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MARCKS protein is a potential target in a naturally occurring equine model of neutrophilic asthma

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Abstract

Background Asthma is a chronic inflammatory airway disease that affects millions of people worldwide. Horses develop asthma spontaneously and serve as a relevant model for multiple phenotypes and endotypes of human asthma. In horses with equine asthma (EA), environmental organic dust triggers increased inflammatory cytokines, excess airway mucus, reversible bronchoconstriction, and airway inflammation. In horses with severe EA (sEA), lower airway inflammation is invariably neutrophilic, making sEA a potential model for severe neutrophilic asthma in humans. Alveolar macrophages (AM) and airway neutrophils contribute to lower airway inflammation and tissue damage through the release of cytokines and toxic mediators including reactive oxygen species. Previous work shows that the Myristoylated Alanine Rich C Kinase Substrate (MARCKS) protein is increased in activated macrophages and neutrophils and is an essential regulator of inflammatory functions in these cell types. We hypothesized that MARCKS protein would be increased in bronchoalveolar lavage (BAL) cells from horses with EA, and that in vitro inhibition of MARCKS with a specific inhibitor peptide known as **M**yristoyl**A**ted **N-**terminal **S**equence (MANS), would diminish cytokine production and respiratory burst.

Methods BAL cells from two populations of healthy and asthmatic horses were evaluated for cytology and MARCKS protein analysis. Isolated alveolar macrophages and peripheral blood neutrophils were stimulated with zymosan to evaluate MARCKS inhibition in cytokine secretion and respiratory burst.

Results We found increased levels of normalized MARCKS protein in total BAL cells from horses with asthma compared to normal horses. MARCKS inhibition with the MANS peptide had no effect on zymosan-stimulated release of tumor necrosis factor alpha (TNFα) or interleukin-8 (IL-8) from alveolar macrophages but did attenuate zymosan-stimulated respiratory burst in both alveolar macrophages and peripheral blood neutrophils.

Conclusions These findings point to a possible role for MARCKS in the pathophysiology of neutrophilic equine asthma and support further investigation of MARCKS as a novel anti-inflammatory target for severe neutrophilic asthma.

Keywords Asthma, Neutrophil, Horse, Inflammation, Reactive oxygen species

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Background

Asthma is a heterogenous syndrome of chronic lower airway inflammation characterized clinically by cough, shortness of breath, chest tightness and wheeze caused by bronchoconstriction, airway hyper-responsiveness, excess mucus, and airway remodeling. There are multiple phenotypes of asthma, including early versus late onset, allergic versus non-allergic, steroid resistant versus steroid sensitive, and asthma of variable disease severity [1]. While rodent models have contributed to the understanding of some aspects of asthma pathophysiology, there are attributes of these models that do not translate well to human asthma [2]. For example, mice do not develop asthma spontaneously. Further, there is increasing recognition that mice are useful models for specific characteristics of human asthma, rather than of the disease overall [3]. Additionally, there are limitations of existing mouse models for investigations of asthma that is non-allergic/non-eosinophilic or poorly-responsive to steroids [4]. Finally, noted differences in lung structure, the distribution of airway inflammation and lack of increased airway smooth muscle in mouse models, limits their application in research involving chronic airway remodeling and acute-on-chronic disease exacerbation [5]. As a result of these limitations, there is a growing interest within the research community and funding agencies such as NIH in naturally-occurring diseases in larger animals as relevant models of human diseases such as asthma [6–8]. Large animal models can provide advantages compared to small animals as related to imaging, pathology, surgical treatments, sample volume, longitudinal studies, and more. Naturally occurring diseases in these animal populations also better reflect the kinds of variables that have the potential to influence study findings (e.g. genetic, immune, history of disease and environmental exposures), like human patients. Specifically with asthma there is a clear benefit of spontaneous animal models as relates to: the understanding of pathogenetic mechanisms and identifying potential new therapeutic targets; the similarity to humans regarding initiating events and triggers; the effects of true chronicity (years vs. weeks); and the manifestation of different asthma phenotypes.

Aside from humans, cats and horses are the only other animal species known to develop asthma "naturally" [6]. In horses, there are two clinically-recognized phenotypes of Equine Asthma (EA) [9]. Mild/moderate EA (mEA) is characterized by decreased athletic performance, intermittent cough, increased tracheal mucus and mild lower airway inflammation that can be mastocytic, eosinophilic, neutrophilic or mixed cell types. Horses with mEA show no signs of respiratory compromise at rest. By contrast, horses with severe EA (sEA) experience episodes of asthma exacerbation characterized by frequent cough, increased respiratory rate and/or effort at rest and abnormalities on tracheal and lung auscultation (i.e., mucus rattle, crackles and/or wheezes). This severe EA phenotype in horses, as in humans, involves the larger neutrophilic contribution together with the "classic" asthma characteristics of goblet cell metaplasia, basement membrane thickening, and, of course, airways hyperreactivity [7, 10].

Several aspects of mEA and sEA have been proposed as appropriate for modeling human asthma phenotypes including non-allergic, poorly steroid-responsive and adult-onset [11]. Organic-dust induced asthma is another human asthma phenotype for which EA is an excellent translational model [12]. Organic dust consists of a mixture of many potential antigens, including particulate matter, endotoxins or lipopolysaccharide (LPS), peptidoglycans, noxious gases, and β-D-glucans [13]. In asthmatic horses, inhalation of organic dust triggers respiratory epithelial cells and alveolar macrophages to release proinflammatory mediators and chemoattractants, leading to an influx of neutrophils. Together, alveolar macrophages and neutrophils produce proinflammatory cytokines and reactive oxygen species, along with other inflammatory substances, leading to a cycle of inflammation and tissue damage. In fact, there is strong evidence that production of reactive oxygen species and resultant enhanced oxidant stress plays a major role in the development of the asthma phenotype [14–17].

Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) has been identified as an essential regulator of neutrophil and macrophage inflammatory functions and a promising target for various airway diseases. MARCKS is a 32 kDa substrate for Protein Kinase C (PKC) expressed ubiquitously in eukaryotic cells. In cells at rest, MARCKS localizes to the inner leaflet of the plasma membrane, crosslinking actin filaments. MARCKS binds the plasma membrane via its highly basic serine-rich effector domain (ED), which interacts electrostatically with acidic lipids in the cell membrane, and via the myristoylated N-terminus, which inserts hydrophobically into the lipid bilayer [18]. While localized to the plasma membrane, MARCKS ED binds to and sequesters up to four molecules of phosphatidylinositol 4,5-bisphosphate (PIP2). MARCKS-PIP2 binding is disrupted when cells receive activation signals that induce PKC-mediated MARCKS phosphorylation or calcium/calmodulin binding of the ED. MARCKS is then displaced to the cytosol, resulting in actin reorganization and release of PIP2 molecules [19, 20]. Due to its critical role in actin reorganization and PIP2 molecule sequestration, MARCKS has been recognized as playing an important role in diverse biological processes including cell motility, phagocytosis, membrane trafficking and

secretion [18, 21]. MARCKS is also a key regulator of neutrophil and macrophage functions including migration, adhesion, degranulation, cytokine secretion and respiratory burst [22–25]. A MARCKS-specific inhibitor peptide known as MANS (Myristoylated N-terminal Sequence) is identical to the first 24 amino acids of the N-terminal region of MARCKS and has been reported to attenuate equine neutrophil migration, adhesion and respiratory burst [24], LPS-induced cytokines in canine neutrophils [25] and migration of primary murine macrophages [26].

Early MARCKS studies showed that levels of this protein were significantly increased in neutrophils following LPS and TNFα stimulation, coinciding with increased MARCKS phosphorylation [27]. In a recent study using a murine model of steroid-resistant asthma, Wang and colleagues showed that suppressing MARCKS phosphorylation attenuated asthma symptoms and severe neutrophilic inflammation [28]. Given the important role that MARCKS plays in the inflammatory functions of neutrophils and macrophages and the animal model evidence supporting MARCKS as a potential target for asthma, we hypothesized that MARCKS protein levels would be elevated in BAL cells of asthmatic horses compared to normal horses and that MARCKS inhibition would attenuate inflammatory responses of equine alveolar macrophages and neutrophils, specifically release of pro-inflammatory cytokines and generation of ROS.

