Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS) Protein Regulation of Human Neutrophil Migration

Rachael E. Eckert¹, Laura E. Neuder¹, Joungjoa Park³, Kenneth B. Adler^{2,3}, and Samuel L. Jones^{1,3}

¹Department of Clinical Sciences, ²Department of Molecular Biomedical Sciences, and ³Center for Comparative Medicine and Translational Research, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina

Neutrophil migration into infected tissues is essential for host defense, but products of activated neutrophils can be quite damaging to host cells. Neutrophil influx into the lung and airways and resultant inflammation characterizes diseases such as chronic obstructive pulmonary disease, bronchiectasis, and cystic fibrosis. To migrate, neutrophils must reorganize the actin cytoskeleton to establish a leading edge pseudopod and a trailing edge uropod. The actin-binding protein myristoylated alanine-rich C-kinase substrate (MARCKS) has been shown to bind and cross-link actin in a variety of cell types and to co-localize with F-actin in the leading edge lamellipodium of migrating fibroblasts. The hypothesis that MARCKS has a role in the regulation of neutrophil migration was tested using a cell-permeant peptide derived from the MARCKS myristoylated aminoterminal (MANS peptide). Treatment of isolated human neutrophils with MANS significantly inhibited both their migration and $\beta 2$ integrin-dependent adhesion in response to N-formyl-methionyl-leucyl-phenylalanine (fMLF), IL-8, or leukotriene (LT)B₄. The IC₅₀ for fMLF-induced migration and adhesion was 17.1 μ M and 12.5 μ M, respectively. MANS significantly reduced the F-actin content in neutrophils 30 seconds after fMLF stimulation, although the peptide did not alter the ability of cells to polarize or spread. MANS did not alter fMLF-induced increases in surface $\beta 2$ integrin expression. These results suggest that MARCKS, via its myristoylated aminoterminal, is a key regulator of neutrophil migration and adhesion.

Keywords: inflammation; adhesion; $\beta 2$ integrin; actin

Neutrophils are first responders of the immune system, and rapidly migrate into developing sites of inflammation to phagocytose bacteria and clear tissue debris. Although required in tissues for adequate host defense against a range of infectious pathogens, a major component of the clinical syndrome of inflammatory diseases is excessive neutrophilic inflammation. Influx of neutrophils into the lung and airways characterizes and contributes to inflammation associated with chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis, and even asthma. Further elucidation of the molecular mechanisms involved in neutrophil migration may lead to development of therapeutics that could limit tissue damage caused by neutrophil-based inflammation.

Stimulation of chemoattractant receptors in neutrophils activates numerous responses that require precise signaling and reorganization of the cytoskeleton, including adhesion and

CLINICAL RELEVANCE

We present the novel finding that myristoylated alanine-rich C-kinase substrate (MARCKS) protein is essential for neutrophil migration and adhesion, and that the MARCKS aminoterminal regulates this function. Our findings represent a potentially new therapeutic strategy to treat pulmonary inflammation and inflammatory diseases of other organs or systems.

migration (1–7). Multiple signaling pathways coordinate to direct neutrophil migration and enable activation at appropriate sites. One pathway that promotes migration is mediated by protein kinase C (PKC). PKC is a serine-threonine kinase involved in many cytoskeletal-dependent processes of neutrophils, including actin assembly (8–11), degranulation (12), oxidative burst (13–15), adhesion (16, 17), and migration (17–20). Neutrophils contain a range of PKC isoforms, including the classical Ca²⁺-sensitive PKC α and PKC β , the novel Ca²⁺-independent PKC δ , and the atypical Ca²⁺- and diacylglycerol-independent PKC ζ (21). Through the use of specific inhibitors and activators, the PKC pathway has been shown to be instrumental in neutrophil migration (17–20), yet intracellular targets mediating the activities of PKC have yet to be elucidated.

One potential candidate is the actin-binding protein myristoylated alanine-rich C-kinase substrate (MARCKS). Initially membrane bound, MARCKS is rapidly phosphorylated by PKC after chemoattractant stimulation and translocates to the cytosol (22). MARCKS association with the plasma membrane can also be disrupted by binding of Ca²⁺-activated calmodulin, another signaling molecule shown to be required for neutrophil chemotaxis (23). These mutually exclusive pathways (24) direct the subcellular localization and presumably the involvement of MARCKS in regulation of the actin cytoskeleton. Although mechanisms involved in MARCKS-mediated cytoskeletal rearrangement are unknown, there is compelling evidence to suggest an important association. MARCKS has been shown to bind and cross-link actin in a variety of cell types (25–27), and peptides analogous to the effector domain of MARCKS are able to induce actin polymerization and bundle existing filaments (25, 28, 29). In fibroblasts, MARCKS co-localizes with F-actin in lamellipodia, suggesting a close relationship of MARCKS with the cytoskeletal machinery responsible for directed migration (30).

In this report, we hypothesized that MARCKS, via its myristoylated aminoterminal, could be an important regulator of neutrophil migration and/or adhesion. Using isolated human neutrophils, we demonstrate here, for the first time, that MARCKS is involved integrally in these neutrophil functions. Furthermore, it appears that the myristoylated aminoterminal of MARCKS is critical to these functions, as a peptide identical to the conserved 24-amino acid N-terminus of MARCKS is shown to inhibit neutrophil migration and adhesion.

