A Myristoylated Alanine-Rich C Kinase Substrate–Related Peptide Suppresses Cytokine mRNA and Protein Expression in LPS-Activated Canine Neutrophils

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Myristoylated alanine-rich C kinase substrate (MARCKS) is a ubiquitously expressed protein kinase C substrate that has emerged as a potential therapeutic target for the amelioration of mucin secretion and inflammation in patients with chronic obstructive pulmonary disease. MARCKS also plays a key role in regulating the adhesion, migration, and degranulation of neutrophils. Moreover, given its biological role in epithelial and immune cells, we hypothesized that MARCKS may play an integral role in cytokine secretion by neutrophils. Because the amino terminus of MARCKS is highly conserved across vertebrate species, we successfully applied the well-characterized human MARCKS inhibitory peptide, myristoylated N-terminal sequence (MANS), to attenuate the function of MARCKS in isolated canine neutrophils. Pretreatment of canine neutrophils with MANS peptide significantly reduced both mRNA and protein expression in a broad range of LPS-induced cytokines, including IL-8, a chemokine (C-X-C motif) ligand-1 orthologue, and TNF- α , in comparison with untreated cells or those treated with a control peptide. This reduction in cytokine expression was observed even when neutrophils were treated with MANS 2 hours after LPS exposure. The observed reduction in cytokine secretion was not attributable to protein retention or cell death, but was associated with reduced cytokine transcript synthesis. These observations identify MARCKS protein as a promising therapeutic target in the treatment of inflammatory diseases or syndromes attributed to neutrophil influx and inflammatory cytokine production, such as sepsis, acute lung injury, and acute respiratory distress syndrome.

Keywords: MARCKS; cytokine; inflammation; neutrophil

Neutrophils are “professional” phagocytic cells that provide the host with a first line of defense against acute bacterial and fungal infection. After recruitment to a site of infection, neutrophils adhere to the capillary endothelium, migrate through vessel walls and interstitial tissues, and then phagocytose, kill, and destroy invading microorganisms, using a wide array of

CLINICAL RELEVANCE

Our observations identify the myristoylated alanine-rich C kinase substrate protein as a promising therapeutic target in the treatment of inflammatory diseases or syndromes attributed to neutrophil influx and inflammatory cytokine production, such as sepsis, acute lung injury, and acute respiratory distress syndrome.

proinflammatory mediators and proteolytic enzymes. During this series of events, neutrophils damage normal tissue. Prolonged or exacerbated neutrophil recruitment and activation causes excessive and often irreversible tissue damage that has been directly linked to patient morbidity and mortality in acute lung injury (ALI), acute respiratory distress syndrome (ARDS), septicemia with multiorgan failure, and ischemia-reperfusion (1). Mortality rates in these syndromes range from 40 to 70%, and are predominantly attributed to irreversible systemic tissue damage. The identification of molecular mechanisms defining neutrophil migration and cytokine release is paramount in the treatment of patients with these acute inflammatory syndromes.

Recent observations suggest that the myristoylated alanine-rich C kinase substrate (MARCKS) protein plays a critical role in neutrophil function, and thus may offer a therapeutic target for the treatment of neutrophil-associated pathology. MARCKS is a ubiquitously expressed protein kinase C (PKC) substrate that mediates a variety of cellular functions, including cell migration, adhesion, and cytoskeletal reorganization (2). The inhibition of MARCKS with an experimental N-terminus mimic peptide, named the myristoylated N-terminal sequence (MANS) peptide, reduces both the adhesion and migration of human neutrophils (3). Further, in a murine model of neutrophil elastase-induced bronchitis, the inhibition of MARCKS with the MANS peptide reduced both neutrophil influx into the lung as well as chemokine (C-X-C motif) ligand-1 (CXCL1), IL-1 β , IL-6, macrophage chemotactic protein-1 (MCP-1), and TNF- α concentrations in bronchoalveolar lavage (BAL) fluid (4). The underlying cause of cytokine reduction in this study was undefined, and was hypothesized to be attributable to either the reduction of cytokine-producing neutrophils or a direct effect of MARCKS inhibition on the secretion of cytokines by resident stromal or immune cells.

