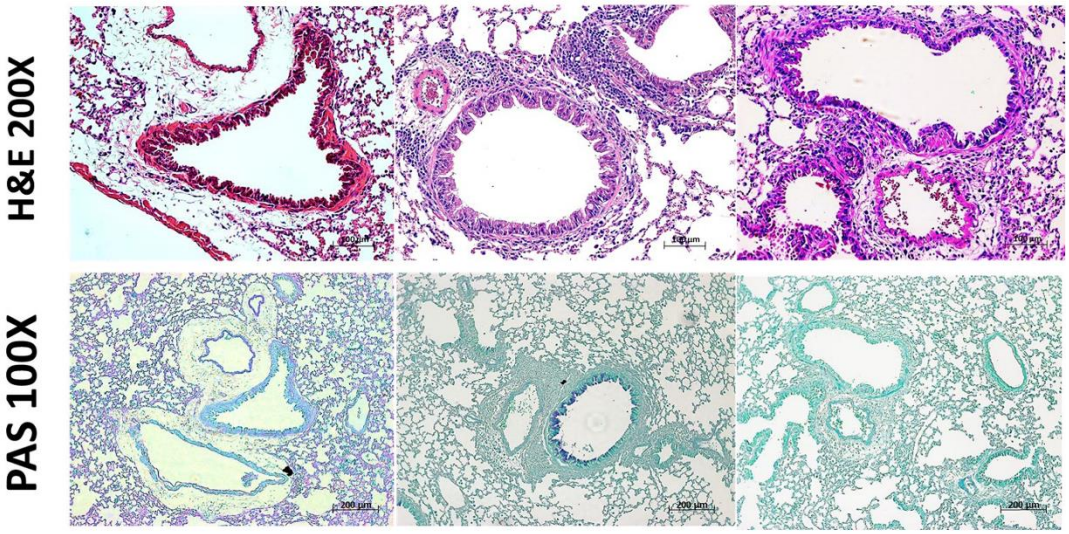
A

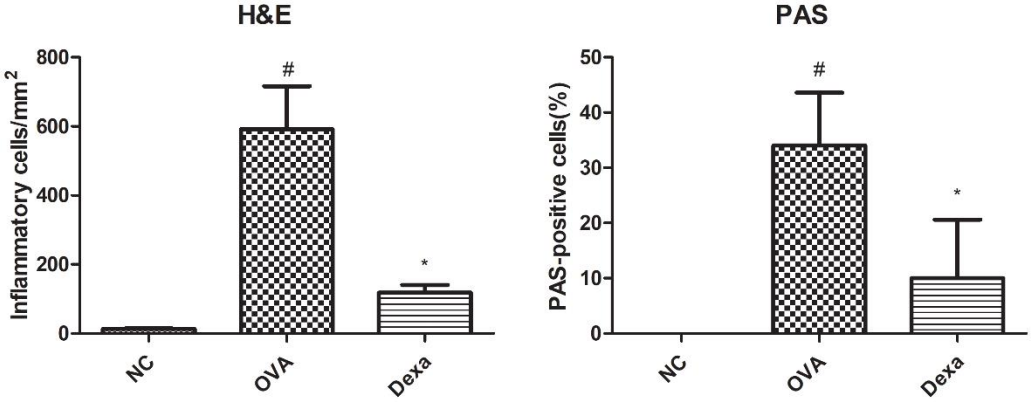

OVA/Alum
i.p.
Dexa
Sacrifice
1 4 7
12-16
4% OVA exposure

B

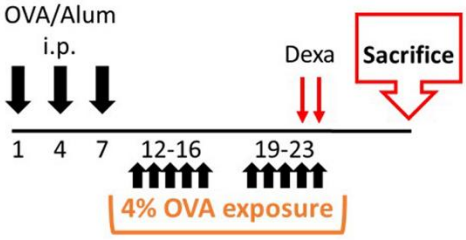

Neutrophil
Eosinophil
Cell number (X10^5)
Negative
Positive
Dexa
#
*

C

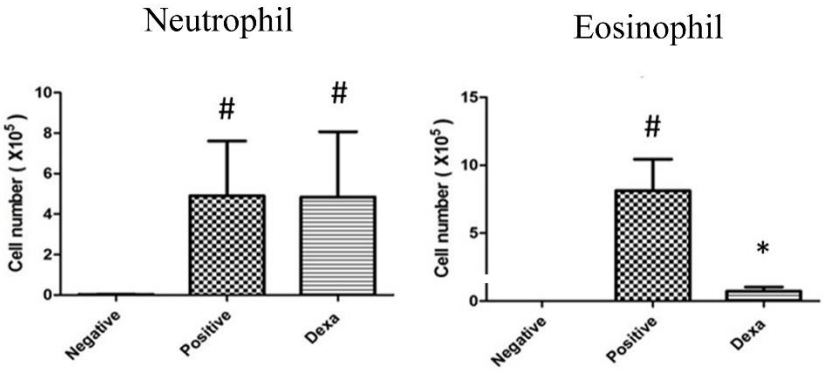

H&E 200X
PAS 100X


H&E
PAS
Inflammatory cells/mm^2
PAS-positive cells(%)
NC
OVA
Dexa
#
*

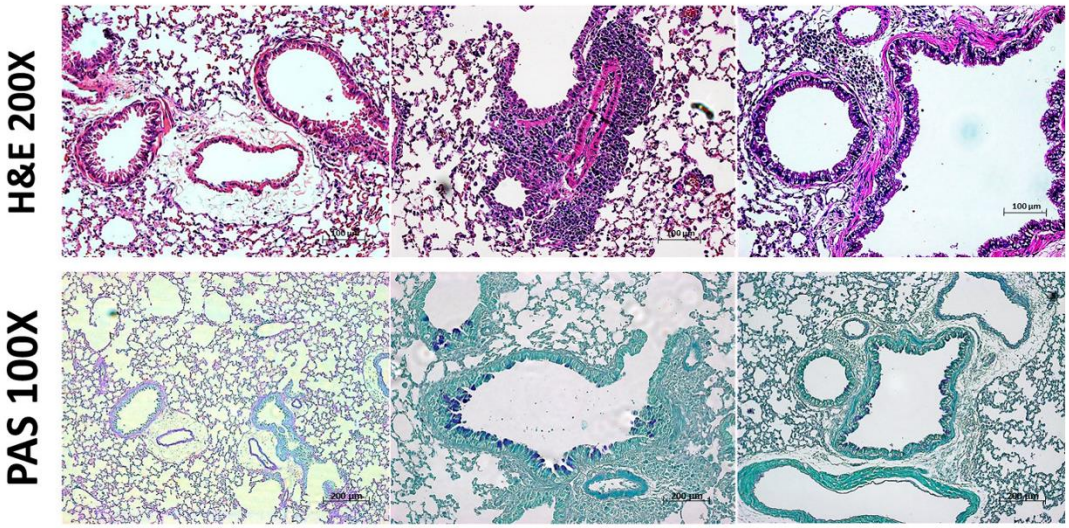
D

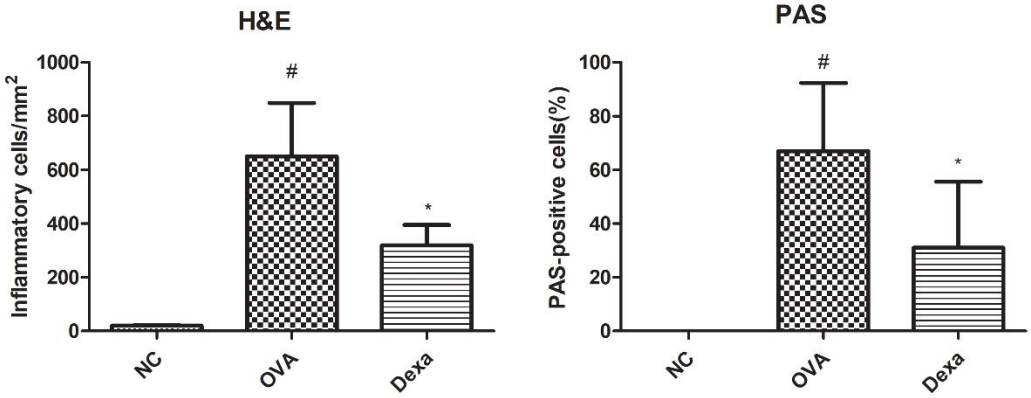

OVA/Alum
i.p.
Dexa
Sacrifice
1 4 7 12-16 19-23
