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Targeting the phosphorylation site of MARCKS alleviates symptoms of steroid-resistant like asthma in a murine model

Running title: Targeting p-MARCKS alleviates steroid-resistant asthma

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Protein kinase C is the target for the tumour-promoting phorbol esters, such as tetradecanoyl-β-phorbol acetate (TPA, also known as phorbol 12-myristate 13-acetate). Classical protein kinase C isoforms: PKCα, PKCβ, and PKCγ are activated by Ca2+ and diacylglycerol, and may be inhibited by GF109203X, calphostin C, Gö 6983, chelerythrine and Ro31-8220.Novel protein kinase C isoforms: PKCδ, PKCε, PKCη, PKCθ and PKCμ are activated by diacylglycerol and may be inhibited by calphostin C, Gö 6983 and chelerythrine. Atypical protein kinase C isoforms:PKCι, PKCζ.

Abstract

BACKGROUND

Myristoylated alanine-rich C kinase substrate (MARCKS), a protein kinase C (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 **M**ARCKS **p**hosphorylation **s**ite (MPS peptide) and assessed its therapeutic potential against steroid-resistant asthma.

METHODS

Mice were sensitized with ovalbumin (OVA), alum, and challenged with aerosolized OVA 5 times a week for one month. The mice were intratracheally administered MPS peptides 3 times a week, 1 h before OVA challenge. Asthma symptoms and cell profiles in the bronchoalveolar lavage (BAL) were assessed, and key proteins were analyzed using western blotting.

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 hyper-responsiveness (AHR). 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 (Duox-1) and iNOS, in lung tissues. Compared to Dexa, MPS peptides inhibited C5a production and attenuated interleukin-17A (IL-17A) and KC production in the airway more effectively, thus suppressing asthma symptoms.

CONCLUSIONS

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

Key words: Myristoylated alanine-rich C kinase substrate (MARCKS), **M**ARCKS **p**hosphorylation **s**ite (MPS) peptide, Neutrophilic inflammation, Steroid-resistant asthma

Abbreviations:

AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage fluid; C5a, complement component 5a; Ca^{2+}, calcium iron; DAG, diacylglycerol; Dexa, dexamethasone; Duox, dual oxidase; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GATA-3, GATA binding protein 3; HBE, human bronchial epithelial; HBSS, hank's balanced salt solution; H/E, hematoxylin/eosin; IFN-γ, interferon gamma; IHC, immunohistochemistry; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; iNOS, inducible NOS; i.p.,

intraperitoneal injection; IP_3, inositol 1, 4, 5-triphosphate; i.t., intratracheal instillation; IVIS, *In vivo* imaging system; MAN, N-terminal MARCKS-related; MARCKS, myristoylated, alanine-rich c-kinase substrate; Mch, methacholine; MDR, multiple drug resistance; MH, multiple homology; MPS, MARCKS phosphorylation site; MT, masson's trichrome; OVA, ovalbumin; PAS, periodic acid–Schiff; Penh, enhanced pause; PIP_2, phosphatidylinositol 4,5-biphosphate; PKC, protein kinase C; PSD, phosphorylation site domain; PVDF, polyvinylidene fluoride membranes; RI, resistance index; RORγt, RAR-related orphan receptor gamma t; T-bet, T-box transcription factor; T_H, T helper cell; TIMP, tissue inhibitors of metalloproteinase

Introduction

Asthma, which is a chronic inflammatory disease, is considered a health problem worldwide. According to World Health Organization (WHO) estimates, 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, Leung & Bloom, 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 protein kinase C (PKC) protein kinase C and is composed of three highly conserved regions: the N-terminal myristoylated domain, the phosphorylation domain (PSD) and the multiple homology 2 (MH2) domain. MARCKS is anchored to the cell membrane through an amide bond forms between the amino group of the N-terminal residue and the highly basic PSD domain of several positively charged Lysine residues interacting with negatively charged acidic membrane phospholipids (Harlan, 1991; Sundaram, Cook & Byers, 2004; WU, 1982). The PSD domain comprises 25 amino acids containing 4 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, Thai, Yoneda, Adler, Yang & Wu, 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 PIP2-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 **M**ARCKS **p**hosphorylation **s**ite domain (MPS) and investigated its effects on steroid-resistant asthma in a mouse model and the mechanism underlying these effects. We investigated whether the **M**ARCKS **p**hosphorylation **s**ite could be utilized as a novel therapeutic target for steroid-resistant asthma.

Methods

Reagents and chemicals

Ovalbumin (OVA) (grade V), methacholine, and dexamethasone (Dexa) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hank's balanced salt solution (HBSS) was obtained from Invitrogen (Carlsbad, CA, USA). Alum adjuvant was purchased from Thermo Fisher Scientific (AlumImuject; Pierce Chemical, Rockford, IL, USA). The **M**ARCKS **p**hosphorylation **s**ite (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, USA).

Animals

Six-week-old female BALB/cJ mice were purchased from the National Laboratory Animal Center, National Research Laboratories, Taiwan. (RRID:IMSR_JAX:000651). All mice were housed in a pathogen-free environment at the animal center 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 7 per cage under a 12 h light–dark cycle, and fed for 8 weeks. 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).

Animal experiments

On day 0, 6-week-old female BALB/c mice were immunized with 50 μg of OVA and 2 mg of alum adjuvant dissolved in 200 μL of PBS through intraperitoneal injection (i.p.). 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 one month as previously described (Lee et al., 2013); (**Fig. 1 A**). On days 12, 14, 16, 19, 21, 23, 26, 28, 30, 33, 35, and 37, the mice were administered 0.46 mg·kg^{-1} 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} Dexa intratracheal instillation (i.t.) 1 h before OVA exposure. Each group was composed of 5–7 mice. On Day 38, the mice were euthanized via carbon dioxide (CO_2) exposure, and chronic inflammatory responses were noted (**Fig. 1 B**).