(Received in original form October 15, 2008 and in final form May 29, 2009)

This work was supported by Grant # R37 HL36982 from the National Institutes of Health and by a Pilot Grant from the NCSU Center for Comparative Medicine and Translational Research.

Correspondence and requests for reprints should be addressed to Samuel L. Jones, D.V.M., Ph.D., CVM Research Building, Room 244, 4700 Hillsborough St., Raleigh, NC 27606. E-mail: sam_jones@ncsu.edu

Am J Respir Cell Mol Biol Vol 42, pp 586–594, 2010

Originally Published in Press as DOI: 10.1165/rcmb.2008-0394OC on July 2, 2009

Internet address: www.atsjournals.org

MATERIALS AND METHODS

Reagents

Ficoll-Paque Plus and Dextran T500 were from Amersham Biosciences (Piscataway, NJ). Dimethyl sulfoxide (Me₂SO), N-formyl-methionyl-leucyl-phenylalanine (fMLF), phorbol 12-myristate 13-acetate (PMA), Triton-X 100, pepstatin, HEPES, o-phenylenediamine (OPD) dihydrochloride, and poly-L-lysine were from Sigma Chemical Co. (St. Louis, MO). Powdered phosphate-buffered saline (PBS) and Hanks' balanced salt solution (HBSS) were from Life Technologies (Grand Island, NY). Ethylenediamine tetraacetate dihydrate (EDTA) was from Fisher Scientific (Atlanta, GA). Leukotriene B₄ (LTB₄) was from Cayman Chemical (Ann Arbor, MI). Fetal calf serum (FCS) was obtained from Hyclone (Logan, UT). Calcein, Alexa Fluor 546-phalloidin, Alexa Fluor 647-phalloidin, Rabbit anti-fluorescein isothiocyanate (anti-FITC) Ab, and Alexa Fluor 488-goat-anti-rabbit Ab were obtained from Molecular Probes (Invitrogen, Carlsbad, CA). Diisopropylfluorophosphate (DFP) and human recombinant IL-8 were from BD Biosciences (San Diego, CA). Monoclonal antibody IB4 (anti-β2, CD18) was prepared as described (31). Monoclonal anti-MARCKS and anti-tubulin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-labeled sheep-anti-mouse antibody was from eBioscience (San Diego, CA). Dako mounting medium was from Dako North America Inc. (Carpenteria, CA). Goat serum was from Jackson ImmunoResearch (West Grove, PA).

Peptides

Synthesis of the cell-permeant peptide derived from the MARCKS myristoylated aminoterminal (MANS) and RNS peptides was performed by Genemed Synthesis, Inc. (San Francisco, CA). The MANS peptide is identical to the first 24 amino acids of the MARCKS protein: myristic acid-GAQFSKTAACKGEAAERPGEAAVA. The RNS peptide is a randomly scrambled control: myristic acid-GTAPAAEGAGAEVKRASAEAKQAF. Both peptides have been previously described (27). Viability of neutrophils after peptide treatment was confirmed by trypan blue dye exclusion.

Preparation of Neutrophils

The NC State University Institutional Review Board (IRB) approved these studies. Human leukocyte-rich plasma was separated from whole blood using dextran sedimentation. Neutrophils were isolated from plasma using a Ficoll gradient. Approximately 6 ml of plasma was layered on 5 ml of sterile, endotoxin-free Ficoll-Paque solution and spun at 1,800 rpm for 20 minutes. Neutrophils were used if they demonstrated greater than 98% viability, as determined by exclusion of trypan blue dye incorporation. Red blood cells were lysed by hypotonic lysis, and remaining neutrophils were washed once with HBSS. Cells were resuspended in HBSS with 20 mM HEPES, 8.9 mM sodium bicarbonate, 1 mM Ca²⁺, and 1 mM Mg²⁺ before assays (HBSS⁺⁺). All data replicates were from separate donors.

Confocal Microscopy

Freshly isolated human neutrophils (0.5×10^6) in HBSS⁺⁺ with 1% bovine serum albumin (BSA) were pretreated with 25 μM FITC-MANS or FITC-RNS peptide for 30 minutes at 37°C. Cells from each treatment group were then added to glass coverslips in 24-well tissue culture plates. Cells were allowed to adhere to the coverslips before fixation with 0.5 ml 3% paraformaldehyde solution (25 mM PIPES, pH 7.0, 50 mM KCl, 3 mM MgCl₂, 3% paraformaldehyde wt/vol, 10 mM EGTA in sterile water) for 20 minutes. Coverslips were washed twice with sterile PBS (pH 7.4) and blocked with 10% goat serum in PBS (pH 7.4) with 0.05% Triton for 1 hour at room temperature (RT). The supernatant was removed and coverslips were incubated in primary antibody (1:200 rabbit-anti-FITC antibody) overnight at 4°C. Cells were washed again before staining with 1:200 Alexa Fluor 647-phalloidin and 1:500 Alexa Fluor 488-goat-anti-rabbit Ab diluted in PBS for 2 hours at RT. Coverslips were washed seven times for 5 minutes each with PBS (pH 7.4) before mounting with Dako mounting medium. Confocal imaging was conducted using a C1 Nikon confocal microscope at the Laboratory for Advanced Electron and Light Optical Methods (LAELOM) at the North Carolina State University, College of Veterinary Medicine.