To elucidate whether MARCKS may be directly involved in cytokine secretion and production, we evaluated the effects of MARCKS inhibition on LPS-induced cytokine production from isolated neutrophils. MARCKS protein is highly conserved between vertebrate species, and importantly, the neutralization-sensitive N-terminal sequence of the protein (from which the MANS peptide is designed) is nearly identical between species,

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allowing the use of multiple animal models for evaluating the function of this protein. In this study we chose to use the dog as a relevant large animal “model” for the study of inflammation. The rationale for using dogs in this study was multifold. Clearly, important reasons included the availability of a large quantity of highly purified neutrophils (5) and the potential for the use of clinically ill veterinary patients in subsequent studies if the inhibition of MARCKS was identified as a viable therapeutic option to treat inflammatory disease, but even more relevant is the thinking that naturally occurring animal diseases in veterinary species could represent far better translational models of human disease than induced diseases in mice (6, 7) for both therapeutic evaluation and use in therapeutic discovery attempts.

Using isolated canine neutrophils, we demonstrate here, for the first time, that the inhibition of MARCKS with MANS peptide reduces the secretion of a broad range of LPS-induced proinflammatory cytokines, including IL-8, a CXCL1 orthologue, and TNF- α . Importantly, this reduction is maintained even when the inhibitory peptide is administered 2 hours after the initiation of LPS-induced inflammation. Finally, we demonstrate that the inhibition of cytokine secretion by MANS is not attributable to cytokine retention or cell death, but is the result of reduced cytokine protein production associated with reduced cytokine transcript levels.

MATERIALS AND METHODS

Bioinformatics and Phylogenetics

Sequence alignments were generated with ClustalW (8). For phylogenetic analyses, members of the MARCKS and MARCKS-related protein families were aligned by ClustalW (<http://www.ch.embnet.org/software/ClustalW.html>), and neighbor-joining trees were constructed from pairwise Poisson correction distances with 2,000 bootstrap replications via MEGA4 software (9). MARCKS sequences were acquired from GenBank and included the following Accession numbers: human (NP_002347), dog (XP_855257), mouse (NP_032564), *Xenopus* (NP_001080075), and chicken (NP_990811). MARCKS-related protein family members were similarly acquired from GenBank and included the following Accession numbers: human (NP_075385), dog (XP_854637), mouse (NP_034937), and chicken (NP_001074187).

Preparation of Canine Neutrophils

All experiments involving dogs were performed in accordance with relevant institutional and national guidelines and regulations, and were approved by the Institutional Animal Care and Use Committee of North Carolina State University. Peripheral blood samples were collected from healthy dogs via jugular venipuncture into Vacutainer tubes containing acid citrate dextrose (ACD; Becton-Dickinson, Franklin Lakes, NJ). Canine neutrophils were prepared as previously described (5). In brief, whole blood was centrifuged over endotoxin-tested Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) at $400 \times g$ for 25 minutes, and neutrophils were harvested from the bottom of the tube below the Histopaque gradient. Erythrocytes were lysed with ammonium chloride lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA), and cells were washed twice in endotoxin-free PBS (Gibco, Carlsbad, CA). The purity of neutrophils was determined by the microscopic examination of stained cytospin preparations and by flow cytometry, using a monoclonal antibody specifically against canine neutrophils (catalogue number CADO48A; VMRD, Inc., Pullman, WA). The purity of canine neutrophils was greater than 98%.

Western Blotting

Untreated and Phorbol 12-myristate 13-acetate (PMA)-treated canine neutrophils were lysed in RIPA lysis buffer (Pierce, Rockford, IL) containing a cocktail of protease inhibitors (1 mM iodoacetamide, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 100 $\mu\text{g}/\text{ml}$ pepstatin A, 1 mM phenylmethanesulfonyl fluoride, and 5 mM diisopropylfluorophosphate; Sigma-

Aldrich, St. Louis, MO) on ice for 30 minutes. Lysates were centrifuged at $12,000 \times g$ for 10 minutes at 4°C . Supernatants were recovered and treated with $5 \times$ reducing sample buffer (Pierce, Rockford, IL) at 95°C for 5 minutes. The lysates were then subjected to SDS-PAGE (NuPAGE 4–12% Bis-Tris Gel; Invitrogen, Carlsbad, CA), and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% milk-Tris-buffered saline with 0.05% Tween 20 (TBST), membranes were incubated with goat anti-human-MARCKS antibody N-19 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse-phospho-MARCKS (Ser152/156) antibody (Cell Signaling Technology, Danvers, MA) at 4°C overnight. After five consecutive 5-minute washes, membranes were incubated in horseradish peroxidase (HRP)-labeled secondary antibodies for 1 hour. Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used for signal detection.