Methods

Horse population A– North Carolina State university (NCSU)

All procedures performed for the purposes of this study were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC 16-0740O and 19–779). Clinical trial number: not applicable. Horses included in this population were university owned adult teaching horses or client-owned horses referred for evaluation of respiratory disease. All horses lived in the southeastern United States. The horses were 6 to 24 years of age, and of mixed breed and gender (Table 1). The horses received no medications during the study. All horses received a physical exam, BAL and respiratory score, as previously described [29].

Horse population B– Equine respiratory tissue bank (ERTB)

BAL cell lysates from an additional 24 horses with EA were obtained from the Equine Respiratory Tissue Biobank (ERTB) (JP Lavoie). These samples were used to conduct a separate secondary investigation of MARCKS protein levels in the BAL cell lysates of asthmatic horses who had also received pulmonary function testing. The horses of the subpopulation were 9 to 24 years of age and of mixed breeds and gender (Table 2). Each horse received a physical examination, lung function test including Delta PpL, pulmonary resistance and elastance, and a BAL, except for three horses where lung function test was not performed. Delta PpL was computed as the difference between airway pressure and pleural pressure. Pleural pressure was measured indirectly as esophageal

Table 1 Demographic and BAL cytology data from population A

Horse	Breed	Age (years)	Sex	Clinical Score	Neutrophil %	Lymphocyte %	Macrophage %	Mast %	Eos %	Group
1 A	Thoroughbred (TB)	10	F	1	6.3	56.3	37	0.3	0	Normal
2 A	Quarter Horse (QH)	20	F	2	2.7	387	57.3	1.3	0	Normal
3 A	Paint	19	F	1	3	44.7	50.7	1.3	0.3	Normal
4 A	Warmblood cross	13	F	1	1.7	48	47.3	2.3	0.7	Normal
5 A	Quarter Horse	6	F	0	1.7	40	54	3.6	0.7	mEA
6 A	Quarter Horse	16	F	1	5	32.3	59.3	3.4	0	mEA
7 A	Tennessee Walking Horse	9	M	2	15	16	66	2	1	mEA
8 A	Quarter Horse	18	F	1	2.7	45.3	47	5	0	mEA
9 A	Paint	10	F	0	10.8	42	45	1.7	0.5	mEA
10 A	Quarter Horse	21	F	0	17.3	47.7	34.3	0.7	0	mEA
11 A	Quarter Horse	24	F	1	12	48.3	28.7	8	3	mEA
12 A	Quarter Horse/Thoroughbred	12	M	0	9.3	45	39.7	6	0	mEA
13 A	Quarter Horse/Thoroughbred	13	M	0	2	30.3	58.4	9	0.3	mEA
14 A	Quarter Horse/Thoroughbred	7	M	0	2.3	40	52.4	5.3	0	mEA
15 A	Pony	11	F	3	22	42	31.7	1.3	3	sEA
16 A	Quarter Horse	19	F	1	27.8	36	33.6	2.3	0.3	sEA
17 A	Quarter Horse	21	F	0	22.3	43.7	31.3	2.3	0.3	sEA
18 A	Walking Horse	20	M	6	12.3	35	49.3	3	0.3	sEA
19 A	Paint	16	F	5	53.7	5	39	0.7	1.7	sEA

Table 2 Demographic, BAL cytology and pulmonary function test data from population B

Horse	Breed	Age (yr)	Sex	Symptoms Y/*N*	Neutrophil %	Lymphocyte %	Macrophage %	Mast %	Eos %	Delta PpL	Resistance	Elastance	Group
1B	Standardbred	13	F	N	2.75	51.5	45.75	0	0	No lung function test			Normal
2B	TB Cross	20	F	N	2	59.75	37.75	1	0	5	0.2	0.4	Normal
3B	Arabian	18	M	N	1.75	41.5	53.5	0	3.25	9.15	0.873	1.096	Normal
4B	Standardbred	9	F	N	3.75	46	50	0.25	0	7.36	0.61	0.78	Normal
5B	Morgan	19	M	N	3	46	47.5	0.25	3.25	5.813	0.432	0.672	Normal
6B	Standardbred	19	F	N	1	48.5	50.5	0	0	5.63	0.438	0.309	Normal
7B	Quarter Horse	20	F	N	1.25	50.5	48.25	0	0	No lung function test			Normal
8B	Quarter Horse	14	M	Y	8.75	41.5	49.75	0	0	56.955	2.732	7.073	sEA
9B	Standardbred	15	F	Y	14.25	58	22	0.5	0	58.766	4.317	6.059	sEA
10B	Belgian	22	F	Y	15.75	46.5	37	0.25	0.05	28.37	1.49	0.71	sEA
11B	Canadian	15	F	Y	14.75	70.5	14.75	0.25	0	52.885	3.027	4.492	sEA
12B	Arabian	15	F	Y	10	38.5	66.25	0.25	0	No lung function test			sEA
13B	Arabian x QH	16	F	Y	10.75	63	25.25	0.25	0.75	31.547	1.936	1.995	sEA
14B	Quarter Horse	13	F	Y	14.5	57.5	27.5	0.75	0	29.19	1.63	2.2	sEA
15B	Standardbred	19	F	Y	16	54.5	29	0.75	0	64.63	4.1	7.56	sEA
16B	Quarter Horse	11	M	Y	7.75	60.75	30.25	1.25	0	30.27	3.08	2.61	sEA
17B	Quarter Horse	20	F	Y	12.5	66.25	19.25	2	0	89.449	2.775	16.166	sEA
18B	Standardbred	24	F	Y	29	52	18	1	0	23.370	2.088	2.166	sEA
19B	Cross	20	M	Y	63.5	16.5	20	0	0	29.288	2.37	1.813	sEA
20B	Paint Horse	12	M	Y	36.25	38	23.75	1	0	58.527	4.100	3.600	sEA
21B	QH Cross	20	F	Y	29.5	50.25	19.75	0.5	0	38.312	2.105	3.115	sEA
22B	Canadian	9	F	Y	27.75	42.25	29.5	0.5	0	69.875	3.829	6.087	sEA
23B	Paint Horse	16	F	Y	34.75	42.25	22.5	0.25	0	74.47	3.64	12.58	sEA
24B	Quarter Horse	15	M	Y	81.5	9	9	0.5	0	35	1.99	0.51	sEA

pressure using an esophageal balloon catheter. Pulmonary resistance and elastance were calculated from the airflow data that was measured using pneumotachograph mounted on the face mask and connected to a differential pressure transducer.

Disease classifications

In population A, horses were classified as normal, mild/moderate EA or severe EA based on history, physical exam, respiratory score and BAL cytology (Table 3). In non-performance horses (i.e. university-owned horses), diagnosis of mEA was based on no respiratory abnormalities at rest, no history of asthmatic episodes, and mild/moderate lower airway inflammation on BAL cytology. In population B, horses were classified as healthy or sEA by the same criteria as population A, with the addition of conventional pulmonary function testing results. Horses with history, physical exam and diagnostics consistent with asthma that presented with labored breathing at rest were diagnosed with sEA, regardless of BAL cytology. Fractional numbers on differential were rounded to the nearest whole number.

Bronchoalveolar lavage

For Population A, BAL samples were collected as described previously [30]. Briefly, after intravenous premedication with sedative (0.005-0.01 mg/kg detomidine and 0.02–0.04 mg/kg butorphanol), a local anesthetic gel (lidocaine) was applied at the nostril. The BAL was then performed with a 3-meter cuffed Bivona® tube, with two 150-250mL boluses of sterile isotonic saline solution. For Population B, BAL samples were collected as described previously [31]. Briefly, horses were sedated and a fiberoptic flexible endoscope was passed through the nares and into the right lung until wedged in the wall of a bronchus. Two 250 mL boluses of warm sterile isotonic saline solution were instilled and aspirated via the endoscope's biopsy channel. Recovered BALF samples were pooled, placed on ice and processed within one hour of collection for downstream analysis, or kept at room temperature for ex vivo experiments.