4% OVA exposure

E


Neutrophil
Eosinophil
Cell number (X10^5)
Negative
Positive
Dexa
#
*

F


H&E 200X
PAS 100X


H&E
PAS
Inflammatory cells/mm2
PAS-positive cells(%)
NC
OVA
Dexa
#
*

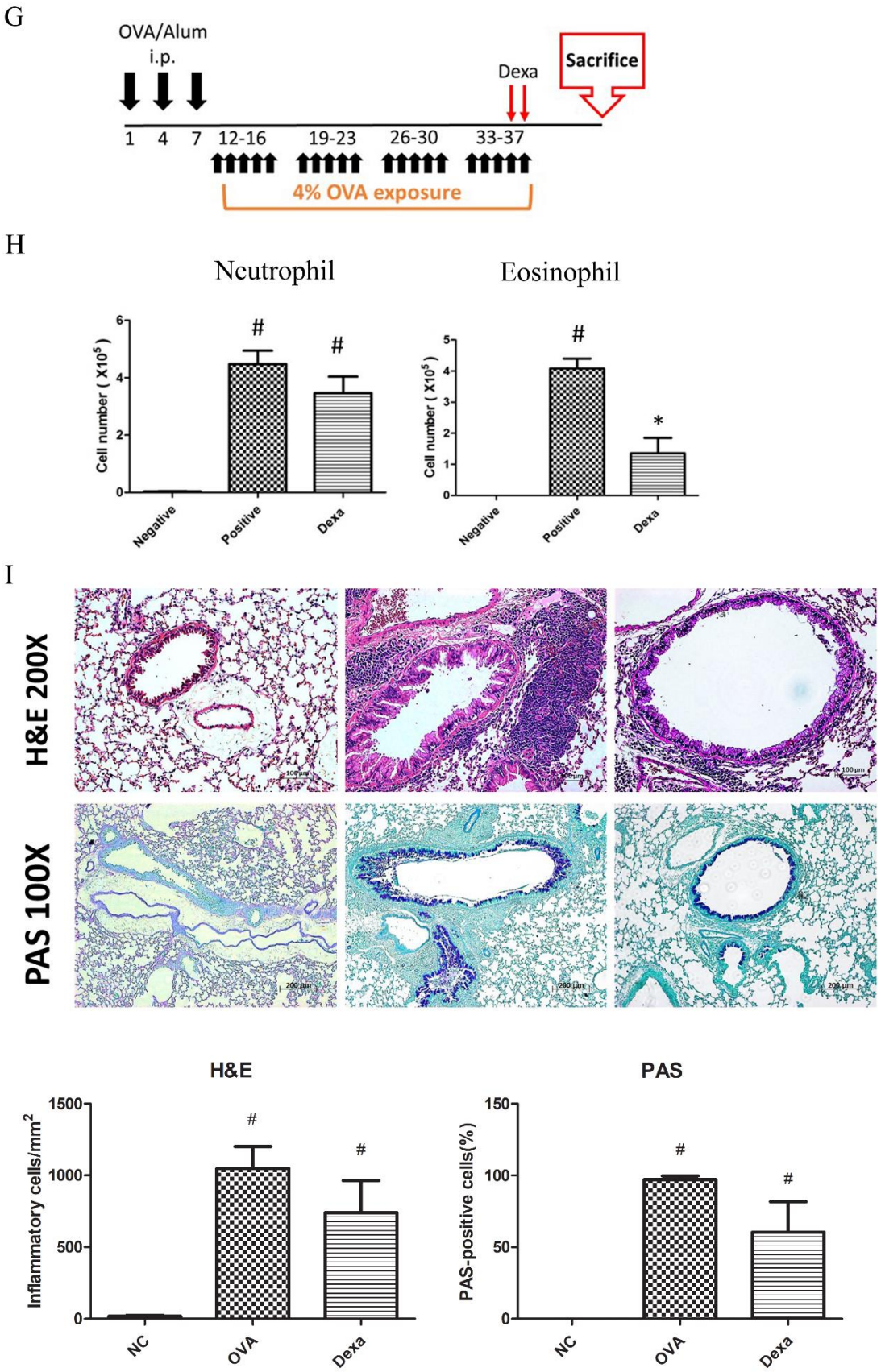


Figure S3. Pulmonary inflammation and pathology at different times of OVA exposure.

(A, D, G) Mice at 6 weeks of age were sensitized with OVA-alum. On day 12, the mice were exposed to PBS or 5 mL 4 % OVA for five days per week for 1, 2, and 4 weeks, respectively. (B, E, H) Mice were intratracheally instilled with 1 mg/kg of dexamethasone twice, at 24 h and 48 h before dissection. On day 17, 24, and 38, the mice were sacrificed and differential cell counts in the BAL were assessed by May-Giemsa staining. (C, F, I) Lungs were subjected to hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson's trichrome (MT) staining. High magnification (bar represents 100 μm for H&E and MT, 200 μm for PAS). Data are expressed as mean ± S.D. ($n \geqq 5$). $^{\#}P < 0.05$, compared to the NC group.; $^{*}P < 0.05$ compared to OVA group.