Peptides

The cell-permeant MARCKS myristoylated N-terminus mimic peptide (MANS peptide) and the scrambled missense control peptide (RNS peptide) were synthesized by PolyPeptide Laboratories (San Diego, CA). The sequence of the MANS peptide is identical to the first 24 amino acids of human MARCKS (i.e., MA-GAQFSKT-AAKGEEAAERPGEEAAVA, where MA = N-terminal myristate chain). The control RNS peptide has the same amino acid composition as the MANS peptide, but is arranged in a random order (i.e., MA-GTAPAAEGAGAEVKRASAEAKQAF).

Treatment of Canine Neutrophils with the MANS Peptide

Freshly isolated canine neutrophils were seeded at a density of 2×10^5 cells/well in 96-well plates and cultured in RPMI-1640 supplemented with endotoxin-tested 10% FBS (Gibco), 1 mM sodium pyruvate, 15 mM HEPES, 55 μM 2-mercaptoethanol, 10 IU/ml penicillin, and 10 $\mu\text{g}/\text{ml}$ streptomycin. To enhance cell viability, 30 pg/ml of recombinant canine granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN) were added to the culture medium, as previously described (5). Cells were treated with the MANS or RNS peptide either before or after stimulation with LPS, and assessed for cytokine and chemokine production, as will be described.

To evaluate the effects of pretreating cells with MANS before LPS exposure, neutrophils rested in culture medium for 30 minutes, were incubated with the MANS or RNS peptide for 30 minutes, and were treated with crudely purified LPS from *Escherichia coli* strain O111:B4 (which contains agonists for Toll-like receptor [TLR] 2, TLR4, and TLR9 (10); catalogue number L3024; Sigma-Aldrich) for 18 hours (see Figure E1A in the online supplement). Cell culture supernatants were harvested and evaluated for cytokine and chemokines production, as will be described.

To evaluate the effects of treating cells with MANS after LPS exposure, neutrophils rested for 1 hour, were incubated with crudely purified LPS for 2 hours, and were treated with the MANS or RNS peptide for 16 hours (Figure E1B). Cell culture supernatants were harvested and evaluated for cytokines and chemokines, as will be described.

To evaluate the effects of MANS treatment on cytokine retention and production, canine neutrophils were prepared as already described, except that cells were treated with Brefeldin A solution (eBioscience, San Diego, CA) to facilitate cytokine retention, and cells were harvested from culture 16 hours after treatment (Figures E1C and E1D). Intracellular IL-8 production was evaluated via flow cytometry.

Measurement of Cytokines and Chemokines

Cell culture supernatants were harvested and evaluated for the production of IL-8, MCP-1, GM-CSF, TNF- α , and a canine orthologue of CXCL1 with a Milliplex Map canine cytokine/chemokine kit (Millipore), according to the manufacturer's instructions. The dynamic ranges (i.e., upper and lower limits of detection) for cytokines were 8,617.65 and 63.90 pg/ml for IL-8, 52,164.38 and 2.91 pg/ml for the CXCL1 orthologue, 42,466.72 and 3.24 pg/ml for TNF- α , and 7,972.61 and 64.59 pg/ml for MCP-1.

Flow Cytometry

Neutrophils were permeabilized using BD Cytofix/Cytoperm Fixation/Permeabilization Solution (BD Biosciences, Mountain View, CA),

according to the manufacturer's instructions. After permeabilization, cells were incubated with biotinylated anti-canine IL-8 monoclonal antibody (clone number 258911; R&D Systems) in $1 \times$ Perm/Wash buffer on ice for 30 minutes. Cells were then washed with $1 \times$ Perm/Wash buffer and incubated with Streptavidin-PerCP (Biolegend, San Diego, CA) for 15 minutes on ice. Anti-human-CD25 monoclonal antibody BC96 (Biolegend) was used as an isotype control for intracellular staining. Cell death was evaluated by Pacific-Blue Labeled Annexin V (Biolegend), according to the manufacturer's instructions. Cell staining was analyzed on an LSR II flow cytometer (BD Biosciences, San Jose, CA), using FACSDiva software (BD Biosciences).