Cytological analysis

Cells (100 µL) were collected on object slides by cytospin centrifugation with 50 µL 30% bovine serum albumin (BSA) and stained by Wrights-Giemsa. Differential count was performed using a light microscope by counting 400 cells/slide by a certified clinical pathologist blinded to samples.

Table 3 Reference ranges and EA phenotypes based on equine BAL cytology

Classification of EA by BAL cytology	% Neutrophils	% Mast Cells	% Eosinophils
Normal	≤ 6%	≤ 2%	≤ 1%
Mild/Moderate	≥ 7% but ≤ 20%	(and/or) ≥ 3%	(and/or) > 1%
Severe	≥ 20%		

Lysate preparation

For samples from Population A, one Protease inhibitor (cOmplete™ ULTRA, Roche) tablet was added per 50mL BAL samples processed for cell lysate. The BALF was filtered through sterile gauze to remove mucus and was spun at 1500 rpm for 10 min. The mixed BAL cells were resuspended in Hank's balanced salt solution (HBSS). The cells were lysed with ice cold RIPA buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM sodium pyrophosphate and 50mM sodium fluoride] containing protease inhibitors (cOmplete™ ULTRA tablets mini, Roche) and phosphatase inhibitors (Pierce™ Phosphatase Inhibitor Mini Tablets, Thermo Fisher) for 30 min on ice. After lysis, cell solutions were spun at 13,000 rpm for 10 min at 4 °C. Supernatants were collected stored at -80 °C. For samples from Population B, cell lysate was prepared using TRIzol™ Reagent (Invitrogen™) and total cell protein was recovered from the phenol-ethanol supernatant by isopropanol precipitation.

Immunoblotting

Protein concentrations in cell lysates were measured using BCA Protein Assay Reagent (Pierce). Cell lysate was mixed with NuPAGE LDS Sample Buffer (4X) (Thermo Fisher) and Sample Reducing Agent (10X) (Thermo Fisher) and boiled for 10 min. Equal amount of protein was analyzed in 4–12% SDS-PAGE with MES running buffer. Resolved samples were transferred to Immobilon-P PVDF membrane (Millipore) and blocked for 1 h with 5% non-fat dry milk (for total MARCKS (Abcam (ab72459)) or 5% BSA (for Phospho-MARCKS (Ser159/163) (D13D2 Cell Signaling Technology)) with Tween-20 (TBS/T; 136 µM NaCl, 20 µM Tris-base (pH: 7.6) and 0.1% Tween-20 v/v) prior to overnight incubation with the 1:1000 dilution of primary antibody in TBS/T at 4 °C. Membranes were washed with TBS/T and incubated with 1:2000 dilution of anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology) in 5% non-fat dry milk in TBS/T for one hour, washed three times for 5 min each, developed using Bio-Rad Clarity Western ECL Substrate, and imaged using a Bio-Rad ChemiDoc. Images were analyzed using ImageJ and relative protein ratios were calculated as previously described [32].

ELISA

Analysis of MARCKS was carried out using equine-specific MARCKS ELISA kit (MyBioSource) per the manufacturer's protocol. MARCKS ELISA results were

normalized to the total protein concentrations determined using either BCA Protein Assay Reagent (Pierce), or nanodrop (used due to limited quantity of lysate from the ERTB). Equine-specific TNFα (R&D systems) and IL-8 (Genorise) ELISAs were carried out per the manufacturer's protocol. For the TNFα ELISA, cell culture supernatants were diluted 1:100 prior to analysis.

Alveolar macrophage isolation

Alveolar macrophages were isolated from different individual, non-asthmatic, horses. The BALF was filtered through sterile gauze to remove mucus and was spun at 1500 rpm for 10 min. The mixed BAL cells were resuspended in Hank's balanced salt solution (HBSS), and following total cell count and differential count determination, centrifuged at 1500 rpm for 10 min. Based on the calculated total alveolar macrophage count, mixed BAL cells were resuspended in RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% heat inactivated fetal calf serum (Gibco). Alveolar macrophages were then seeded into plates at equivalent densities (between 700,000 and 1.0×10^6 cells depending on BALF macrophage %) and incubated for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times to remove nonadherent cells, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 h. This isolation method routinely yielded 96–98% macrophages based on Wright's Geimsa staining.

Zymosan stimulation of alveolar macrophages

Alveolar macrophages were seeded in 24 well plates based on total alveolar macrophage count and incubated in air at 37 °C with 5% CO_2 for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times so that non-adherent cells would be removed, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 h. The media was changed to RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% equine whole serum and the cells were then primed with 1 ng/mL equine GM-CSF (Kingfisher Biotech). Following 30-minute incubation at 37 °C with 5% CO_2, cells were stimulated with 100 µg/mL zymosan for 6 h. For MARCKS inhibition, immediately after priming, cells were treated with 10, 25, 50 or 100 µM MANS peptide, 100 µM control Random N-terminal Sequence (RNS) peptide or vehicle (PBS) for 30 min at 37 °C followed by zymosan for the pretreatment groups. For post-treatment groups, appropriate concentrations of MANS, RNS, or control were added 1 h following zymosan stimulation. After 6-hour incubation at 37 °C with 5% CO_2, the plate was centrifuged 300 x G for 5 min, supernatants collected into Eppendorf tubes, then centrifuged again at 1300 x rpm for 3 min to pellet remaining cellular components. Cell-free supernatants were collected and frozen at -80 °C until ELISA analysis.

Peripheral blood neutrophil isolation

Peripheral blood neutrophils were isolated from equine whole blood using density gradient centrifugation method. Heparinized whole blood was collected from donor horses via jugular venipuncture. The collected whole blood was kept at room temperature in sterile conical tubes to allow erythrocytes to settle at the bottom. The supernatant (leukocyte-rich plasma) was collected, placed onto Ficoll-Paque Plus (GE Healthcare) layer. Following centrifugation, erythrocyte contamination within the neutrophil pellet was removed by 1-minute hypotonic lysis. This isolation method routinely yields samples that are 95% neutrophils with 98% viability as determined by exclusion of trypan blue dye.

Respiratory burst

Production of reactive oxygen species (ROS) was determined by luminol-enhanced chemiluminescence. Alveolar macrophages were seeded into 96-well plate at a density of 100×10^3 cells per well and incubated at 37 °C with 5% CO_2 for an hour to allow alveolar macrophages to adhere to the bottom of the well. The wells were then washed three times so that nonadherent cells would be removed, and the remaining alveolar macrophages were incubated at 37 °C with 5% CO_2 for at least 20 h. The media was changed to RPMI 1640 medium without phenol red (Thermo Fisher) supplemented with 10% equine whole serum and the cells were then primed with 1 ng/mL equine GM-CSF (Kingfisher Biotech). For the MARCKS inhibition experiment, immediately after priming, the cells were treated with 10, 25, 50 or 100 µM MANS peptide, 100 µM control RNS peptide or vehicle for 30 min at 37 °C. The cells were then stimulated with 100 µg/mL zymosan (Millipore Sigma) or vehicle. 1mM luminol was added to each well, and luminescence was measured every 15 min for 5 h using a Synergy HTX Multi-Mode Microplate Reader (Biotek). Peripheral blood neutrophils were also stimulated with 100 µg/mL zymosan or vehicle as described previously [24] and detected using luminescence.

Statistical analyses

All analyses were performed using GraphPad Prism (GraphPad Software). Data were tested for normality by Shapiro-Wilk test. One-way ANOVA with Tukey's post-hoc test was used to analyze normally distributed data. Nonparametric Kruskal-Wallis ANOVA with Dunn's post-hoc test was used to analyze non-normally distributed data. Chi-square test was performed to determine whether there was a significant difference in the

sex distribution among groups. Correlation between the normalized MARCKS and the BALF cytology results was determined by Pearson's correlation coefficient analysis. For all analyses, $p < 0.05$ was considered statistically significant.

Results

MARCKS protein is increased in BAL cell lysates from horses with mild/moderate and severe asthma compared to healthy horses.