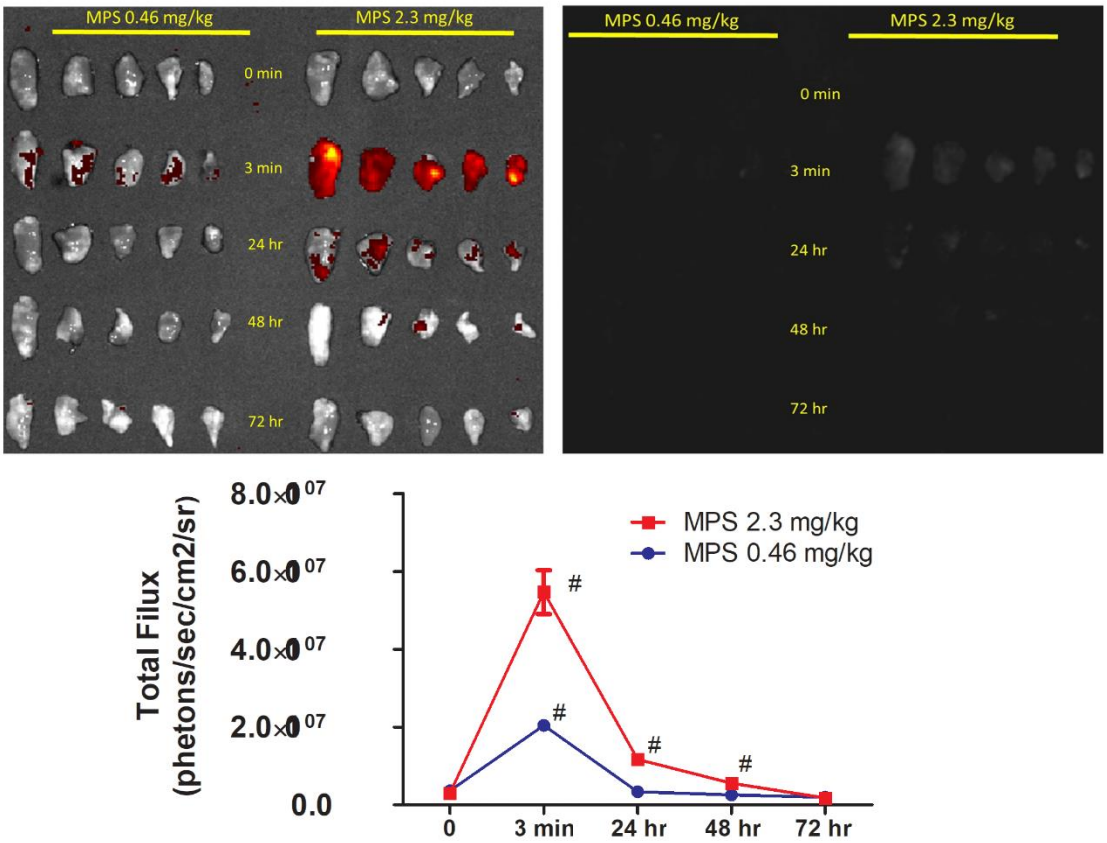


Figure S4. Retention time of MPS peptides in the airway. MPS-FITC peptides (0.46 mg/kg and 2.3 mg/kg) were intratracheally instilled in mice at different time points (0 min, 3 min, 24 h, 48 h, and 72 h), and the lungs were then dissected and observed under an IVIS System to measure and quantify the fluorescence intensity. Data are expressed as mean ± S.D. (n ≧ 5). $^{\#}P < 0.05$, as compared with the NC.

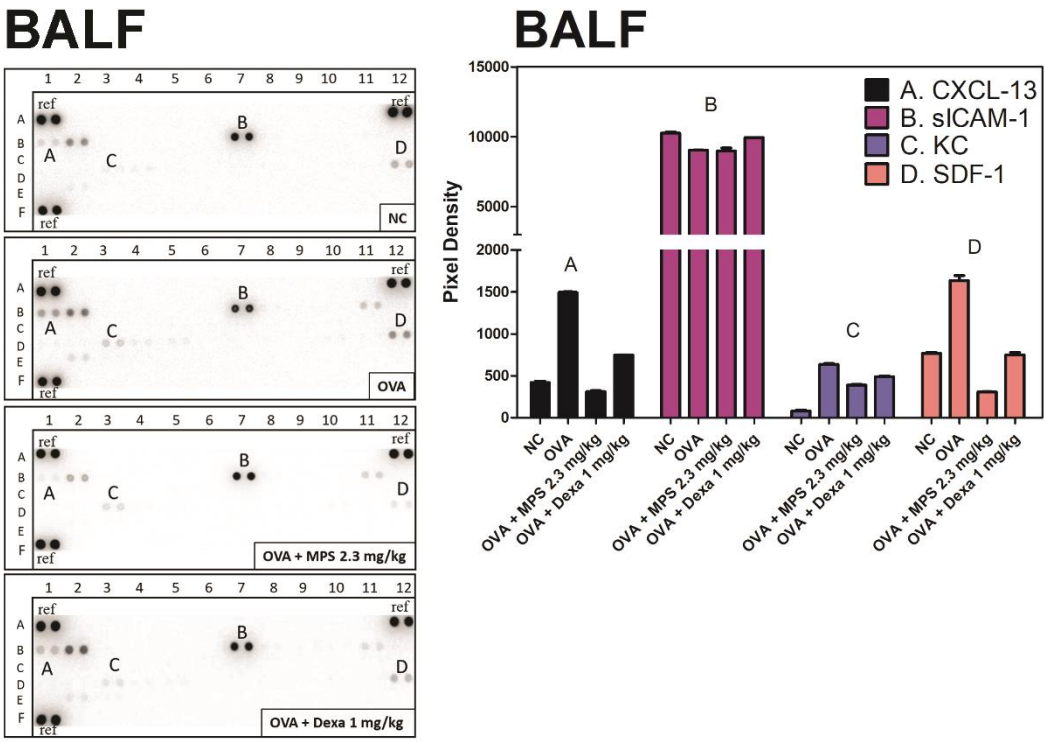


Figure S5. Cytokine expression after MPS treatment using a cytokine array in a murine model of steroid-resistant asthma. BAL fluid (BALF) was analyzed with a mouse cytokine array to screen for 40 cytokines, chemokines, and complement and acute-phase proteins in the BAL. Pixel density plots of chemokine (C-X-C motif) ligand (CXCL)-13 (A), soluble intercellular adhesion molecule-1 (sICAM-1) (B), KC (C) and stromal cell-derived factor (SDF)-1 (D) in BALF, and their quantification using Image J.