RNA Isolation and Real-Time RT-PCR

Canine neutrophils rested in medium for 1 hour were pretreated with MANS peptide or RNS peptide (100 μ M) for 30 minutes, and were exposed to crudely purified LPS from *Escherichia coli* (O111:B4, 100 ng/ml) for 4 hours. RNA was extracted from treated cells (ZR Whole-Blood RNA Miniprep; Zymo Research, Irvine, CA), and cDNA was synthesized (RT2 First Strand Kit; SABiosciences, Frederick, MD). Transcript levels were quantified using real-time PCR with SYBR Green qPCR Master Mix (SABiosciences). Primer pairs were used to amplify canine IL-8 (ACTTCCAAGCTGGCTGTTGC and GGCCACTGTCAATCACTCTC), TNF- α (CCAAGTGACAAGCCA GTAGC and TCTTGATGGCAGAGAGTAGG), and β -actin (GACCCTGAAGTACCCCATTTGAG and TTGTAGAAGGTGTGGT GCCAGAT). Thermal cycling conditions involved 95°C for 10 seconds and 63°C for 30 seconds, for a total of 50 cycles. IL-8 and TNF- α mRNA concentrations were normalized to β -actin concentrations, and fold changes in expression levels were determined using the $2^{-\Delta\Delta CT}$ method (11).

Statistical Analysis

Treatment group and peptide dosage effects were analyzed according to one-way ANOVA with a Student-Newman-Keuls *post hoc* test, using Prism 5 (GraphPad Software, San Diego, CA). In cases where cytokine concentrations were below the assay lower limit of detection (LLOD), values were assigned that were one half of the LLOD (as already defined) for the purposes of statistical analysis (12–17). The level of significance used was $P \leq 0.05$. Error bars represent the SEM.

RESULTS

The Functional Domains of MARCKS Protein Are Highly Homologous across Species

A comparison of MARCKS protein sequence across dog, human, and mouse revealed three evolutionarily conserved domains: (1) the N-terminal myristoylated domain, (2) the multiple homology 2 (MH-2) domain, and (3) the phosphorylation site (PSD) domain (Figure 1A) (2). Canine MARCKS shares 66.3% identity and 72.4% similarity with the human MARCKS protein. To confirm that the predicted canine MARCKS is indeed orthologous to human MARCKS, a phylogenetic comparison was performed between MARCKS and the MARCKS-related protein from dogs, humans, and other species. Phylogenetic analysis revealed that the predicted canine MARCKS sequence belongs to MARCKS, and not to the MARCKS-related protein family (Figure 1B). These observations support the classification of the predicted sequence as canine MARCKS.

MARCKS is expressed in human neutrophils (3, 18), and antibodies that recognize human MARCKS have been shown to recognize MARCKS in the Madin-Darby canine kidney (MDCK) epithelial cell line according to Western blot analysis (19). Based on sequence analysis, the N-terminus of canine MARCKS differs by a single conserved residue (E²¹ in humans, and D²¹ in dogs). MARCKS expression in primary canine neutrophils was demonstrated by Western blot analysis with an antibody against the amino terminus of human MARCKS (Figure 1C). A protein of

the predicted molecular weight (MW; ~ 68 kD) was observed in canine neutrophils, and was of similar MW to that detected in human neutrophils. This observation and the identification of two canine expressed sequence tags (EST) verifying the amino-terminus sequence of canine MARCKS (GenBank DN354260.1 and DN416117.1) suggest that the N-terminal region of MARCKS is nearly identical between humans and dogs at the DNA and protein levels.

The PSD domain of canine MARCKS was similarly probed using an anti-human phospho-MARCKS antibody. Neither human nor canine neutrophils exhibited detectable concentrations of phospho-MARCKS in the absence of stimulation, but neutrophils from both species rapidly phosphorylated MARCKS upon stimulation with PMA or LPS (Figure 1D). Neither the MANS nor the RNS peptide affected the PMA-induced or LPS-induced phosphorylation of MARCKS (data not shown).