To investigate whether there were alterations in MARCKS protein in horses with asthma, nineteen horses were sampled (Population A). Results of physical examination and BAL cytology for population A are provided in Table 1. Four horses were classified as normal, ten had mild/moderate inflammation, no respiratory signs at rest and no history of severe asthma episodes, consistent with mEA, and five had signs of current or historical signs of labored breathing at rest, cough and neutrophilic BAL cytology, consistent with sEA. Due to the lack of performance horses and pulmonary function data in population A, we chose to also analyze samples from horses whose patient information, diagnostic results and airway samples were available through a biobank. The advantage of this population is that all samples came from sEA horses in exacerbation, but with variable degrees of airway inflammation, and sEA diagnosis was verified by increased pulmonary resistance and decreased compliance using conventional pulmonary function testing. The results from population B are provided in Table 2. Out of the 24 horses, seven horses were healthy and 17 had sEA. In population B, ΔPpL and pulmonary resistance were significantly different between the healthy and severe asthma groups (Figure S1).

In horses from population A, levels of normalized MARCKS protein were significantly increased in BAL cell lysates from healthy horses compared to mEA and sEA horses (Fig. 1A). In horses from population B, levels of normalized MARCKS protein were significantly increased in BAL cell lysates from healthy compared to sEA horses (Fig. 1B). In population A, there was no significant correlation between normalized MARCKS and percentage of specific cell types in the BALF (Supplemental Fig. 2A-E). Similarly, no significant correlation was found in between normalized MARCKS and absolute counts of any specific cell types in the BALF (Supplemental Fig. 2F-J). In population B, there were significant positive correlations between normalized MARCKS and % BALF neutrophils and a trend towards positive correlation between MARCKS and absolute neutrophil counts (Supplemental Fig. 3A, F). A significant negative correlation was found between MARCKS and % BALF macrophages (Supplemental Fig. 3D).

To further verify the results of the equine MARCKS ELISA, we used immunoblotting to determine whether total and/or phospho-MARCKS was increased in BAL cells from horses with EA. Qualitative analysis of representative immunoblot support increased total MARCKS

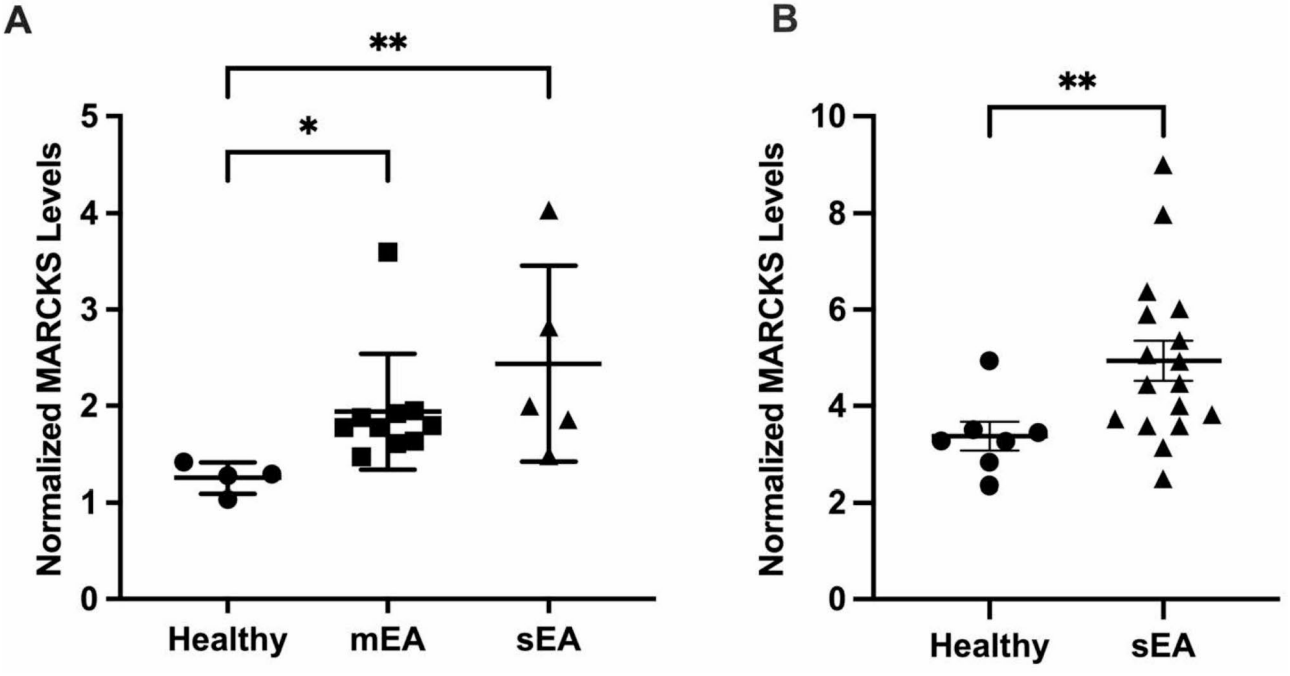

Fig. 1 MARCKS protein is increased in BAL cell lysates from horses with sEA. MARCKS levels in BAL cell lysates from Population A ($n = 19$) (**A**) and Population B ($n = 24$) (**B**). Data were tested for normality. Data from Population A was analyzed using nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test. Data from Population B was analyzed using Welch's t-test. *$p < 0.05$, **$p \leq 0.006$

expression in BAL samples from horses with EA compared to healthy horses (Fig. 2). Interestingly, phospho-MARCKS did not appear to be increased in our samples.

MARCKS inhibition with the MANS peptide attenuates zymosan-induced ROS in alveolar macrophages and neutrophils, but not release of cytokines.

Airway neutrophilia is marked in horses with sEA, and these neutrophils express mRNA for cytokines including TNFα and IL-8 [33–35]. Previous studies have shown that inhibition of MARCKS with the MANS peptide is able to inhibit in vitro mRNA and protein expression of the pro-inflammatory mediators IL-8 and TNFα by canine neutrophils [25] and interleukin-6 (IL-6) and TNFα by murine peritoneal macrophages [36]. Zymosan is a cell wall preparation of *Saccharomyces cerevisiae*, which is primarily composed of β1,3-glucan core linked to chitin and β1,6-glucans. Beta-glucans are one of the environmental triggers of EA [37] and are also common triggers for organic-dust induced asthma in people [12]. Therefore, zymosan was selected as a potentially relevant stimulant for in vitro modeling of organic-dust induced lower airway inflammation in asthma. Zymosan induced significant cytokine production of both TNFα and IL-8 in alveolar macrophages. However, MANS peptide treatment had no significant effect on levels of these cytokines in cellular supernatant (Fig. 3). Staurosporine-mediated PKC inhibition significantly decreased TNFα production (Fig. 3A). While not significant, there was a trend towards decreased IL-8 release in MANS treated cells (Fig. 3B).

Reactive oxygen species production (ROS) by innate immune cells, such as neutrophils and macrophages, contributes to the progression of inflammation in the lung. Excessive ROS production results in oxidative stress and tissue damage that encourages disease progression [38–40]. Given that ROS production is such an important driver of lung inflammation, the effect of MARCKS inhibition on ROS production was evaluated in zymosan-stimulated alveolar macrophages and neutrophils using luminescence. Zymosan elicits a robust respiratory burst response in alveolar macrophages (Fig. 4) and neutrophils (Fig. 5). At peak stimulation, MANS peptide treatment attenuated zymosan-stimulated ROS production in a concentration dependent manner in both cell types. The PKC inhibitor staurosporine also significantly inhibited ROS (data not shown), while the control peptide RNS had no effect. Taken together, these data demonstrate that MARCKS inhibition with MANS peptide attenuates zymosan-induced ROS production in both alveolar macrophages and peripheral blood neutrophils.