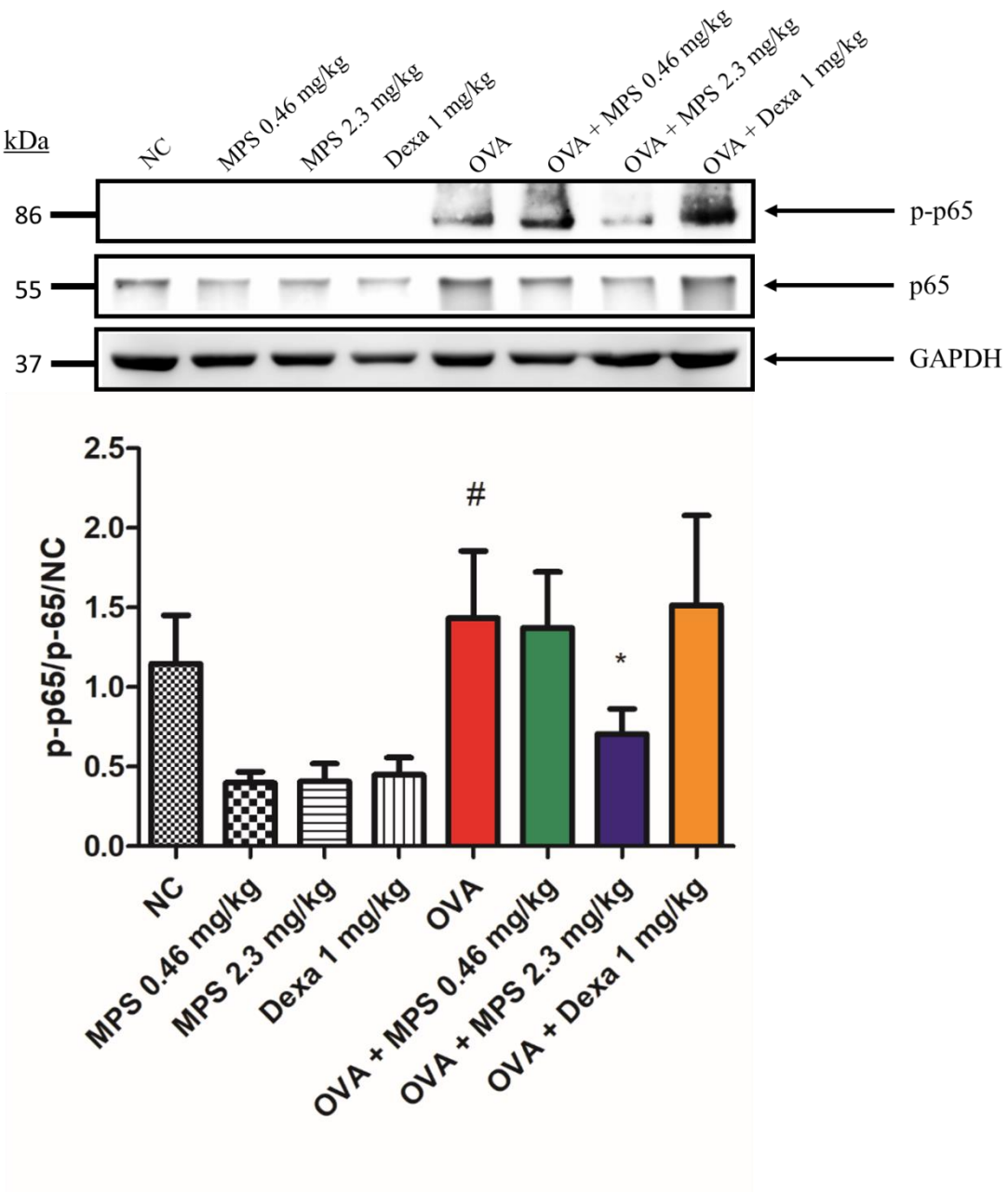


Figure S6. The effect of phospho-p65 and p-65 in mice with steroid-resistant like asthma. Lung proteins were subjected to western blot analysis to assess the expression of p-p65, p-65, and GAPDH. The proteins were quantified using Image J. Data are expressed as the mean ± S.D. (n = 5). $^{\#}P < 0.05$, as compared with the NC group. $^{*}P < 0.05$, as compared with the OVA group.