Pretreatment of Neutrophils with the MANS Peptide Significantly Reduces the Secretion of Several LPS-Induced Proinflammatory Cytokines

The high level of homology between the N-terminus of human and canine MARCKS justified our use of the well-characterized MARCKS inhibitory peptide MANS (3, 4, 20, 21) in canine cells. The cell-permeant MANS peptide is identical to the amino terminus of human MARCKS, and has previously been shown to inhibit MARCKS-associated neutrophil degranulation (21) and airway mucin secretion (4, 21). To elucidate whether MARCKS is directly implicated in cytokine secretion, purified canine neutrophils were pretreated with a titrating dose of the MANS peptide or control RNS peptide before activation with LPS. Neither the MANS peptide nor the RNS peptide affected baseline cytokine secretion from purified neutrophils (Figure E2). Neutrophils secreted minimal amounts of cytokines in the absence of stimulation, but after activation by LPS, secreted high concentrations of multiple proinflammatory cytokines, including the CXCL1 orthologue, IL-8, TNF- α , and MCP-1. The treatment of neutrophils with 100 μ M MANS before the addition of LPS significantly reduced the secretion of the CXCL1 orthologue, IL-8, and TNF- α , compared with neutrophils treated with an equivalent dose of the control RNS peptide (Figures 2A–2C). Although the pretreatment of neutrophils with the MANS peptide trended toward lowering MCP-1 concentrations, differences were not significant between treatment groups (Figure 2D).

The MANS Peptide Maintains Its Ability to Diminish Cytokine Secretion When Administered 2 Hours after LPS Activation

To determine whether MARCKS inhibition remains effective in reducing cytokine secretion during ongoing inflammation, titrating doses of the MANS or RNS peptide were added to neutrophil cultures 2 hours after the addition of LPS. The secretion of the CXCL1 orthologue, IL-8, and TNF- α into cellular supernatants of LPS-pretreated neutrophils was again significantly lower in cells treated with 100 μ M of the MANS peptide, compared with supernatants of cells treated with the RNS peptide (Figures 3A–3C). Similar to observations involving the pretreatment of neutrophils with peptides, the addition of MANS peptide to LPS-pretreated neutrophils did not significantly diminish the secretion of MCP-1 (Figure 3D).

MARCKS Inhibition Reduces the Production (Rather Than Release) of Neutrophil Cytokines

As previously mentioned, MARCKS is known to play an integral role in cell motility and migration (3). Mechanistically, the effect

Figure 1. Myristoylated alanine-rich C kinase substrate (MARCKS) and phospho-MARCKS are detectable in canine neutrophils. PMA or LPS exposure provokes MARCKS phosphorylation. (A) The functional domains of MARCKS protein are highly conserved. A protein alignment of the predicted canine MARCKS with human and murine MARCKS is shown. Identities (exact match) are in *black*, and similarities are in *gray*. Regions corresponding to the amino-terminus, multiple homology-2 domain (MH-2) and the phosphorylation site domain (PSD) are indicated above the alignment. (B) Phylogenetic comparison of the predicted canine MARCKS protein to members of the MARCKS and MARCKS-related protein families. Branch lengths are measured in terms of amino-acid substitutions, with the scale indicated below the tree. (C and D) Anti-human MARCKS antibodies detect human and canine MARCKS. (C) Equivalent total proteins from canine neutrophils (lane 1) and human neutrophils (lane 2) were electrophoresed and subjected to Western blot analyses with an antibody against the N-terminus of human MARCKS. (D) Equivalent total proteins from untreated canine neutrophils (lane 1), canine neutrophils treated with PMA for 20 minutes (lane 2), untreated human neutrophils (lane 3), human neutrophils treated with PMA (100 nM; lane 4), untreated canine neutrophils (lane 5), and canine neutrophils treated with LPS (100 ng/ml) for 20 minutes (lane 6) were subjected to Western blot analyses, using an antibody against human phospho-MARCKS (Ser^{152/156}). Western blots are representative of cells from four dogs, and show the results of two separate studies merged into a single image, as indicated by the *solid vertical line* between lanes 4 and 5.

of MARCKS on cellular migration is likely exerted at the level of cytoskeletal rearrangement, because MARCKS has been shown to associate with actin as it recycles on and off the plasma membrane (2). Because cytoskeletal function is without doubt involved in the expulsion of cytokines from neutrophils, we suspected that the disruption of MARCKS by the MANS peptide was likely decreasing cytokine secretion by enhancing the retention of cytokines within cells. To evaluate cytokine retention, LPS-induced IL-8 was measured by intracellular flow cytometry in the presence or absence of the MANS or RNS peptide. Surprisingly, intracellular IL-8 concentrations were significantly lower in cells treated with 100 μ M MANS peptide before LPS exposure, in comparison with those pretreated with the control RNS peptide (*bar graph* in Figure 4A, *flow histograms* in Figure E3). The MANS peptide was also able to reduce IL-8 production significantly after 2 hours of LPS pretreatment (Figure 4B). The treatment of neutrophils with the MANS peptide slightly reduced the percentage of annexin V⁺

neutrophils, thereby ruling out the induction of cell death as an explanation for the reduced concentrations of intracellular cytokines (Figure E4). Collectively, these data suggest that the reduction of cytokines from MANS peptide-treated canine neutrophils cannot be attributed to an increased retention of cytokines, but rather is associated with decreased protein production.