Discussion

MARCKS protein has been linked to the pathophysiology of numerous lower airway diseases including several types of lung cancer, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS) and asthma [41]. Potential roles for MARCKS in these diseases include cellular responses involving metastasis and migration [42], mucin secretion [43], cytokine production [36] and leukocyte degranulation [23]. Importantly, peptide inhibitors of the MARCKS protein are an exciting novel therapeutic strategy that have shown benefit in animal models of asthma [44] and acute lung injury [45] and in Phase II clinical trials in patients with COPD [46] or with ARDS [47]. The goals of this study were to determine whether normalized levels of MARCKS and/or phospho-MARCKS protein were altered in a naturally occurring equine model of asthma and whether zymosan-stimulated inflammatory responses of relevant innate immune cells (i.e. alveolar macrophages and neutrophils) were blocked by a MARCKS-specific inhibitor peptide known as MANS. In two geographically distinct populations of horses with sEA, we found that the level of MARCKS protein (normalized) was

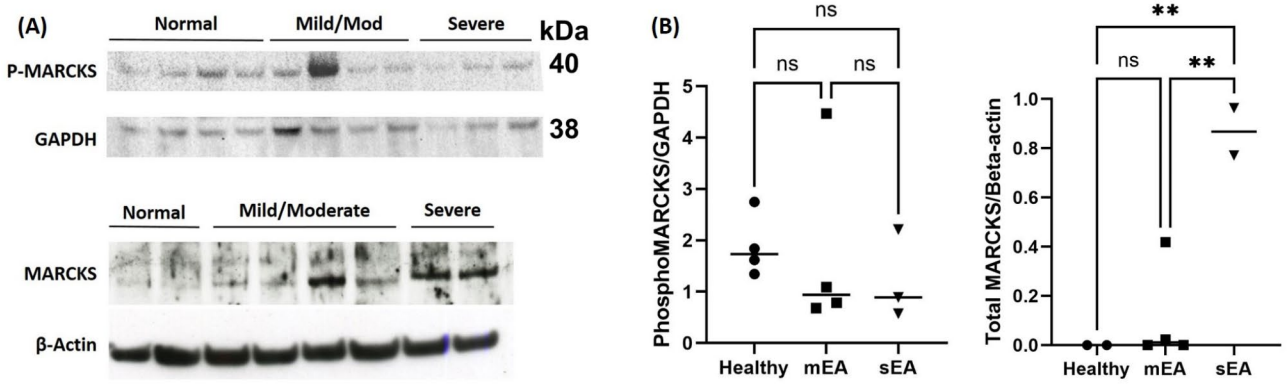

Fig. 2 MARCKS protein is increased in equine BAL cell lysates from horses with sEA. (**A**) BAL cell lysates were randomly selected from Population A for immunoblot of total MARCKS from healthy horses ($n=2$) and horses with mild/moderate ($n=4$) and severe EA ($n=2$) and for phospho-MARCKS from normal horses ($n=4$) and horses with mild/moderate ($n=4$) and severe EA ($n=3$). (**B**) Relative protein ratios were analyzed by one-way ANOVA with Tukey's multiple comparisons test. $^{**}p<0.005$

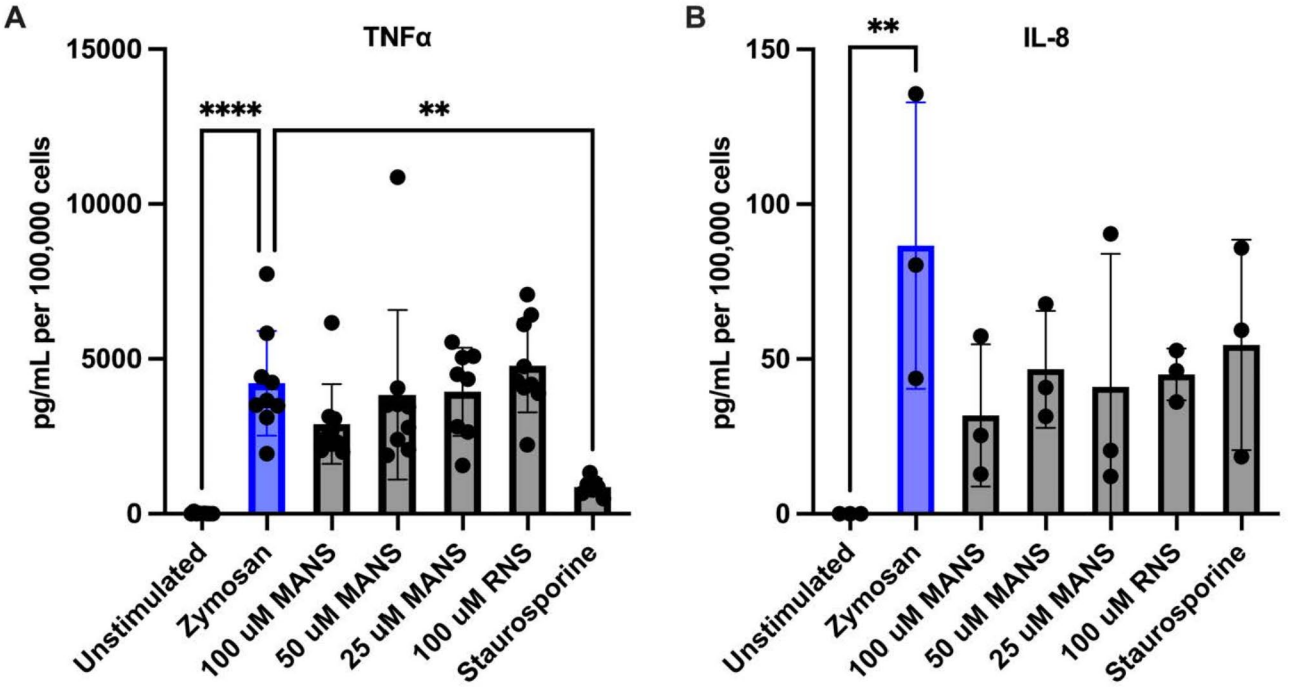

Fig. 3 MANS peptide treatment does not significantly affect cytokine secretion of zymosan-stimulated equine alveolar macrophages. Isolated alveolar macrophages were treated with indicated concentrations of MANS or RNS peptide 30 min before stimulation with 100 ug/mL zymosan. Supernatants were collected for analysis by equine specific ELISA for (**A**) TNFα and (**B**) IL-8. Data are represented as mean ± SD, ($n = 7–9$) for (**A**), $n = 3$ for (**B**). Data were analyzed by one-way ANOVA with Dunnett's multiple comparisons test. *$p < 0.05$, **$p < 0.005$, ****$p < 0.0005$

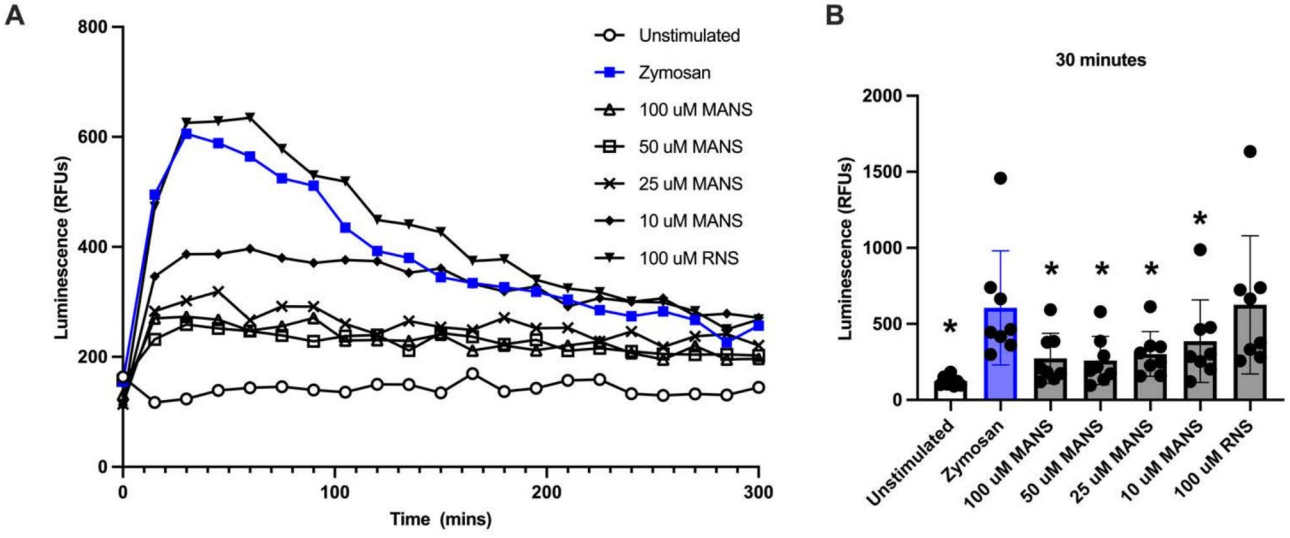

Fig. 4 MANS peptide treatment significantly attenuates ROS production in zymosan-stimulated equine alveolar macrophages. (**A**) Stimulation with 100 ug/mL zymosan caused robust ROS production by alveolar macrophages. Lines represent the median of all individuals ($n = 8$). (**B**) Pretreatment with MANS peptide significantly attenuated ROS production by zymosan-stimulated alveolar macrophages at 30 min. Data analyzed by repeated measures one-way ANOVA with Dunnett's multiple comparisons test, $n = 8$. *$p < 0.05$ when compared to zymosan stimulation