Bronchoalveolar lavage (BAL) and lung histology

Bronchoalveolar lavage was performed by injecting 1 ml of HBSS and gently aspirating 1 ml of BAL. The procedure was performed thrice. BAL samples were centrifuged at 1500 rpm and 4℃ for 5 min. Supernatant from the first BAL sample was stored at –20℃ for cytokine analysis by enzyme-linked immunosorbent assay (ELISA). BAL cells were counted using a

hematocytometer. Cytospin slides of BAL cells were stained with May-Giemsa stain. Cell profile of 300 cells in at least four fields of view was determined under a light microscope (Lee et al., 2013; Lee et al., 2010). Lungs were fixed with 10% formalin, and sectioned for hematoxylin/eosin (H/E) staining, periodic acid–Schiff (PAS) staining and Masson's trichrome (MT) staining was performed to quantify the number of infiltratory 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).

Immunohistochemistry (IHC)

Lung sections were de-paraffinized, hydrated and epitope retrieval was performed according to manufacturer's protocol (Thermo Quanto Detection system, CA, USA). Briefly, the lung sections were stained with hydrogen peroxide block and protein block and stained with rabbit polyclonal anti-Ser152/156 phosphorylated Myristoylated alanine-rich C kinase substrate (MARCKS) (cat#07-1238, Millipore, Bedford, MA, USA) at 1:100 dilution rate and phosphatidylinositol 4,5-biphosphate (PIP_2) antibody (ab811039, Abcam, Cambridge, MA. Afterwards) at a 3 μg/ml dilution rate. Sections were then stained with secondary HRP-labeled polymer and DAB (Antibody amplifier + System-HRP, Thermo), and counterstained with hematoxylin. Immunostained sections were viewed under a Zeiss microscope (Stuttgart, Germany).

Cytokine Assay

Bronchoalveolar lavage (BAL) supernatants were stored at –20°C prior to analysis via enzyme-linked immunosorbent assay (ELISA), performed according to the manufacturer's instructions. Standards were prepared from recombinant mouse interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-13, IL-17, IL-33, eotaxin, and KC (R&D systems, Minneapolis, MN, USA).

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 polyvinylidene fluoride membranes (PVDF) and probed with anti-Ser159/163 phosphorylated MARCKS antibody (ab81295, RRID:AB_2773726; Abcam, Cambridge, MA) at a 1:2000 dilution for western blotting. Anti-FOXP3 at a concentration of 4 μg/ml (ab20034, RRID:AB_445284; Abcam), anti-MARCKS at 1:500 dilution, anti-DUOX-1 (dual oxidase 1) at 1:500 dilution, anti-DUOX-2 (dual oxidase 2) at 1:2000 dilution, anti-total PKC-βII at 1:500 dilution (protein kinase C beta), anti-GATA-3 at 1:500 dilution, anti-RORγt at 1:500 dilution, anti-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-C5a (Genetax 11625, RRID:AB_1949789), anti-Ser660 phosphorylated PKC-βII at 1:1000 dilution (Cell signaling #9371, RRID:AB_2168219), anti-iNOS at 1:500 dilution (inducible NOS), 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 horseradish peroxidase-conjugated secondary antibodies, signals were visualized using a Millipore ECL kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions.

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 seconds for 3 min. The baseline for resistance index (RI) was determined using PBS as a control, and 500 μL each of varying methacholine concentrations (0, 3.125, 6.25, 12.5 mg/mL) 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 (Penh) (Reddy, Lakshmi & Reddy, 2012).

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, 3 min, 6, 24 and 48 h following intratracheal instillation of FITC-conjugated MPS (2.3 $mg \cdot kg^{-1}$) into BALB/c mice. At specified time periods following intratracheal instillation of MPS, the mice were sacrificed and the lungs isolated for fluorescence imaging. Fluorescence intensities of MPS peptides were analyzed using accessory software (Living Image Version 4.0; Caliper Life Sciences). Ratio of fluorescence intensities in the MPS-treated group and those in the control group were calculated using Image J software. (ImageJ, RRID:SCR_003070)

Statistical analysis

Results are presented as mean ± standard deviation (S.D.) for $N \geq 5$. The program 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, MT pathology index, AHR, western blotting, and cytokine levels. In Fig. 1C and D, was analyzed using a paired Student's *t*-test. Statistical significance was set at $P < 0.05$.

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 et al., 2017).

Results

Elevation of MARCKS phosphorylation in the lungs in a murine model of steroid-resistance like asthma

To create a murine steroid-resistant asthma model, mice were sensitized with ovalbumin (OVA) combined with alum, and exposed to OVA for 4 weeks **(Figs. 1A, 1B**). MARCKS was highly expressed in the lungs of OVA-immunized mice (**Fig. 1C**). It was also expressed in bronchial epithelial cells (green arrows) and inflammatory cells (red arrows) (**Fig. 1D**). Steroid-resistant like mice exhibited severe airway inflammation (**Figs. 2C, D**), mucus production (**Fig. 2E**), collagen deposition (**Fig. 2F**), and hyper-responsiveness (AHR) (**Fig. 2G**). The mice were resistant to dexamethasone (Dexa) treatment.

M*ARCKS* ***p****hosphorylation* ***s****ite (MPS) peptide inhibits asthma symptoms by interfering with MARCKS phosphorylation in a murine steroid-resistant like asthma model.*

N-terminal MARCKS-related (MANS) peptides attenuated hypersecretion of mucus in the airway (Singer et al., 2004) and also chronic airway inflammation (Agrawal & Dickey, 2007). This indicated interference in MARCKS function by a peptide may show therapeutic potential against allergic inflammatory diseases. We designed a peptide that targets the

MARCKS phosphorylation site to investigate potential therapeutic effects of MPS on steroid-resistant asthma (**Fig. 2A**). To determine the retention time of MPS, MPS peptides conjugated to fluorescein isothiocyanate (FITC) (2.3 $mg \cdot kg^{-1}$) were intratracheally instilled (i.t.) in mice and MPS levels measured at different time points. The fluorescence of MPS peptide reached its peak at 3 min (**Fig. 2B)**, declined sharply, sustained at 48 h, and disappeared at 72 h (Fig. S4). The fluorescence of low dose MPS peptide (0.46 mg/kg) reached its peak at 3 min but disappeared at 24 h (left panel); (Fig. S4). We investigated the ability of 3 designed MPS peptides in human bronchial epithelial cell line (HBE) 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 (Fig. S1). Peptide fluorescence was sustained for 48 hours in HBE cells (data not shown). Based on these results, the time interval between i.t. instillation of MPS and the relevant assays was set as 48 h. Following i.t. instillation of MPS 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 (**Figs. 2C, D, E, F, G**). Analysis of inflammatory cell profiles of BAL showed that MPS inhibited infiltration of neutrophilia, eosinophilia, and lymphocytes in steroid-resistant like mice. Dexa was able to suppress the infiltration of eosinophilia and lymphocytes, but not that of neutrophilia (**Fig. 2C**). To determine whether suppression of

asthma symptoms following MPS 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, but was unaffected by Dexa treatment (**Fig. 2H**). Thus, targeting p-MARCKS with MPS peptides may block asthma symptoms.