The MANS Peptide Reduces LPS-Induced Cytokine mRNA Synthesis in Neutrophils

To determine whether MANS peptide-induced reduction in cytokine production was associated with changes in mRNA transcript levels, we measured IL-8 and TNF- α mRNA concentrations in MANS and RNS peptide-pretreated canine neutrophils. MANS peptide pretreatment at a concentration of 100 μ M significantly reduced LPS-induced IL-8 (Figure 5A) and TNF- α

Figure 2. Treatment of neutrophils with the MARCKS-inhibiting myristoylated N-terminal sequence (MANS) peptide before LPS exposure significantly reduces the secretion of several cytokines. Canine neutrophils were treated with titrating doses of the MARCKS inhibitory peptide MANS or the control peptide RNS for 30 minutes. Cells were then treated with 100 ng/ml *Escherichia coli* LPS (O111:B4) for 18 hours. Cell culture supernatants were harvested and analyzed for concentrations of (A) a chemokine (C-X-C motif) ligand-1 (CXCL1) orthologue, (B) IL-8, (C) TNF-α, and (D) macrophage chemotactic protein-1 (MCP-1). The release of all cytokines except MCP-1 was significantly attenuated by the MANS but not the RNS peptide. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, $n = 6$ (cells derived from six different dogs).

(Figure 5B) transcript levels, compared with both RNS control peptide or no peptide treatment control.

DISCUSSION

This study sought to determine whether the inhibition of MARCKS function exerts a direct effect on cytokine production and secretion by canine neutrophils after LPS-induced activation. We observed that the inhibition of MARCKS protein with the MANS

peptide resulted in a significant reduction in secretion of a broad range of LPS-induced proinflammatory cytokines from isolated neutrophils. Importantly, the MANS-induced reduction of cytokine secretion remained achievable 2 hours after the onset of LPS-induced inflammation. The reduction of cytokine secretion was not attributable to cytokine retention or cell death, but is likely the result of reduced cytokine production, because a concomitant reduction in cytokine transcript levels was observed.

Figure 3. When administered 2 hours after LPS-induced activation, the MARCKS-inhibiting MANS peptide reduces cytokine secretion. Canine neutrophils were treated with 100 ng/ml *Escherichia coli* LPS (O111:B4) for 2 hours, and then treated with titrating doses of MANS peptide or RNS control peptide for 16 hours. Cell culture supernatants were harvested and evaluated for secreted (A) CXCL1 orthologue, (B) IL-8, (C) TNF-α, and (D) MCP-1. The MANS peptide but not the RNS peptide significantly attenuated the release of all cytokines except MCP-1. * $P < 0.05$ and *** $P < 0.001$, $n = 6$ (cells derived from six different dogs).

Figure 4. The MANS peptide reduces intracellular IL-8 protein. Neutrophils were treated with titrating doses of MANS or RNS peptide either (A) before or (B) 2 hours after LPS stimulation. Intracellular IL-8 mean fluorescence intensity (MFI) was determined by flow cytometry. MANS, but not RNS, significantly decreased IL-8 within the cells. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, $n = 5$ (cells derived from five different dogs).

Because neutrophil cytokine secretion is a major cause of tissue damage in multiple acute inflammatory diseases, a more thorough understanding of the mechanism of cytokine production is vital for the identification of novel therapeutic targets. Previous studies identified MARCKS as a putative therapeutic target in the treatment of chronic obstructive pulmonary disease (COPD) and chronic bronchitis because of its ability to decrease mucin secretion. Interestingly, in these experiments, MARCKS inhibition was associated with a reduction in proinflammatory cytokines. Foster and colleagues observed a 50–60% reduction in CXCL1, IL-1 β , IL-6, MCP-1, and TNF- α in BAL fluid after administration of the MANS peptide in a neutrophil elastase-induced murine bronchitis model (4). A significant reduction of neutrophil migration into inflamed lungs was also observed, and whether the reduction of cytokines in BAL fluid was attributable to the absence of cells, or was a direct effect of MARCKS inhibition on cytokine production, remained unclear. To determine whether MARCKS inhibition exerts a direct impact on cytokine production, we used isolated canine neutrophils and a previously characterized LPS model of induced inflammation (5).