significantly increased in the BAL cell lysates of asthmatic horses compared to healthy horses. This is the first study to report elevated levels of MARCKS protein in airway lavage cells in a naturally occurring large animal model of severe neutrophilic asthma. This may seem surprising, since there have been several previous studies that have compared proteomic profiles of airway samples from healthy vs. asthmatic horses and humans, and MARCKS was not identified as differentially expressed by these studies. However, most of these studies analyzed BALF supernatant rather than cells or tissues [48, 49], and because MARCKS is an intracellular protein, it

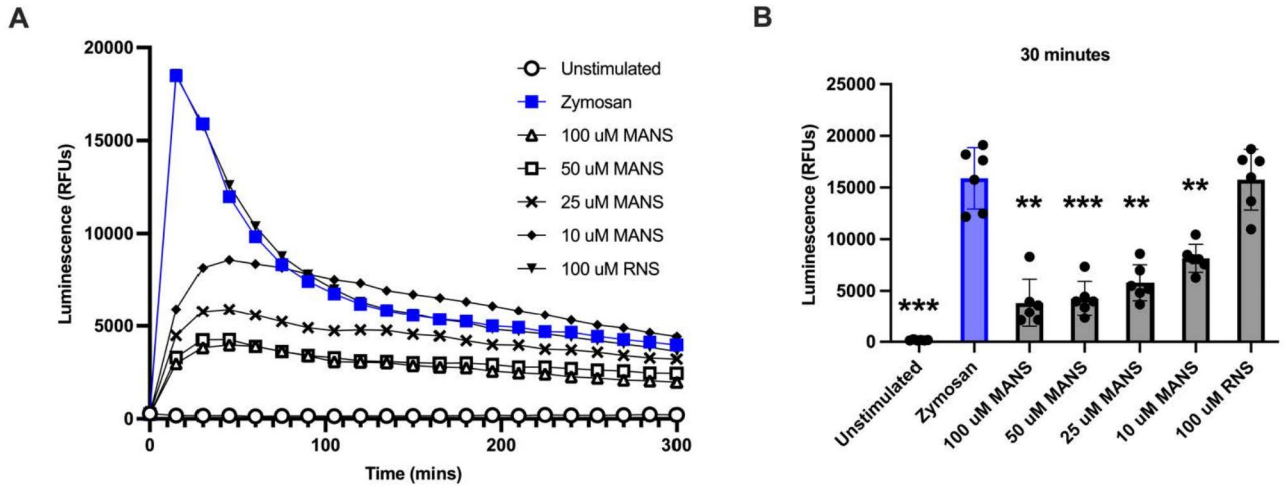

Fig. 5 MANS peptide treatment significantly attenuates ROS production in zymosan-stimulated equine neutrophils. (**A**) Stimulation with 100 ug/mL zymosan caused robust ROS production by peripheral blood neutrophils. Lines represent the median of all individuals ($n=6$). (**B**) Pretreatment with MANS peptide significantly attenuated ROS production by zymosan-stimulated neutrophils at 30 min. Data analyzed by repeated measures one-way ANOVA with Dunnett's multiple comparisons test, $n=6$. *$p<0.05$, **$p<0.005$, ***$p<0.001$ when compared to zymosan stimulation

was not detected in airway lavage fluid. One recent study did find that MARCKS protein was increased in extracellular vesicles (EVs) released from LPS-stimulated neutrophils from severely asthmatic horses in crisis [50]. Neutrophil EVs have been linked to airway smooth muscle hyperplasia and asthma pathogenesis [51]. Because EVs reflect the contents of their cells of origin, it is probable that increased MARCKS in neutrophil derived EVs was due to an increase in MARCKS protein in the LPS-stimulated neutrophils, which has been previously documented [27]. While we did find that MARCKS protein was elevated in BAL cells from horses with asthma, we did not measure MARCKS mRNA transcripts; therefore, we did not determine whether the MARCKS gene is differentially expressed in equine asthma. However, several previous studies have investigated differential gene expression in lower airway cells from humans and horses with and without asthma. While one of these studies used a human microarray to report downregulation of MARCKS mRNA in horses with severe asthma compared to healthy horses [52], several other studies found that MARCKS mRNA expression did not differ between health and disease [48, 53, 54]. This is in seeming contrast to the early MARCKS studies by Thelen et al. which reported rapid increase of MARCKS protein (45 min) following stimulation of human neutrophils with LPS [27]. MARCKS is known to undergo several types of post-translational modifications, which could explain differences in levels of MARCKS protein without differences in MARCKS mRNA [18]. Additionally, further clarification could come from investigation of mRNA at the level of individual cells (i.e. single cell RNAseq), rather than transcriptomics of mixed BAL cells.

A critical regulator of MARCKS function is phosphorylation by PKC [55, 56], which results in the release of actin and PIP2. MARCKS plays an essential role in many actin- and PIP2-dependent events such as migration [20, 22, 57, 58], adhesion [24, 59], cytokine secretion [60] and respiratory burst [24], all of which are important inflammatory cellular functions. A recent study demonstrated increased phospho-MARCKS in the lung homogenates of mice using a murine model of steroid-resistant asthma [28]. MARCKS phosphorylation has also been shown to be increased in respiratory epithelium in a murine model of allergic asthma [61]. In this model, pre-treatment with a MARCKS ED targeting peptide effectively blocked MARCKS phosphorylation, attenuated migration of dendritic cells, reduced Th2-related lymphocyte activation and eliminated clinical signs of asthma [61]. Interestingly, our results did not detect increased phospho-MARCKS in horses with asthma. This finding seems to contrast with the study by Wang et al.; however, in the murine study, the investigators analyzed phospho-MARCKS in lung homogenate, rather than isolated BAL cells. Since MARCKS is a ubiquitously expressed protein, the lung homogenate sample from Wang et al. contained many more types of cells than our BAL lysates. Additionally, Wang and colleagues were using an induced-animal model designed to cause symptomatic disease, while the samples we used for immunoblotting were from horses with naturally occurring asthma that had not been induced to exacerbation and had variable clinical signs at the time of sample collection. Further, our ability to detect increased MARCKS phosphorylation could have been affected by timing, since previous studies have shown the cycle of phosphorylation/de-phosphorylation varies significantly by cell type [62–66]. Finally, PKC

is known to phosphorylate MARCKS at 3 to 4 serines within the ED, depending on the species [67]. Available antibodies with cross-reactivity for horses are limited. For this study, we used a phospho-MARCKS antibody targeting Ser159/163. Further work is needed to determine the potential significance of other MARCKS phosphorylation sites in equine airway cells. In future studies we will investigate multiple MARCKS phosphorylation sites in BAL and respiratory epithelial cells in asthmatic horses with controlled timing of asthma exacerbation.