Neutrophil Cell Fractionation

Neutrophils were suspended in HBSS⁺⁺ (25×10^6 cells/ml) and treated with MANS peptide (50 μM) or RNS peptide (50 μM) or no peptide at 37°C for 10 minutes. Vehicle (PBS) or PMA (50 ng/ml) were added to cells and incubated at 37°C for 5 minutes. After incubation, phosphatase inhibitor cocktail-1 (Sigma) was added to the cells. Cells were centrifuged at 1,000 rpm for 5 minutes at 4°C for collection, and subcellular fractionation was performed as described (32).

Cells were washed three times with ice-cold PBS, then lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and a protease inhibitor cocktail) was added. Cells were sonicated three times for 15 seconds and centrifuged at $500 \times g$ for 2 minutes to remove cell debris and nuclei. Supernatants were transferred to Eppendorf tubes and centrifuged at $20,000 \times g$ for 45 minutes at 4°C. The supernatants of the high-speed centrifugation were collected as the cytosolic fraction. Lysis buffer containing 1% Triton X-100 was added to the remaining pellets, and the mixture was sonicated briefly and centrifuged at $20,000 \times g$ for 30 minutes at 4°C. The supernatants resulting from the second high-speed centrifugation were collected as the membrane fraction. The protein concentration in each sample was measured with a Bio-Rad Protein assay (Bradford assay) following the manufacturer's instructions (Bio-Rad, Hercules, CA).

Samples containing extracted cytosol and membrane proteins were mixed with 2× sample buffer and boiled for 5 minutes. After flash spinning, samples containing 0.4 μg of total protein were loaded into wells and subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane using a Semi-dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's instructions. After transfer, the membrane was rinsed with PBS and blocked in 3% BSA for 1 hour at room temperature. The membrane was then incubated with the antibody against MARCKS (1:500) diluted in PBS/0.05% Tween 20 (PBST) with 1% BSA overnight at 4°C. After washing with PBST, the membrane was incubated with secondary antibody (donkey anti-goat IgG-HRP) diluted in PBST with 1% BSA for 1 hour at room temperature. After washing 3 × 15 minutes with PBST, proteins on the membrane were detected using an Amersham ECL kit followed by exposure to Hyperfilm ECL (Amersham Bioscience, Buckinghamshire, UK). The cytosol membrane was stripped and probed with a monoclonal anti-α-tubulin antibody.

Migration Assay

Neutrophils were labeled with the cell-permeant fluorescent dye calcein (1 μg/ml) for 30 minutes at RT. Cells were then washed and resuspended in HBSS⁺⁺ with 2% FCS. Cells were treated with the indicated concentration of peptide or PBS for 30 minutes at 37°C. A total of 1×10^4 cells were placed on a 2-μm pore size membrane of a ChemoTx plate (Neuro Probe, Inc., Gaithersburg, MD). Lower wells of the plate were filled with HBSS ± chemoattractant. Standard wells contained 1×10^4 cells. Cells were allowed to migrate for 1 hour at 37°C. After incubation, cells on the top of the filter were washed away with PBS and 0.5 mM EDTA added to the top of the filter for 5 minutes to detach adherent cells. The plate was then centrifuged at 1,000 rpm for 1 minute, the filter removed, and fluorescence measured in the lower wells (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices, Toronto, ON, Canada). Percent migration was determined by dividing the fluorescence of each well by the fluorescence of the standard wells containing 1×10^4 cells.

Adhesion Assay

Neutrophils (1×10^7 /ml) were suspended in HBSS and incubated with calcein (1 μg/ml) for 30 minutes at RT. Cells were washed and resuspended in HBSS⁺⁺ to a final concentration of 2×10^6 /ml. Cells were pretreated with a range of concentrations (0–50 μM) of peptide or PBS for 30 minutes at 37°C. Cells from the various treatment groups (1×10^5) were added in triplicate to Immulon 2 plates (Dynatech, Chantilly, VA) coated with 5% FCS in PBS or with immune complexes (IC), as previously described (7). After incubation at 37°C for 10 minutes, adhesion was stimulated with 100 nM fMLF for 3 minutes or 10 ng/ml PMA for 30 minutes at 37°C. IC-stimulated groups were incubated for 30 minutes at 37°C. Fluorescence was measured (485 nm excitation, 530 nm emission wavelengths) using an fMax fluorescence plate reader (Molecular Devices) before and after serial washes with 150 μl PBS to

dislodge nonadherent cells. Fluorescence after washing was divided by fluorescence before washing to calculate percent adhesion.