Dogs were chosen as the source of neutrophils because of the availability of a large quantity of highly purified neutrophils (5) and the potential for the use of clinically ill veterinary patients with acute lung disease in subsequent studies if the inhibition of MARCKS was identified as a viable therapeutic option for treating inflammatory lung disease. Naturally occurring disease

Figure 5. The MANS peptide reduces specific cytokine mRNA synthesis. Neutrophils were treated with titrating doses of MANS and RNS peptide 30 minutes before LPS activation. (A) IL-8 and (B) TNF- α mRNA synthesis was quantified by real-time PCR 4 hours after LPS activation. MANS, but not RNS, significantly decreased the mRNA synthesis of both cytokines. * $P < 0.05$, $n = 8$ (cells derived from eight different dogs).

in veterinary species represents a novel approach to therapeutic discovery, because there is a growing recognition that induced animal models of disease (e.g., murine models) can result in an oversimplification of disease pathogenesis and thus provide limited utility in therapeutic evaluations (6, 7). Further, regulatory barriers to the *in vivo* use of investigational therapeutics in veterinary patients are less extensive than in humans, making the use of these patients in therapeutic discovery a valuable resource to evaluate relevant clinical outcomes in affected patients. For these reasons, canine neutrophils were selected as the model of choice in these studies.

Through sequence and phylogenetic analyses, we found that the three critical regions of the MARCKS protein (the N-terminus, the MH-2 domain, and the PSD domain) are highly homologous between humans and dogs. We verified by Western blotting that beyond gene sequence homology, the N-terminus and the PSD domain at the protein level were also highly similar. Based on the known function of these domains in human MARCKS, we think that canine MARCKS likely associates with the plasma membrane, and functions as a substrate for PKC or as a binding site for calcium/calmodulin (2).

The role of MARCKS protein as a PKC substrate and binding site for calcium/calmodulin firmly places this protein in the signal transduction pathways known to be essential for inflammatory

cytokine production (22). To evaluate whether MARCKS plays a direct role in cytokine secretion and production, we assessed the ability of the MANS peptide to alter cytokine secretion before and after the treatment of neutrophils with LPS. We found that both the pretreatment and post-treatment of neutrophils with the MANS peptide reduced the LPS-induced secretion of IL-8, the CXCL1 orthologue, and TNF- α . MCP-1 concentrations tended to be decreased in the presence of the MANS peptide, but were not significantly reduced compared with control samples. Previous experiments in our laboratory found canine MCP-1 production to be mechanistically uncoupled from factors regulating IL-8, CXCL1 orthologue, and TNF- α production (5). Because IL-8 and the CXCL1 orthologue are used by neutrophils in an autocrine or paracrine manner (23–25), their production and secretion may be tightly regulated by multiple signaling pathways (including the triggering receptor expressed on myeloid cells-1 [TREM-1]-mediated pathway and the MARCKS-mediated pathway) in neutrophils. In contrast, because MCP-1 is a chemoattractant for monocytes and T cells (26, 27), its production and expulsion by neutrophils may be primarily regulated by early TLR signaling. Collectively, these data suggest alternate pathways of cytokine regulation in canine neutrophils, namely, one that may be independent of MARCKS protein function, and another that is dependent on MARCKS protein function.