The level of MARCKS protein was further analyzed to evaluate correlations between the protein levels and the BALF cytology results. The overall BALF cellularity increases in horses with EA [68], but differential cell counts do not reflect the change in the overall cellularity. Also, as % neutrophils increase in the differential cell counts, % macrophages and lymphocytes decrease [9]. Therefore, the correlations between protein levels and absolute and differential % cell types were analyzed. Given the fact that MARCKS protein is known to increase in activated neutrophils and macrophages and that neutrophils are increased in the airways of horses with sEA, we hypothesized that the level of MARCKS protein would be positively correlated with % and absolute neutrophils in BAL samples from horses with EA. Indeed, this hypothesis was confirmed in Population B, where we found a significant positive correlation between normalized MARCKS levels and % BALF neutrophils. This finding is consistent with early reports on MARCKS that showed rapid induction of MARCKS phosphorylation (i.e., < 30 s) and synthesis (45 min) following stimulation of human neutrophils with TNFα or LPS and that MARCKS constitutes 90% of all proteins synthesized by neutrophils in response to TNFα or LPS [27]. Interestingly, we did not find the same significant correlation in Population A. There are atleast two possible explanations for the differences in findings between our two horse populations. First, all of the horses in Population B were in acute asthma exacerbation, while horses in Population A had variable symptoms at the time of sample collection. Because BAL neutrophils are correlated with asthma severity in horses [71], this variability in disease exacerbation in Population A could have affected both neutrophil numbers and MARCKS levels. Second, bronchoconstriction and mucus plugging can impede cell recovery during sample collection. This could have reduced the BAL neutrophil numbers in severely affected animals in Population A, which would have altered the "apparent" correlation between MARCKS and neutrophils. In future studies, we will ensure that all horses are in exacerbation at the time of collection by triggering the onset of asthma symptoms with organic dust. In follow up studies, we also plan to specifically investigate MARCKS expression and phosphorylation by cell type, in asthmatic and non-asthmatic horses.

One of the limitations of our study is the lack of pulmonary function data to aid in the phenotypic classification of horses in Population A. While conventional pulmonary function testing is well described and validated for the diagnosis of EA, there are no commercially available products and use of this equipment is primarily limited to specialty hospitals and research centers. Portable, non-invasive methods for pulmonary function testing in horses are currently under development. Next to pulmonary function testing, BALF cytology is considered a useful method to detect lower airway inflammation in horses. BAL is a minimally invasive procedure that can be performed with standing sedation and requires minimal equipment. However, BALF cytology for asthma diagnosis does have limitations. While reference ranges for normal BAL cell percentages have been established for horses, it is not uncommon to collect a sample on a clinically healthy "control" and discover the BALF cytology is slightly "abnormal". Or the reverse is also possible. BAL cytology from horses with clinically severe asthma may not reflect the expected level of airway neutrophilia. Like human asthma, these individuals are classified as paucigranulocytic. Previous research has also shown that BALF cytology does not always correlate with lung biopsy findings [69]. According to current consensus definitions, diagnosis of mEA is based on decreased or impaired athletic performance, occasional cough, increased inflammation on BAL cytology and no signs of respiratory infection or respiratory abnormalities (i.e. increased nostril flare, tachypnea, abdominal breathing effort, lung auscultation abnormalities) at rest; and diagnosis of sEA is based on a history or presenting complaint of recurrent cough, labored breathing (i.e. increased nostril flare, tachypnea, abdominal effort), crackles and/or wheezes on lung auscultation, neutrophilic inflammation on BAL cytology and no signs of respiratory infection [9]. While the horses grouped as mEA in Population A did have evidence of lower airway inflammation on BALF cytology, some of these horses were university-, rather client-, owned and used for teaching, which precluded our ability to know whether their airway inflammation was performance limiting. It is for this reason that we included analysis of a separate population of horses with pulmonary function test results available to further confirm asthma diagnosis. In the future, non-invasive options for pulmonary function testing in horses will improve researchers' ability to correlate lower airway inflammation in non-exercising horses with altered pulmonary function and asthma diagnosis. This will improve the utility of this naturally occurring model for translational research.

Following exposure to organic dust, alveolar macrophages become activated and release pro-inflammatory

mediators including IL-6, C-X-C motif chemokine ligand 1 (CXCL1) and C-X-C motif chemokine ligand 2 (CXCL2) [70], as well as reactive oxygen species [39]. In response to these signals, large numbers of neutrophils are recruited to the airways. In severely asthmatic horses, this increase in airway neutrophils is coincident with the onset of asthma exacerbation and the degree of airway neutrophilia is associated with severity of clinical signs [71]. Concomitantly, the severity of clinical signs of EA decrease as airway neutrophils decrease [72]. Along with neutrophils, alveolar macrophages are also thought to play a role in the pathophysiology of sEA. In a crossover study using moldy hay challenge of sEA horses, Aharonson-Raz et al. showed that depletion of pulmonary intravascular macrophages with gadolinium chloride attenuated asthma symptoms, reduced airway neutrophilia, and decreased proinflammatory cytokine mRNA in BAL cells [73]. MARCKS protein has also been identified as a potential contributor to asthma pathogenesis. In a murine model of neutrophilic airway inflammation, Damera et al. reported that MARCKS inhibition significantly attenuated neutrophilic infiltration and significantly reduced BALF levels of CXCL1, IL-6 and TNFα [60]. In another murine model of severe steroid resistant asthma, Wang et al. showed that intratracheal treatment with a peptide targeting the MARCKS phosphorylation-site domain decreased neutrophilic airway inflammation, which resulted in decreased asthma symptoms [28]. Taken together, these data point to neutrophils, AMs and MARCKS as potential contributors to the pathophysiology and symptoms of severe asthma. Therefore, targeting the inflammatory functions of neutrophils and macrophages, potentially by targeting MARCKS, could be a viable strategy for the management of severe neutrophilic asthma.

Given the increased levels of MARCKS in the asthmatic BAL, and MARCKS known role in inflammatory functions, MARCKS could be involved in asthma pathogenesis. As we have published previously, MARCKS is integrally involved in many neutrophil and macrophage inflammatory responses, including migration [22, 24, 26, 58], release of inflammatory mediators [23, 25], and the respiratory burst [24, 59]. Thus, we hypothesized that inhibition of MARCKS with the MANS peptide would attenuate secretion of proinflammatory cytokines in alveolar macrophages and ROS production in AMs and peripheral blood neutrophils. Alveolar macrophages were treated with MANS peptide 30 min before stimulation with zymosan. Although zymosan stimulated significant production of TNFα and IL-8, there was no significant difference in MANS or RNS treated cells stimulated for up to 6 h. Our findings are in contrast to previous studies showing that MARCKS protein does play a role in cytokine production in neutrophils and macrophages. Specifically, Li et al. showed that pretreatment of canine neutrophils with the MANS peptide inhibited in vitro mRNA and protein expression of the pro-inflammatory cytokines IL-8 and TNF-α, and Lee et al. showed MANS treatment decreased IL-6 and TNFα mRNA in murine peritoneal macrophages [25, 36]. One potential explanation for our disparate findings is differences in the method of cell stimulation. The studies by Li et al. and Lee et al. used LPS to stimulate cytokine production while we utilized zymosan. LPS is known to stimulate cytokine production via TLR4 receptor signaling, while opsonized zymosan is phagocytosed by both neutrophils and macrophages, and engages numerous receptors including CD11b/CD18 (complement receptor 3), Dectin1, TLR-2 and TLR-6 [74]. Differences in stimulant activation pathways and cell signaling could explain differences in our findings compared to other studies. Interestingly, in a murine model of neutrophilic asthma, Wang et al. showed that treatment with a MARCKS phosphorylation blocking peptide (MPS) did attenuate airway cytokines including C5a, IL-17 A and KC. In future work we would like to expand our investigation of cytokines to include other pro-inflammatory cytokines, such as IL-6, such as IL-10 and non-neutrophilic cytokines, such as IFN-γ. Additional in vivo studies are needed to determine whether MARCKS N-terminus mimetic peptides, such as MANS or BIO-11,006, alter cytokines in the lower airways of asthmatic horses following natural organic dust challenge.