MPS reduces interleukin-17A (*IL-17A), C5a expression and oxidative stress production and contributes to the inhibition of asthma symptoms in a murine steroid-resistant like 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., 2017a; Hansbro et al., 2017b; Linden, 2006; Zijlstra, Ten Hacken, Hoffmann, van Oosterhout & Heijink, 2012). We observed a similar phenomenon. In steroid-resistant-like mice, T_H1-specific cytokine interferon-γ (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 (**Fig. 3A**). Following treatment with 2.3 $mg \cdot kg^{-1}$ MPS, all these cytokines were suppressed. In contrast, Dexa inhibited IFN-γ, IL-4, and eotaxin and slightly attenuated IL-13 and KC production, but did not inhibit IL-17. MPS also inhibited expression of T_H-specific transcription factors, (T-box transcription factor (T-bet) T_H1, (GATA Binding Protein-3 (GATA-3) T_H2 and (RAR-related orphan receptor gamma t (RORγt) T_H17 in lung tissues. By contrast, Dexa inhibited GATA-3 expression and slightly

suppressed T-bet expression but not RORγt (**Fig. 3B**). This trend was similar to that for T_H-specific cytokines. Previous study found that mice subjected to prolonged 3% OVA exposure (3 times a week for a total period of 6 weeks), displayed airway tolerance by reducing 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 lungs. FOXP3 expression in lung tissue was not increased in our steroid-resistant like asthma model (**Fig. 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, cytokines/chemokines arrays were utilized to characterize the cytokines/chemokines profile following MPS and Dexa treatment. Of the 40 cytokines, chemokines and acute-phase proteins that were screened, complement component 5a (C5a) and tissue inhibitors of metalloproteinase (TIMP-1), which were markedly overexpressed in steroid-resistant like mice, were downregulated by MPS but not by Dexa (**Fig. 3C**). C5a is important for recruitment and activation of granulocytes (Ehrengruber, Geiser & Deranleau, 1994; Zeck-Kapp, Kroegel, Riede & Kapp, 1995). In addition, interleukin-1 receptor antagonist, IL-1Ra (receptor antagonist for IL-1β), levels in BAL were lower in Dexa-treated steroid-resistant-like mice than in OVA- or MPS-treated mice (**Fig. 3C**).

Other chemokines including chemokine (C-X-C motif) ligand (CXCL)-13, soluble intercellular adhesion molecule-1 (sICAM-1), KC and the stromal cell-derived factor-1 (SDF-1), were also increased in the OVA treatment group (**Fig. 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 pathologic mechanism which induces steroid-resistant responses in asthma. Therefore, we examined oxidative stress in lung tissues. We found that numerous proteins had been nitrosylated. As well, levels of the oxidative indicator, 3-nitrotyrosine (3-NT), and related enzymes including dual oxidase-1 (Duox-1), Duox-2 and inducible nitric oxide synthase (iNOS) in the lung tissues of the OVA group were increased (**Fig. 4**). The 2.3 $mg \cdot kg^{-1}$ MPS treatment group, but not Dexa treatment, significantly reduced 3-NT, Duox-1 and iNOS expression in the lungs.

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

To clarify mechanisms underlying the effect of MPS on steroid-resistant signaling, histone deacetylase-2 (HDAC-2) protein expression and NF-κB activation, involved in steroid-resistant signaling (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 rescue HDAC-2 reduction (Fig. S2). However, MPS 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 inhibited asthma symptoms by activating NF-κB. A high dose of MPS treatment suppressed high pp65 expression in the OVA-immunized group whereas Dexa treatment did not change pp65 expression (Fig. S6).

Mutation of Ser residues in MPS to Asp or Ala blocks MPS-induced anti-asthma effects in a murine steroid-resistant –like asthma model.

We investigated molecular mechanisms underlying inhibition of asthma symptoms upon MPS treatment. First, 2 different mutant MPS peptides were designed (**Fig. 2A**): A-MPS (4 Ser residues mutated to Ala which cannot be phosphorylated); and D-MPS (2 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-induced inhibition of asthma symptoms, including airway inflammation (**Figs. 5A, B**), mucus production (**Fig. 5C**), collagen deposition (**Fig. 5D**), and AHR (**Fig. 5E, F, G**). Furthermore, A-MPS and D-MPS did not inhibit OVA-induced MARCKS phosphorylation in the lungs (**Fig. 6**). Finally, unlike MPS, A-MPS and D-MPS were unable to suppress an increase in phosphorylated protein

kinase C (PKC) expression in the lungs of murine steroid resistant-like-asthma models (**Fig. 6**).

Discussion

The protein, MARCKS, induces vesicle release during exocytosis and cell migration via actin cytoskeleton remodeling. 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; Ching-Hsien Chen, 2015), renal cell carcinoma (Chen, Fong, Yu, Wu, Trott & Weiss, 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 MARCKS phosphorylation site, alleviated asthma symptoms including airway inflammation, mucus production, airway remodeling, and airway hyper-responsiveness in steroid-resistant models. This suggested that the MARCKS phosphorylation site is a potential target for the treatment of steroid-resistant asthma. These results suggested that MPS 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

phosphatidylinositol 4,5-bisphosphate (PIP_2) (Glaser & Wanaski, 1996; Morton et al., 2012). PIP_2 is then activated by phospholipase C-γ1 and phosphatidylinositol-4, 5-bisphosphate 3-kinase (PtdIns-3-OH kinase) allowing it to activate downstream signaling pathways involving immune cell activation. We also found that MPS attenuated PKC phosphorylation which may reduce MARCKS phosphorylation. However, PKC is activated by calcium ions (Ca^{2+}) and diacylglycerol (DAG), released following MARCKS phosphorylation. Therefore, inhibition of PKC phosphorylation may occur after MPS interferes with MARCKS phosphorylation. This may require further investigation. We also investigated PIP2 expression in the lungs using IHC. PIP2, 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 (Fig. S7). Following MPS treatment, PIP2 level was reduced through attenuation of inflammatory cell infiltration in the lungs. The effect of MPS on PIP2 downstream signaling pathways needs further investigation.