Cell Surface CD18 Expression

Neutrophils were suspended in HBSS with 1 mM Ca^{2+} to a concentration of 4×10^6 cells/ml. Cells were pretreated with 50 μM peptide or PBS for 30 minutes at 37°C . Cells were then left unstimulated ("T = 0") or stimulated with 100 nM fMLF for 3 minutes or 50 ng/ml PMA for 15 minutes at 37°C . Samples were immediately placed on ice, washed once with wash buffer (1% FBS, 0.1% Na Azide in PBS), and centrifuged at 1,200 rpm for 10 minutes at 4°C , before incubation with 10 $\mu\text{g}/\text{ml}$ IB4 antibody (anti-CD18) in 200 μl wash buffer for 40 minutes on ice. Cells were then washed twice in wash buffer before the cell pellet was resuspended in 200 μl of 1:50 dilution of FITC-labeled sheep-anti-mouse antibody for 20 minutes on ice. Cells underwent two final washes before resuspension in 1 ml of sterile PBS and immediate analysis using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence of each treatment population was normalized to the respective unstimulated control ("T = 0").

Fluorescence Microscopy

Freshly isolated human neutrophils (0.5×10^6) in HBSS⁺⁺ with 1% BSA were pretreated with 25 μM peptide or PBS for 30 minutes at 37°C . After peptide treatment, cells from each group were added to FCS-coated glass coverslips in 24-well tissue culture plates. Cells were allowed to adhere to the coverslips before stimulation with 100 nM fMLF for 60 seconds or 10 ng/ml PMA for 15 minutes at 37°C . The supernatant was removed and cells were fixed with 0.5 ml of a 3% paraformaldehyde solution (25 mM PIPES, pH 7.0, 50 mM KCl, 3 mM MgCl_2 , 3% paraformaldehyde wt/vol, 10 mM EGTA in sterile water) for 20 minutes. Coverslips were then washed gently with PBS twice and PBS-protein (0.2% gelatin, 0.2% azide, 0.1% ovalbumin in PBS) twice before extraction with 0.5 ml cold Triton lysis buffer (10 mM PIPES, pH 6.8, 0.5% Triton-X 100 vol/vol, 300 mM sucrose, 100 mM KCl, 3 mM MgCl_2 in sterile water). Cells were washed again before staining with Alexa Fluor 546-phalloidin diluted 1:50 in PBS-protein for 30 minutes at RT. Coverslips were mounted with a thin film of OPD-glycerol (1 mg/ml OPD in glycerol) and CytoSeal 60 (Richard-Allen Scientific, Kalamazoo, MI). Fluorescence of Alexa Fluor 546-phalloidin-stained F-actin was viewed with a Nikon TE-200 inverted epifluorescence microscope using a digital camera and associated software for image capture (SPOT; Diagnostics Instruments, Inc., Sterling Heights, MI). Cells were evaluated for PMA-induced spreading and fMLF-induced polarization. Treatment groups were blinded to the independent slide reader. In the PMA-treated cells, 100 total cells were counted and scored as "spread," as evident by increased surface area, membrane ruffling, and increased formation of F-actin, or "not spread" if they appeared as inactivated, small, round, resting neutrophils. In the fMLF-treated cells, 100 total cells were counted and scored as "unpolarized" for inactivated resting cells, "intermediately polarized" for cells with increased F-actin content and shape change but no clear leading edge, or "polarized" for cells with a redistribution of F-actin to the membrane and a distinct shape change with clear formation of an F-actin-rich leading edge.

F-actin Detection

Human neutrophils were isolated as described and washed once in Buffer A (140 mM NaCl, 1 mM KH_2PO_4 , 5 mM Na_2PO_4 , 1.5 mM CaCl_2 , 0.3 mM MgSO_4 , 1 mM MgCl_2 , 10 mM HEPES, pH 7.4) before final resuspension in Buffer A at a concentration of 5×10^6 cells/ml. Ninety-eight microliters of cell suspension was placed in sterile BD Falcon 5 ml polystyrene round-bottom tubes (BD Biosciences, Bedford, MA). Cells were stimulated with 100 nM fMLF for the indicated time periods before fixation with 100 μl of 3% formaldehyde and 0.1% BSA in PBS for 20 minutes. Cells were washed in Buffer A before permeabilization with 0.1% Triton and 1 unit per reaction of Alexa Fluor 546-phalloidin in 100 μl Buffer A for 30 minutes on ice. Four hundred microliters of PBS was added to each sample before transfer to a fresh tube for FACS analysis. The reported mean fluorescence intensity (MFI) for each sample was divided by the respective "T = 0" unstimulated control to determine the fold change in F-actin levels.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Data were analyzed by Student's two-sample *t* test assuming equal variance, or one-way ANOVA, with *P* < 0.05 considered statistically significant.

RESULTS

Loading Neutrophils with MANS Peptide Dislodges MARCKS from Cell Membranes

We used a cell-permeant myristoylated peptide identical to the first 24 amino acids of the aminoterminal of MARCKS protein (MANS peptide) or a randomly scrambled control peptide (RNS peptide) to investigate the requirement for the aminoterminal region of MARCKS in neutrophil migration. To first determine whether the MANS and RNS peptides were cell-permeant in neutrophils, we pretreated purified cells with FITC-labeled MANS or RNS peptide and then examined the distribution of labeled peptides and F-actin as described in MATERIALS AND METHODS. FITC-labeled peptide and F-actin were imaged using confocal microscopy. Both MANS and RNS peptides were detected within neutrophil cytosol, demonstrating that both the myristoylated peptides are indeed cell-permeant and translocate to the intracellular compartment (Figures 1A and 1B).