Similar to previous studies, MANS peptide treatment attenuated reactive oxygen species production in both alveolar macrophages and peripheral blood neutrophils [24]. Novel to these previous studies, this is the first study to show that a MARCKS-targeting peptide inhibits ROS production triggered by zymosan. Zymosan is a cell wall preparation of *Saccharomyces cerevisiae*, which is primarily composed of β1,3-glucan core linked to chitin and β1,6-glucans, and serves as a useful model for investigating cellular responses to fungal organisms or components. In previous studies, our lab has shown that MANS peptide inhibits insoluble immune complex (IIC)-, but not phorbol 12-myristate 13-acetate (PMA), stimulated ROS production in equine neutrophils. Because PMA-stimulated ROS remained intact, we concluded that MANS peptide treatment does not prevent assembly of the NADPH oxidase enzyme complex. Instead, since inhibition of MARCKS had differential effects on β_2-integrin independent (PMA) vs. β_2-integrin dependent (IIC) respiratory burst, we proposed an essential role for MARCKS in β_2-integrin dependent ROS production. Previous studies have identified a role for MARCKS in macrophage phagocytosis of zymosan particles [75]. B_2-integrins have also been shown to be essential for zymosan-stimulated ROS production in porcine and human

neutrophils [76] but not zymosan-stimulated release of IL-8 in human neutrophils [77]. Based on these data and our findings, inhibition of β_2-integrins is one potential mechanism by which MANS treatment blocks zymosan-stimulated ROS production in these cells. This mechanism could also explain our finding that MANS peptide did not inhibit cytokine secretion. Importantly, previous studies have shown that even without phagocytosis, surface cross-linking of neutrophil β_2-integrins is sufficient to trigger cellular signaling that results in activation of NADPH-oxidase and production of intracellular ROS, and that this signaling involves the actin cytoskeleton [78]. Given MARCKS role in actin regulation [18], this is another potential mechanism that could explain MANS inhibition of zymosan-stimulated ROS production. Additional studies are currently underway to investigate these potential underlying cellular mechanisms.

MANS inhibition of ROS warrants further investigation as a potential therapeutic strategy for asthma. Multiple studies have demonstrated the significant role of ROS in asthma severity, and ROS is a key mediator of airway inflammation, airway hyper-responsiveness, tissue injury, and remodeling [79]. In one study, H2O2 in expired breath condensate was positively correlated with eosinophils, neutrophils, symptom score, and PEFR variability, and was negatively related to FEV1% predicted [80]. In another study, serum ROS levels were significantly associated with the degrees of airway obstruction, WBC counts, neutrophil counts, IL-6, and severe exacerbations in patients with bronchial asthma [81]. Data linking ROS to the pathogenesis of asthma has been recently reviewed, along with various potential mechanisms to target ROS in asthma [ref]. Given the level of interest in ROS targeting in asthma, further investigation of peptide inhibition of MARCKS seems warranted.

While alveolar macrophages and neutrophils play key roles in the initiation and perpetuation of lower airway inflammation in severe asthma, there are other cell types that contribute to the pathophysiology of this disease including airway epithelial cells. As the lung's first line of defense against airborne irritants, airway epithelial cells produce ROS and secrete a variety of proinflammatory cytokines and chemokines that modulate host immune response [82]. Goblet cells within the respiratory epithelium are also responsible for secretion of mucus, which accumulates and contributes to cough and obstruction in asthma. Importantly, MARCKS is known to help regulate mucin secretion in airway epithelial cells, and inhibitor peptides of MARCKS have been shown to decrease excess airway mucus in vivo [43, 44, 83]. In future studies we will broaden our investigation of MARCKS role in the pathophysiology of severe neutrophilic asthma. Using the equine model, we plan to investigate levels of total and phospho-MARCKS in respiratory epithelium cultured from sEA horses during crisis vs. remission. We also plan to conduct in vivo studies in horses with sEA to determine the effect of nebulized MARCKS inhibitor peptides on clinical signs of asthma, as well as airway mucus and inflammation following natural organic dust challenge.

Conclusions

These study results show that MARCKS protein is increased in BAL cells from horses with naturally occurring severe neutrophilic asthma. Further, peptide mediated inhibition of MARCKS in zymosan-stimulated alveolar macrophages and neutrophils significantly attenuates ROS. We assert these data provide compelling evidence for MARCKS as a novel therapeutic target for severe neutrophilic asthma and that further investigation of MARCKS-targeting strategies in a naturally occurring equine asthma model is warranted.

Abbreviations

EA	Equine asthma
sEA	Severe EA
mEA	Mild/moderate EA
AM	Alveolar macrophages
MARCKS	Myristoylated Alanine Rich C Kinase Substrate
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
MANS	MARCKS N-terminal Sequence
TNFα	Tumor necrosis factor alpha
IL-8	Interleukin-8
LPS	Lipopolysaccharide
PKC	Protein Kinase C
ED	Effector domain
PIP2	Phosphatidylinositol 4,5-bisphosphate
ERTB	Equine Respiratory Tissue Bank
PpL	Pleural pressure
HBSS	Hank's balanced salt solution
SDS	Sodium dodecyl sulfate
BSA	Bovine serum albumin
HRP	Horseradish peroxidase
RNS	Random N-terminal Sequence
ROS	Reactive oxygen species
IL-6	Interleukin-6
COPD	Chronic obstructive pulmonary disease
ARDS	Acute respiratory distress syndrome
EVs	Extracellular vesicles
CXCL1	C-X-C motif chemokine ligand 1
CXCL2	C-X-C motif chemokine ligand 2
PMA	Phorbol 12-myristate 13-acetate
IIC	Insoluble immune complex

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03194-w.

Supplementary Material 1: Supplemental Fig.1. Conventional pulmonary function test results from healthy and sEA horses (Population B). (A) Delta PpL, (B) pulmonary resistance, and (C) Elastase were determined in the ERTB horse population. Unpaired t-test with Welch's correction ($n = 21$). $^{**}p < 0.005$, $^{****}p = 0.0001$

Supplementary Material 2: Supplemental Fig.2. Correlations between normalized MARCKS levels and BALF cells in Population A. Correlation between normalized MARCKS levels and BALF % neutrophils (A), % mast cells (B), % eosinophils (C), % macrophages (D), and % lymphocytes (E). Correlation between normalized MARCKS levels and absolute BALF counts

of neutrophils (F), lymphocytes (G), macrophages (H), mast cells (I), and eosinophils (J). No significant correlation was found in between normalized MARCKS levels and the BALF cytology results. p values were calculated with Pearson's correlation coefficient analysis. ***n*** = 18

Supplementary Material 3: Supplemental Fig.3. Correlations between normalized MARCKS levels and BALF cells in Population B. Correlation between normalized MARCKS levels and BALF % neutrophils (A), % mast cells (B), % eosinophils (C), % macrophages (D), and % lymphocytes (E). Correlation between normalized MARCKS levels and absolute BALF counts of neutrophils (F), macrophages (G), mast cells (H), lymphocytes (I) and eosinophils (J). Significant positive and negative correlations are found between % BALF neutrophils and macrophages and normalized MARCKS levels, respectively. p values were calculated with Pearson's correlation coefficient analysis. ***n*** = 24

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Author contributions
Study design– K.U.D., K.B.A., M.K.S. Acquisition, analysis, and interpretation of data– H.E.C., K.U.D., J.P.L, M.K.S. Manuscript drafting and revision– H.E.C., K.U.D., K.B.A., J.P.L. M.K.S.

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Data availability
Any data generated or analyzed during this study that are not included in this published article [and its supplementary information files] are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Procedures and protocols for population A were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC 16-0740O and 19–779). Samples for Population B were obtained from the Equine Respiratory Tissue Bank (ERTB) (Lavoie laboratory).

Consent for publication
Not applicable.

Competing interests
HC - The author declares that they have no competing interests. KD - The author declares that they have no competing interests. KA - Patents planned, issued or pending: Biomarck Corporation, Durham, NC. METHODS FOR REGULATING INFLAMMATORY MEDIATORS AND PEPTIDES USEFUL THEREIN. Inventors: Kenneth Adler, Suji Takashi, Indu Parikh, Linda Martin, and Yuehuva Li.Assignment: BioMarck and NC State University. Filed: 6/4/2009 US Patent: 8,563,689; Granted: October 22, 2013. KD - The author declares that they have no competing interests. MKS - The author declares that they have no competing interests.

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