To understand the mechanisms by which MPS 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 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 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.

Cytokine profile in BAL was analyzed. MPS 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 downregulating PIP2/phospholipase C/inositol 1, 4, 5-triphosphate (IP3), DAG/nuclear factor (NF)-κB, activator protein 1 (AP-1) and nuclear factor of activated T-cell (NFAT) signaling 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). 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 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, 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 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 response and function and recruits granulocytes and monocytes (Guo & Ward, 2005). In our study, C5a increase in the lungs and BAL observed in a murine steroid-resistant like asthma model was inhibited by MPS. However, the levels of C5a, reportedly a steroid-resistant inflammatory mediator, which were shown to be elevated in the BAL of asthmatic patients (NORBERT KRUG, 2001), were shown to be inhibited by steroid treatment in cardiopulmonary bypass patients (Engelman, Rousou, Flack, Deaton, Kalfin & Das, 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.

Author contribution

This study was designed, directed, and coordinated by Reen Wu, and Chen-Chen Lee. Chen-Chen Lee as the corresponding author, provided conceptual and manuscript editing. Chien-Neng Wang performed the experiments, modulated the animal models, analyzed the data, and wrote and edited the manuscript. Yu-Chao Lin and Bo-Chun Chang assisted Chien-Neng Wang to carry out the experiment and data analysis.

Conflict of interest

The authors have a conflict of interest in relation to this work.

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.

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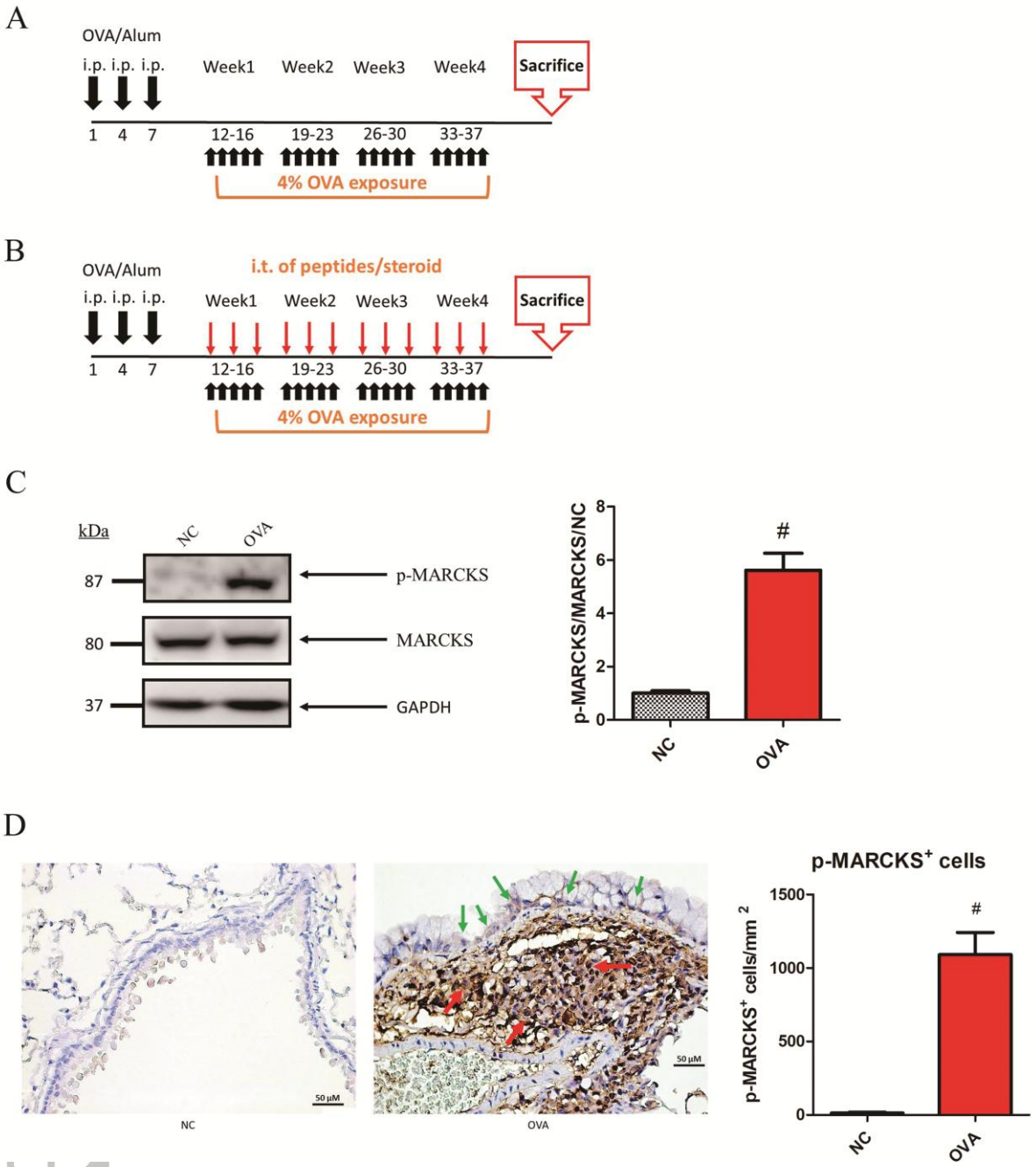


Figure 1. Elevation of phosphorylated p- MARCKS levels in the airways of OVA-immunized mice. (A) OVA-induced steroid-resistance like asthma model. Six-week-old mice were sensitized with OVA/alum, as described in methods. On day 12, mice were exposed to PBS or OVA (4% weight/volume diluted in 0.9% saline; 5 mL/inhalation) 5 days per week for 4

weeks. On day 38, mice were sacrificed, lungs were embedded in paraffin and analyzed via western blot and immunohistochemical (IHC) staining. (B) Brief scheme of the generation of the mouse steroid-resistant like model. Mice were sensitized with aerosolized OVA 5 times/week 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 sacrificed and lungs 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 Image J. **(**D) Paraffin embedded sections of mouse airway tissues were de-paraffinized, IHC stained with anti-Ser152/164 p-MARCKS antibody (green arrows represent epithelial cells and red arrows represent inflammatory cells). The scale bar is 50 μm. Data are expressed as paired *t*-test results for NC and OVA groups (n >11). $^{\#}$ = $P < 0.0001$, as compared to the NC group.