Figure 1. Myristoylated alanine-rich C-kinase substrate (MARCKS) myristoylated aminoterminal (MANS) peptide is cell permeant and dislocates MARCKS from neutrophil cell membranes. (A and B) Purified neutrophils were pretreated with FITC-labeled peptide before staining with rabbit-anti-FITC Ab, Alexa 647-anti-rabbit Ab, and Alexa 647-phalloidin as described in MATERIALS AND METHODS. Cytosolic FITC-labeled peptide (green) and F-actin (red) were imaged using confocal microscopy. (A) MANS and (B) RNS peptides were detected in the cytosol of neutrophils. (C) Purified neutrophils were pretreated with 50 μM of MANS, 50 μM of RNS, or no peptide and then stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) or vehicle (PBS, VC). Cells were fractionated as described in MATERIALS AND METHODS, and MARCKS content in the membrane (M) or cytosol (C) fractions was assessed by Western blot. The cytosol fraction was probed with α -tubulin as a control for total protein content. MANS peptide treatment of VC (unstimulated) neutrophils and PMA stimulation dislocated MARCKS from the membrane to the cytosol. RNS had no effect on MARCKS distribution in either VC or PMA-treated cells. The data shown are representative of three separate experiments.

MANS peptide treatment reduces MARCKS association with mucin granules in airway epithelial cells (33), suggesting that MANS peptide inhibits MARCKS binding to mucin granule membranes. We determined whether MANS peptide treatment affected MARCKS binding to neutrophil cell membranes using a subcellular fractionation technique. MARCKS protein is approximately equally distributed between the cytosol and membrane in unstimulated neutrophils (Figure 1C). Stimulation with PMA resulted in complete translocation of MARCKS protein from the membrane to cytosol. Treatment with MANS peptide resulted in translocation of MARCKS from the membrane to the cytosol in unstimulated neutrophils. MANS had no detectable effect on PMA-induced translocation of MARCKS to the cytosol. In contrast, RNS peptide treatment did not affect the amount of MARCKS detected in either membrane or cytosol fractions from unstimulated or PMA-stimulated neutrophils. Interestingly, in spite of dislocating MARCKS from the cell membranes, MANS peptide treatment had no effect on the kinetics or magnitude of fMLF- or PMA-induced MARCKS phosphorylation (data not shown).

MANS Peptide Significantly Reduces Chemoattractant-Induced Migration

To investigate the role of MARCKS in neutrophil migration, we pretreated purified calcein-labeled neutrophils with a range of concentrations (5–50 μM) of MANS or RNS control peptide and then evaluated the effects on directed migration as described in MATERIALS AND METHODS. Approximately 55% of cells pretreated with PBS ("0 μM ") migrated to lower wells containing the chemoattractant formylated-Met-Leu-Phe (fMLF) (Figure 2A). Only 3% of PBS-treated cells exhibited random migration by migrating into a lower well containing HBSS. Cells pretreated with concentrations of 10 μM or more of MANS peptide demonstrated a concentration-dependent decrease in percent migration, with complete reduction to background levels at a concentration of 50 μM . There was no effect on migration after treatment with concentrations of up to 50 μM of the RNS control peptide. To calculate the IC_{50} , the % reduction in migration of MANS-treated cells compared with vehicle control-treated cells was plotted against MANS concentration. The concentration of MANS that inhibited 50% of fMLF-induced migration was 17.1 μM (data not shown).

We next evaluated the ability of MANS to reduce neutrophil migration in response to other chemoattractant stimuli. The effect on migration induced by endogenous chemoattractants IL-8 or LTB₄ was assessed. Similar to PBS-treated cells, RNS-treated cells attained 60% migration toward fMLF and 80% migration toward IL-8 or LTB₄ (Figure 2B). Alternatively, MANS treatment diminished migration toward fMLF, IL-8, and LTB₄; the percentage of cells able to migrate into the lower wells was reduced to 10–12%. Background migration was similar among the treatment groups.