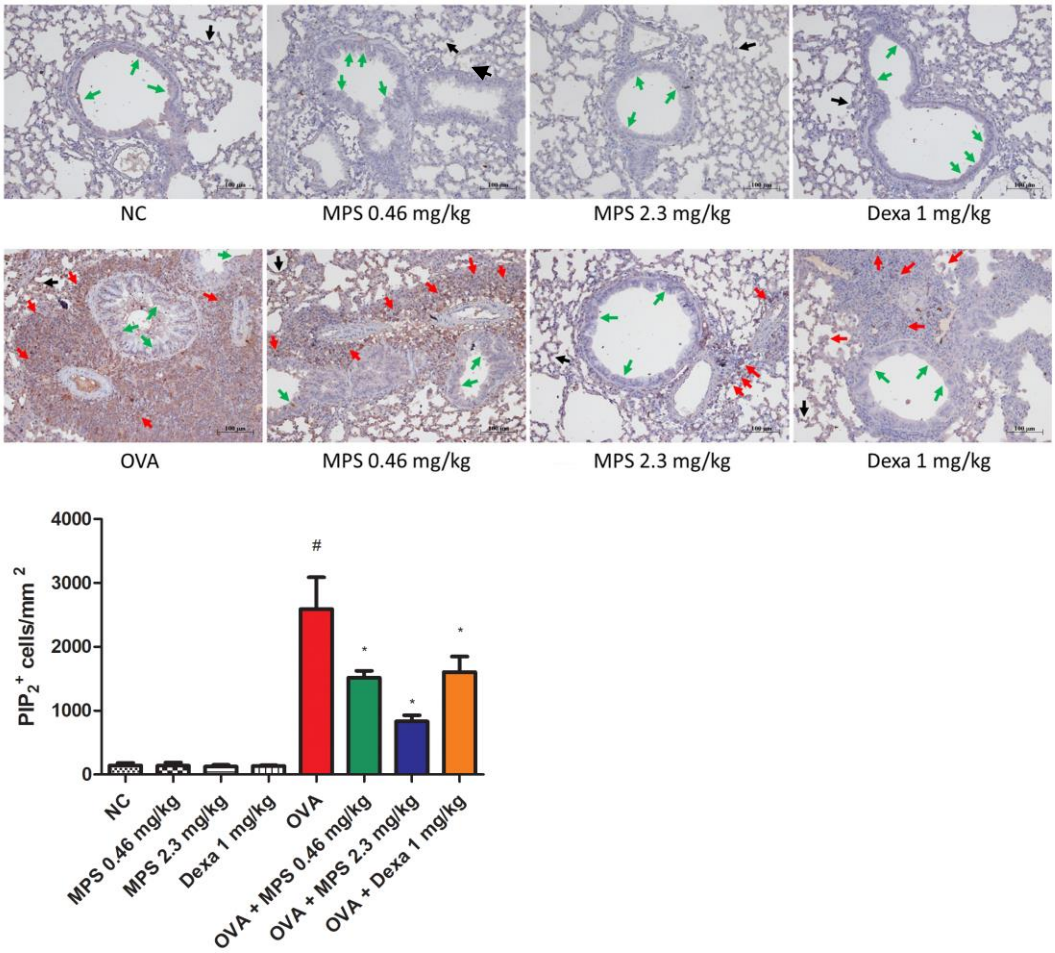


Figure S7. The effect of phosphatidylinositol 4,5-biphosphate (PIP2) levels on the airways of OVA-immunized mice. Mice at 6 weeks of age were sensitized with OVA/alum, as described in **Figure 1B**. Paraffin sections of mouse airway tissues were deparaffinized and IHC stained with PIP_2 antibody (green arrows represent bronchial epithelial cells, black arrows represent alveolar epithelial cells, and red arrows represent for inflammatory cells). High magnification (bar represents 100 μm).