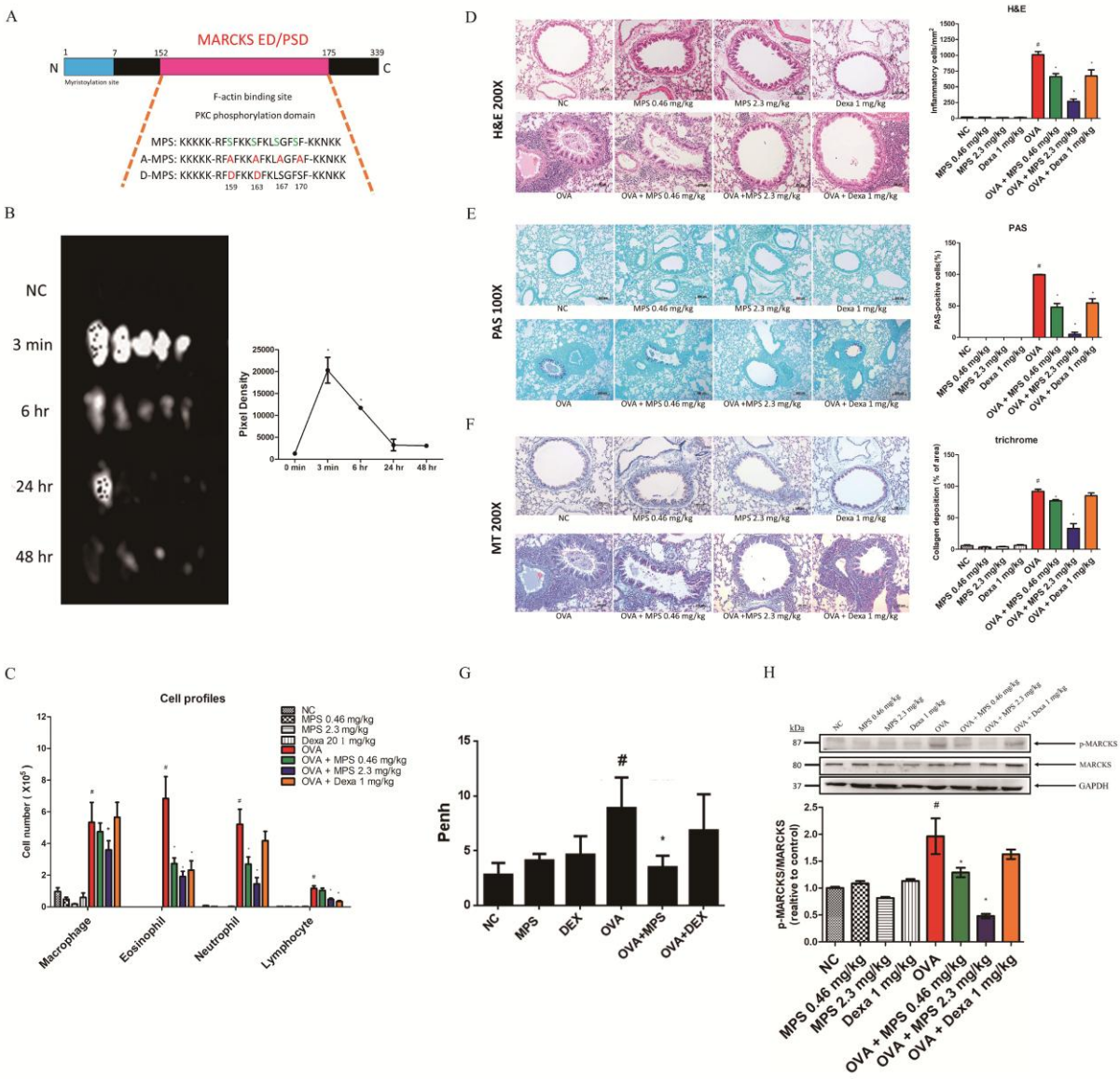


Figure 2. Asthma symptoms were alleviated by a peptide targeting the MARCKS phosphorylation site. (A) Amino acid sequences of MPS, A-MPS, and D-MPS. (B) Retention time of MPS in the airway. MPS-FITC peptides at concentrations of 0.46 mg/kg and 2.3 mg/kg were intratracheally instilled in mice at different time points (0 min, 3 min, 24, 48 and 72 hr), and lungs were dissected and fluorescence was measured under an IVIS System. (C) As described in the methods, mice were sacrificed on day 38 and differential cell counts of

BAL were assessed via May-Giemsa staining. (D-F) Lungs were stained using Hematoxylin and eosin (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 Ser159/163 pMARCKS, MARCKS, and GAPDH expression. Proteins were quantified using Image J. Data are expressed as mean ± S.D. ($n \geqq 5$). $^{\#} = P < 0.05$; $^{*} = P < 0.05$, compared to NC and OVA groups.