MANS Significantly Reduces fMLF-Induced Adhesion

The predominant $\beta 2$ integrin on the surface of neutrophils is CD11b/CD18 (34), which mediates neutrophil adhesion to activated endothelium, fibrinogen, and other matrix proteins derived from plasma, enabling chemotaxis and emigration (35). We investigated the possibility that MANS was disrupting neutrophil migration by interfering with $\beta 2$ integrin-dependent adhesion and, hence, the ability of cells to initiate migration. Approximately 50% of PBS-treated cells ("0 μM ") exhibited adhesion in response to fMLF (Figure 3A). There was no reduction in fMLF-induced adhesion in cells pretreated with 25 μM or 50 μM of RNS. In contrast, there was significant reduction in percent

Figure 2. MANS reduces chemoattractant-induced migration. (A) Purified calcein-labeled neutrophils were pretreated as described in MATERIALS AND METHODS. Data are shown as mean $\pm$ SEM of the percentage of the cells migrating to the lower chamber ($n = 3$). There was no effect on migration after treatment with concentrations up to 50 μM of the RNS control peptide, but, in contrast, there was a concentration-dependent decrease in percent migration after treatment with concentrations of MANS greater than 10 μM , and a complete reduction to background levels at 50 μM . Asterisks denote a significant difference in migration of MANS-treated cells compared with RNS at the same concentration of peptide ($P < 0.05$). (B) Purified calcein-labeled neutrophils were pretreated with 50 μM of MANS, 50 μM of RNS, or PBS before use in a migration assay. Data are shown as mean $\pm$ SEM of the percentage of the input cells migrating to the lower chamber ($n = 3$). RNS- and PBS-treated cells similarly attained approximately 60% migration toward fMLF and approximately 80% migration toward IL-8 or LTB₄. MANS treatment diminished migration toward fMLF, IL-8, and LTB₄ to levels comparable to background migration (HBSS). Asterisks denote a significant difference between percent migration of cells treated with MANS compared with RNS ($P < 0.05$).

adhesion of cells pretreated with MANS peptide. Concentrations of 25 μM and 50 μM MANS resulted in the reduction in adhesion to 24% and 16%, respectively. The concentration of MANS that inhibited 50% of fMLF-induced adhesion (IC_{50}) was 12.5 μM (data not shown).

MANS Reduces both PMA- and IC-Induced Adhesion

We next questioned whether the effect of MANS was specific to the transient adhesion event induced by chemoattractant activation or, instead, was a more general effect and would also

Figure 3. MANS peptide reduces $\beta 2$ integrin-dependent adhesion. Purified calcein-labeled neutrophils were pretreated with the indicated concentrations of MANS, RNS, or PBS ("0 μM ") before use in an adhesion assay. (A) Cells were plated in fetal calf serum (FCS)-coated wells and stimulated to adhere with fMLF (100 nM) for 3 minutes. (B) cells were plated in FCS-coated wells and stimulated to adhere with PMA (10 ng/ml) or allowed to adhere to IC-coated wells for 30 minutes. Data are shown as mean $\pm$ SEM of percentage of the input cells remaining after serial washes ($n = 5$). There was no reduction in adhesion after treatment of cells with 25 μM or 50 μM of RNS peptide compared with PBS-treated cells ("0 μM "). In contrast, there was significant reduction of adhesion of cells pretreated with either 25 μM or 50 μM of MANS peptide. Asterisks indicate a significant difference in adhesion in MANS-treated cells compared with RNS-treated cells at the same concentration of peptide ($P < 0.05$).

impede firm adhesion induced by alternate neutrophil activators, such as phorbol 12-myristate 13-acetate (PMA) or immune complexes (IC). PMA potently activates PKC, while IC-binding ligates and activates Fc γ receptors, both resulting in $\beta 2$ integrin activation and stable adhesion of neutrophils (17). Peptide- or PBS-treated cells were added to FCS-coated wells and subsequently stimulated with PMA, or added to wells coated with stable IC, before serial washes and determination of percent adhesion. RNS-treated cells had levels of adhesion similar to those of PBS-treated cells ("RNS 0 μM ") after both PMA and IC stimulation, with both groups exhibiting 35 to 40% adhesion (Figure 3B). In contrast, MANS-treated cells exhibited a significant reduction in adhesion. Concentrations of 25 μM or 50 μM of MANS resulted in 24% or 18% adhesion after PMA stimulation, respectively. Similarly, 25 μM or 50 μM of MANS peptide reduced IC-induced adhesion to 20% or 12%, respec-

tively. These results suggest that the MANS peptide disruption of $\beta 2$ integrin-dependent adhesion of neutrophils appears to be independent of the stimulus.

MANS Does Not Affect fMLF-Induced Surface $\beta 2$ Integrin Expression

Since MANS has been shown to inhibit exocytic processes such as myeloperoxidase (MPO) release from neutrophils (36) and mucin release from airway epithelial cells (33), we hypothesized that MANS was disrupting adhesion by reducing the ability of neutrophils to up-regulate surface $\beta 2$ integrins. PMA- and fMLF-induced up-regulation of surface $\beta 2$ integrin expression was evaluated using anti-CD18 antibody and flow cytometric analysis as described in MATERIALS AND METHODS. Both PMA and fMLF induced a significant 1.5-fold increase in surface CD18 in PBS control cells compared with unstimulated cells ("T = 0") (Figure 4). RNS-treated cells and MANS-treated cells exhibited a similar increase in CD18 after either PMA or fMLF stimulation, with both groups expressing an approximately 1.5-fold increase over respective peptide-treated unstimulated cells ("T = 0"). These results suggest that MANS does not affect up-regulation of surface $\beta 2$ integrins, and that it is disrupting adhesion through a different mechanism.