MARCKS protein clearly plays a substantial role in neutrophil activation, because the protein constitutes nearly 90% of newly synthesized protein in neutrophils after LPS stimulation (18). This dramatic increase in MARCKS synthesis suggests its utility as a PKC substrate, and its association with cytoskeletal and chaperone proteins is integral to neutrophil function during infection with Gram-negative pathogens (18). Recent data suggest that MARCKS may directly associate with LPS after it is internalized by TLR4, thus playing a role in signal transduction or the regulation of LPS-induced responses (28). Interestingly, in this study by Mancek-Keber and coworkers, the inhibition of MARCKS through the “effector domain” (equivalent to the PSD domain in this study) in MonoMac6 cells with a peptide inhibitor resulted in a decrease in TNF- α secretion, whereas the negation of MARCKS protein production through small interfering (si)RNA in TLR4/MD2-expressing human epithelial kidney (HEK)293 cells exerted the opposite effect, significantly increasing cytokine production (28). Although these data suggest that a role may exist for spatial-temporal MARCKS trafficking in the regulation of cytokine production, the use of different cell lines in these experiments ultimately makes the interpretation of data difficult. Because primary neutrophils are cells that are terminally differentiated and relatively short-lived, gene-silencing approaches using siRNA were not attempted in the present study. It is important to point out that the MANS peptide is specific for MARCKS, because it represents the 24 amino-acid N-terminus of MARCKS. Moreover, the finding that the MANS peptide is effective in attenuating MARCKS function, as opposed to the missense RNS peptide, precludes the nonspecific peptide effects that might occur with a more generalized inhibitor. Thus, our data affirm that interference with MARCKS function markedly reduces cytokine secretion, and extends the role of MARCKS protein beyond neutrophil degranulation (21) and adhesion/migration (3, 4, 29) into cytokine production and the regulation of mRNA transcript synthesis.

Two obvious questions result from the outcomes of our study. The first question relates to where MARCKS exerts its effect in the signaling cascades that define proinflammatory cytokine transcription. Cytokine-mediated inflammation is known to be initiated when highly conserved pathogen-associated molecular patterns, such as LPS, engage the TLRs (30). TLR activation initiates a signal transduction cascade that culminates in NF- κ B

activation and the initiation of cytokine production. TLR activation is the priming event for innate immune cells, whereas the autocrine/paracrine activation of cytokine receptors or the activation of additional immunoreceptor tyrosine-based activation motifs-containing receptors (e.g., Fc Receptor γ and TREM-1) amplifies and perpetuates inflammation (30). In the context of these well-established signaling events, we speculate a plausible role for MARCKS in cytokine production, either in the initial priming event involving NF- κ B, or in signal perpetuation through phospholipase C γ , protein kinase C δ (PKC δ), and the mitogen-activated protein kinase (MAPK) pathway. Cross-talk between NF- κ B and PKC δ is well established (31, 32), and defining the role of MARCKS in proinflammatory cytokine production will likely be confounded by its potential for multiple insertion points in signaling cascades. Despite these clear challenges, the highly relevant concept remains that MARCKS may represent a pivot point whereby inflammation is broadly expanded in its magnitude and duration, making MARCKS a potential therapeutic target for fine-tuning the immune response. Studies are ongoing to address the potential insertion points of MARCKS in signaling cascades leading to cytokine production.

The second question involves elucidating the mechanism of action of the MANS peptide. As a peptide mimic of the N-terminus of MARCKS, the MANS peptide is likely to engage in and disrupt protein–protein interactions that underpin key signal transduction pathways for the cytokine production mediated by the MARCKS protein. Two potential points of intervention have previously been proposed. Eckert and colleagues suggested that the MANS peptide functions as a dominant negative peptide, occupying specific MARCKS binding sites on the plasma membrane, and thereby preventing MARCKS from reassociating with the membrane after phosphorylation (3). In support of this concept, that group showed that the treatment of resting human neutrophils with the MANS peptide resulted in a translocation of MARCKS from the membrane to the cytosol (3). In an alternate hypothesis, Singer and colleagues (20) and Li and colleagues (33) proposed that the inhibition of MARCKS by the MANS peptide is mediated at the level of the cytoskeleton. This is also plausible, given that cytoskeletal proteins regulate lipid raft formation and facilitate the assembly of signaling complexes. Defining the protein–protein interactions of the MANS peptide, and thus identifying which signal transduction pathways are disrupted, will substantially increase our understanding of effective ways to reduce cytokine-mediated inflammation (34).

In conclusion, we show that the disruption of MARCKS function by the MANS peptide inhibits the production of a broad range of cytokines in LPS-activated canine neutrophils, and this reduction of the cytokine “storm” remains possible hours after inflammation is initiated. MARCKS is well-established as a viable therapeutic target for the mucin-associated diseases COPD and cystic fibrosis. Given the newly identified role of MARCKS protein in neutrophil cytokine production, the use of MARCKS as a therapeutic target for acute neutrophil-mediated inflammatory diseases such as ALI, ARDS, septicemia, and ischemia–reperfusion warrants significant consideration.

Author disclosures are available with the text of this article at www.atsjournals.org.

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