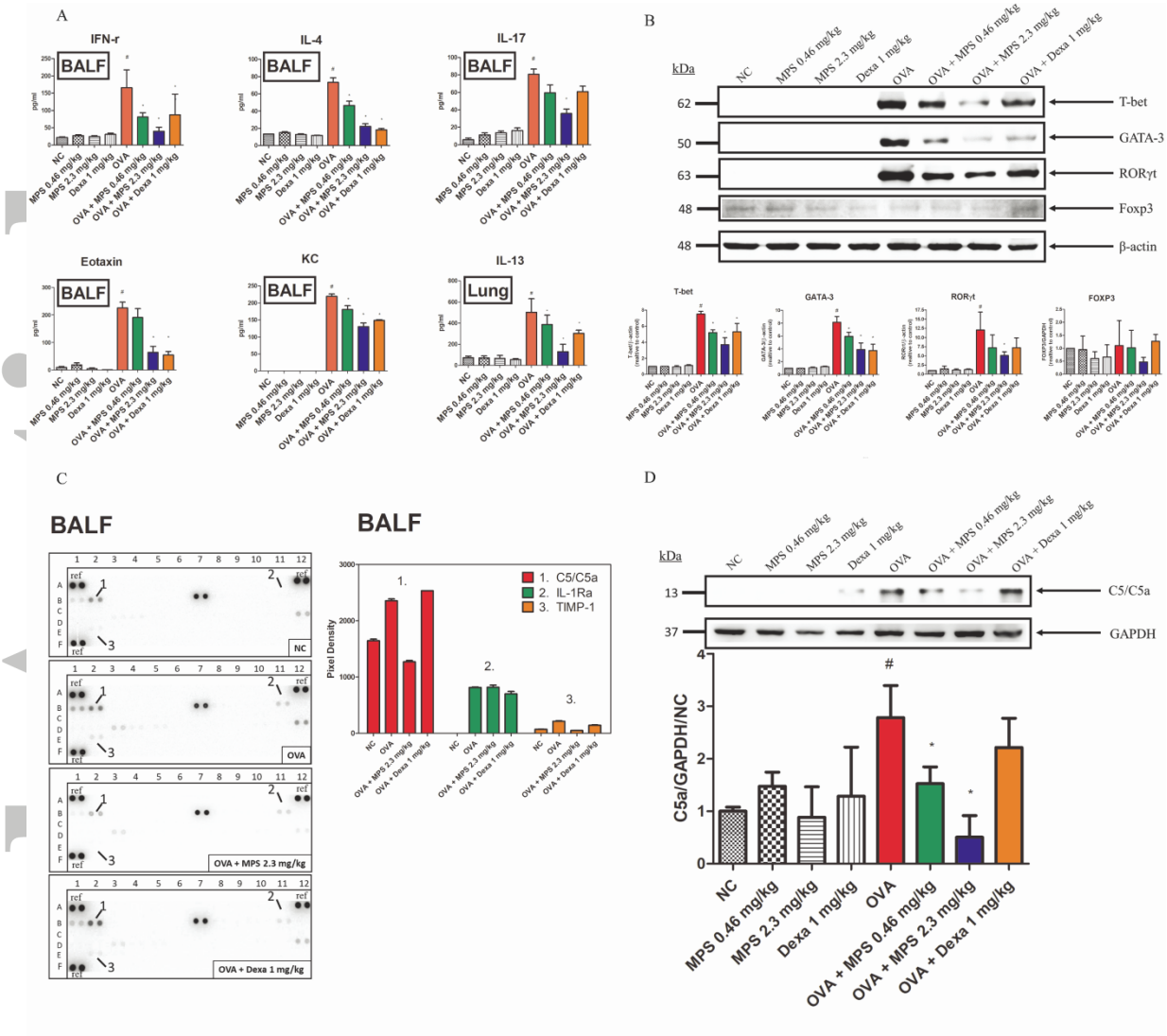
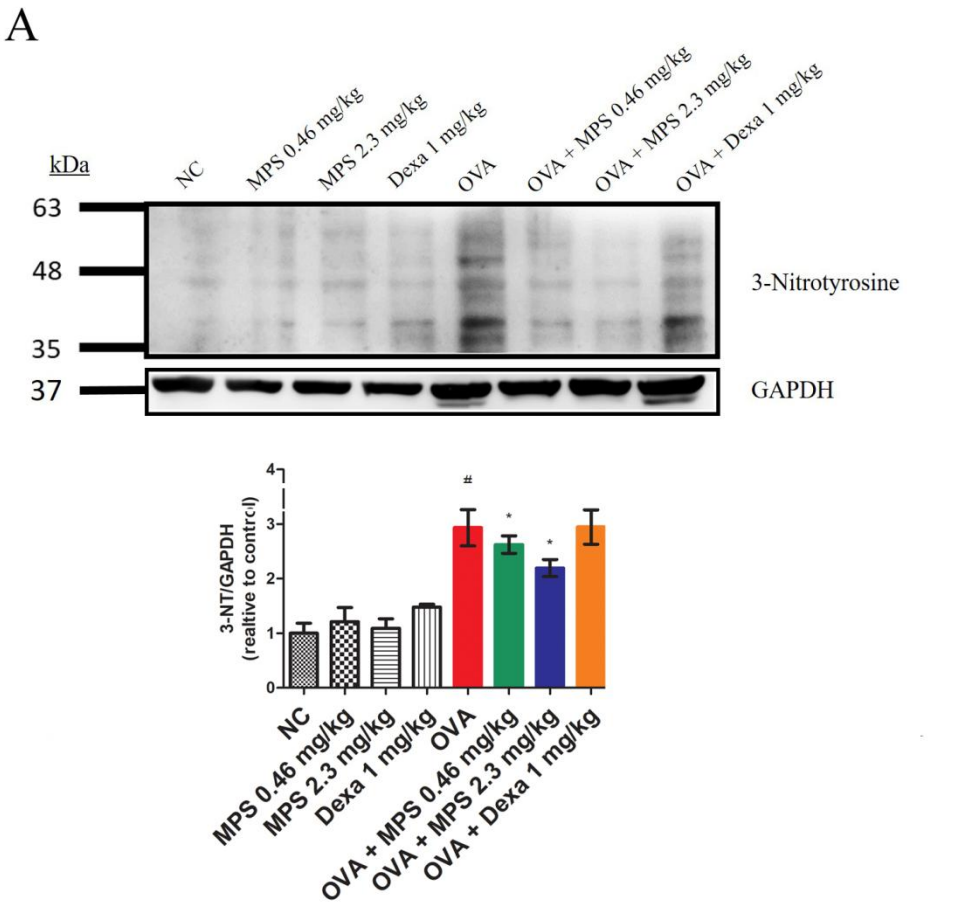


Figure 3. MPS decreased T_H1-, T_H2-, and T_H17-related cytokines and specific-transcriptional factors as well as C5a expression in lungs. (A) On day 38, mice were sacrificed, BAL fluids collected and lung proteins analyzed via ELISA. (B) Proteins were extracted from homogenized lungs and subjected to Western blotting to assess T-bet, GATA-3, RORγt, FOXP3 and β-actin expression. (C) BAL fluids were analyzed 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 analyzed using Image J. (D)

Proteins were extracted from homogenized lungs and subjected to Western blotting to assess C5a and GAPDH expression. These proteins were quantified using Image J. Data are expressed as mean ± S.D. (n $\geqq$ 5). $^{\#}$ = $P < 0.05$, compared to the NC group; * = $P < 0.05$, compared to the OVA group.