MANS Does Not Affect Ability of Neutrophils to Spread

To perhaps reveal a defect in spreading in MANS-treated neutrophils to explain the effect of MANS on migration, we examined the morphology of peptide-treated neutrophils induced to spread through stimulation with PMA. Peptide- or PBS-treated cells were plated on FCS-coated glass coverslips, stimulated with PMA for 10 minutes, and then examined for F-actin distribution using fluorescence microscopy and scored as "spread" or "not spread" according to the criteria outlined in MATERIALS AND METHODS. Approximately 90 to 95% of cells were

Figure 4. MANS does not affect fMLF-induced surface $\beta 2$ integrin expression. Peptide- or PBS-treated neutrophils were stimulated with PMA or fMLF before incubation with IB4 antibody (anti-CD18), secondary antibody, and FACS analysis. Data are shown as mean $\pm$ SEM fold-change in surface CD18 expression ($n = 3$). Both PMA and fMLF induced a significant (~ 1.5 -fold) increase in CD18 on PBS control cells compared with unstimulated cells ("T = 0"). RNS-treated and MANS-treated cells exhibited a similar significant increase in CD18 after either PMA or fMLF stimulation, with both groups expressing approximately a 1.5-fold increase over respective peptide-treated unstimulated cells ("T = 0"). Asterisks indicate a significant difference in $\beta 2$ integrin expression compared with the respective unstimulated peptide treated cells ($P < 0.05$).

fully spread after 10 minutes of PMA stimulation, and there were no significant differences between the treatment groups (Figure 5). Although MANS decreased neutrophil $\beta 2$ integrin-dependent adhesion in a concentration-dependent manner (Figure 3), the mechanism does not appear to involve effects on the ability of the cells to initially spread.

MANS Blunts the Initial fMLF-Induced Rise in F-actin in Neutrophils

MARCKS has been shown to bind and cross-link actin *in vitro* (26) and co-localize with F-actin in the leading edge lamellipodium in fibroblasts (30), suggesting a role for MARCKS in dynamic cytoskeletal rearrangement. To determine whether the effect of MANS on migration was due to disruption of chemoattractant-induced actin polymerization, peptide-treated cells in suspension were stimulated with fMLF and F-actin content was quantified. Stimulation with fMLF resulted in a 2.5-fold increase in F-actin after 30 seconds in PBS-treated control neutrophils, with a rapid return to resting levels by 300 seconds (Figure 6). Cells pretreated with RNS peptide had similar levels of F-actin as control cells at all time points. In contrast, cells pretreated with MANS had less than a 2-fold increase in F-actin levels at 30 seconds after fMLF stimulation, which was significantly different from PBS- and RNS-treated cells, yet had similar levels at 60, 180, and 300 seconds where F-actin content continued to decline toward basal levels. MANS may interfere with the early immediate polymerization event after chemoattractant stimula-

tion. However, as cells are polarizing, MANS-treated cells appear to overcome the effect and achieve equivalent levels of F-actin.

MANS Does Not Affect the Ability of Cells to Polarize

It was possible that the ability of MANS peptide to inhibit early F-actin polymerization accounted for the effects of the peptide on migration and adhesion by altering the F-actin distribution in polarized cells. To further explore the effect of MANS peptide inhibition of peak F-actin polymerization on subsequent F-actin organization, neutrophils were stimulated with fMLF for 60 seconds, the time point at which cells achieve maximum polarization of the actin cytoskeleton, and F-actin distribution in the cells was assessed using fluorescence microscopy. No qualitative differences in F-actin distribution were observed among treatment groups (Figure 7). The extent of polarization in peptide- or PBS-treated cells was scored as "unpolarized," "intermediately polarized," or "polarized," as defined in MATERIALS AND METHODS. In all treatment groups, less than 10% of cells were "unpolarized" after 60 seconds of fMLF stimulation (Figure 7). There were no notable differences between groups in ability to polarize, as 55 to 65% of cells and 30 to 40% of cells were "polarized" or "intermediately polarized," respectively, after fMLF stimulation. Although the MANS peptide inhibits chemotaxis, these results suggest that it does not appear to affect the ability of neutrophils to organize the actin cytoskeleton and appropriately polarize in response to chemoattractant stimulation.

DISCUSSION

We show here that a cell-permeant peptide derived from the MARCKS myristoylated aminoterminal (MANS peptide) inhibits chemoattractant stimulated migration and adhesion in

Figure 5. MANS does not affect PMA-induced spreading. Peptide- or PBS-treated neutrophils were plated on FCS-coated glass coverslips, stimulated with PMA, then fixed, permeabilized, stained with Alexa-phalloidin-546 for F-actin, and visualized using fluorescence microscopy. One hundred total cells were counted and scored as "spread" evident by increased surface area, membrane ruffling, and increased formation of F-actin, or "not spread" if they were inactivated, small, round, resting neutrophils. (A) Representative "spread" neutrophils after peptide or PBS treatment at $\times 40$ magnification. (B) Percentage of total cells spread was calculated and data shown are mean $\pm$ SEM ($n = 3$). Approximately 90 to 95% of cells were fully spread after 10 minutes of PMA stimulation, and there were no differences between the treatment groups.