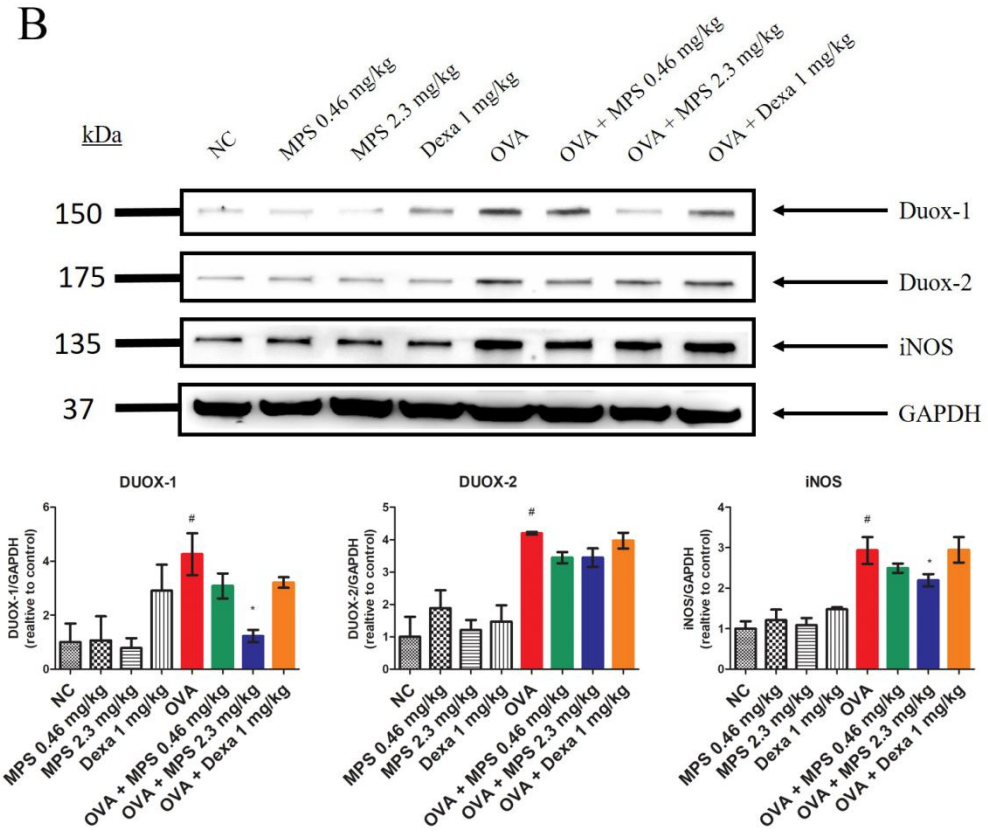


Figure 4. Suppression of oxidative stress in lung tissues from steroid-resistant like mice. Proteins were extracted from homogenized lungs and subjected to Western blotting to assess the expression of (A) 3-nitrotyrosine (3-NT) and (B) Dual oxidase-1 (Duox-1 and -2), inducible nitric oxide synthase (iNOS) and GAPDH. These proteins were quantified using Image J. Data are expressed as mean ± S.D. (n = 5). $^{\#}$ =$P < 0.05$, as compared with the NC group; * = $P < 0.05$, as compared with the OVA group.

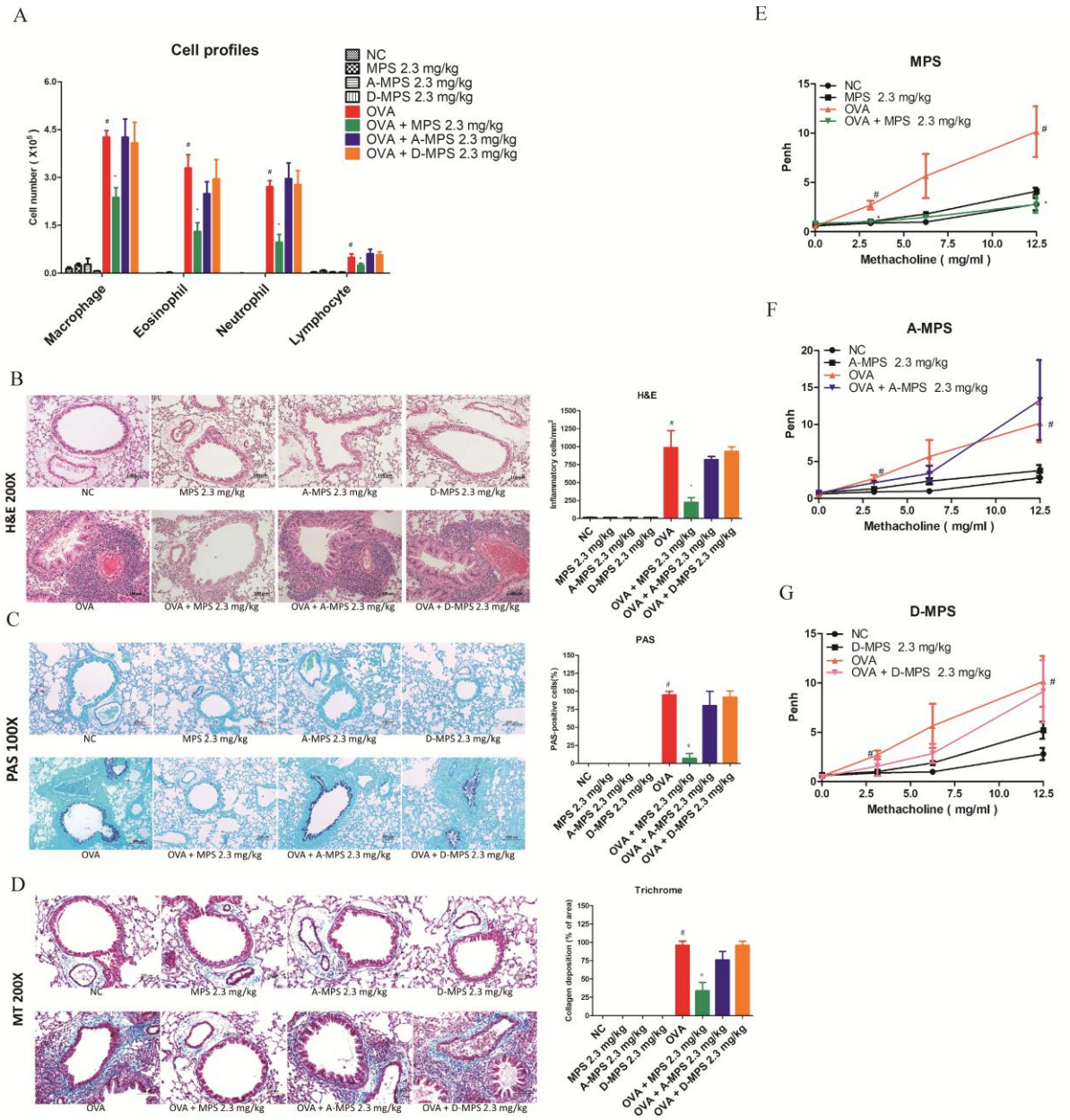


Figure 5. The effects of MPS, A-MPS, and D-MPS on asthma symptoms in steroid-resistant like mice. (A) BAL differential cell counts were assessed via May-Giemsa staining. (B-D) Lung sections were stained using H&E, PAS, and MT staining to assess inflammatory cell infiltration, mucus production and collagen deposition around the airways, respectively. High magnification bar represents 100 μm for H&E and MT; 200 μm for PAS. (E-G) Airway

resistance was determined through whole-body plethysmography. Data are expressed as mean ± S.D. of measured pulmonary resistance values (RL) ($n \geqq 5$). $^{\#}$ =$P < 0.05$, compared to the NC group; * = $P < 0.05$, compared to the OVA group.

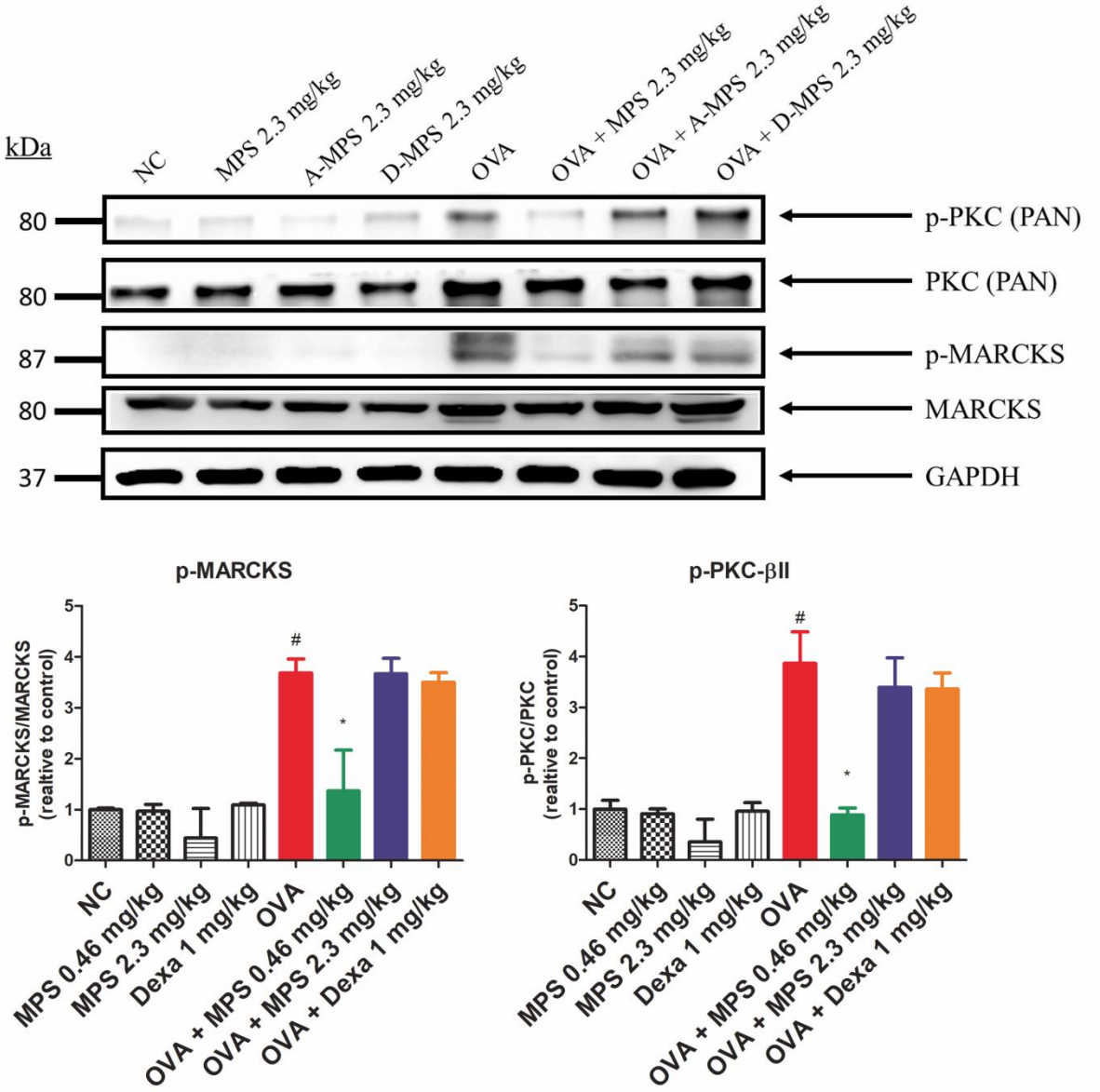


Figure 6. Suppression of p-MARCKS expression by MPS in steroid-resistant like mice. Lung proteins were subjected to western blot to assess the expression of phospho- protein kinase C (p-PKC), total PKC-βII, p-MARCKS, MARCKS and GAPDH. Proteins were quantified using Image J. Data are expressed as mean ± S.D. (n = 5). $^{\#}$ = $P < 0.05$, compared to NC group; * = $P < 0.05$, compared to the OVA group.