Figure 6. MANS blunts the initial fMLF-induced increase in F-actin content in neutrophils. Peptide- or PBS-treated neutrophils were stimulated with fMLF before fixation, permeabilization, and staining with Alexa Fluor-546-phalloidin and FACS analysis. Data are shown as mean $\pm$ SEM of the fold-change in F-actin levels ($n = 3$). fMLF stimulation of PBS control cells resulted in an approximately 2.5-fold increase in F-actin content compared with unstimulated cells ("T = 0") at 30 seconds, with a return to resting levels by 300 seconds. RNS-treated cells had similar levels of F-actin at all time points compared with PBS controls. In contrast, cells pretreated with MANS had a significant decrease in F-actin levels at 30 seconds after fMLF stimulation compared with PBS- and RNS-treated cells, but not at 60, 180, or 300 seconds, where F-actin content continued to decline toward basal levels. Asterisk indicates a significant difference in F-actin concentration in MANS-treated compared with RNS-treated cells ($P < 0.05$).

Figure 7. MANS does not affect fMLF-induced polarization. Peptide- or PBS-treated neutrophils were plated on FCS-coated glass coverslips, stimulated with fMLF for 60 seconds, then fixed, permeabilized, stained with Alexa-phalloidin-546 for F-actin and visualized using fluorescence microscopy. One hundred total cells were counted and scored as “unpolarized” if they were inactivated resting cells, “intermediately polarized” evident by increased F-actin and shape change, with no clear leading edge, or “polarized” as evident by redistribution of F-actin to the membrane and a shape change with clear formation of an F-actin-rich leading edge. (A) Representative “polarized” neutrophils after peptide or PBS treatment at $\times 40$ magnification. (B) Percentage of cells “unpolarized,” “intermediately polarized,” and “polarized” was calculated and data shown are mean $\pm$ SEM ($n = 3$). In all treatment groups, less than 10% of cells were “unpolarized” after 60 seconds of fMLF stimulation. There were no notable differences between groups in their ability to polarize, as 55 to 65% of cells and 30 to 40% of cells were “polarized” or “intermediately polarized,” respectively, after fMLF stimulation.

primary human neutrophils, with an IC_{50} of 17.1 and 12.5 μM , respectively. MANS treatment dislocated MARCKS protein from cell membranes in unstimulated neutrophils, but did not affect PMA- or fMLF-induced MARCKS phosphorylation. MANS treatment also reduced the peak increase in F-actin 30 seconds after fMLF stimulation, but did not affect F-actin concentration at later time points when cells were polarizing, nor the ability of the cells to up-regulate surface $\beta 2$ integrin expression. Treatment with MANS peptide did not appear to affect the ability of neutrophils to polarize or spread. These data demonstrate that the MARCKS function is essential for neutrophil migration and that the aminoterminal appears critical for MARCKS function, at least in part by regulating appropriate MARCKS binding to cell membranes.

Stimulation of neutrophils with fMLF results in rapid phosphorylation of MARCKS (with maximal phosphorylation by 40 s) and translocation of MARCKS to the cytosol before dephosphorylation by 4 minutes and its subsequent return to the plasma membrane (22). MANS has been shown to inhibit MARCKS binding to mucin granule membranes of human bronchial epi-

thelial cells, while not affecting phosphorylation levels (33). We propose that in neutrophils, MANS may occupy specific MARCKS binding sites on the plasma membrane, dislocating endogenous MARCKS from cell membranes in the resting state and preventing MARCKS from reassociating with the membrane after phosphorylation. By forcing MARCKS distribution to the cytosol, MANS may inhibit migration and adhesion by displacing MARCKS to an inappropriate subcellular compartment, thereby disrupting its function.

The results of the studies presented are the first demonstration that MARCKS has an essential role in the mechanism of neutrophil migration and adhesion, and the first to demonstrate that the 24-amino acid N-terminal region regulates MARCKS function in these processes. A major role for the N-terminal region of MARCKS in other functions, such as mucin secretion and inflammatory cell degranulation, was first described in studies from our laboratory (27, 33, 36). To our knowledge, these studies were the first to point out a role for the N-terminal region of MARCKS in any physiological functions. Interestingly, a previous study by Myat and coworkers (30) used a mutant version of MARCKS that was palmitoylated rather than myristoylated at the aminoterminal, thereby anchoring the mutant MARCKS protein in the membrane, even when it was phosphorylated. The palmitoylated MARCKS mutant arrested cell spreading and inhibited adhesion in fibroblasts, suggesting that MARCKS dislocation from the membrane is necessary for the shape change needed for cells to attach to substrate. Our system is quite different, as the MANS peptide *inhibits* MARCKS association with cell membranes rather than anchoring it to membranes, indicating that MARCKS association with cell membranes is necessary for cell migration and adhesion. However, we did not observe an effect on cell spreading or polarization, unlike the study by Myat and colleagues cited above. Together, these two systems demonstrate that normal regulation of MARCKS association with and disassociation from cell membranes is necessary for MARCKS function, and suggest that MARCKS must dislocate from the cell membrane for cell spreading to occur during adhesion, but that MARCKS association with cell membranes is essential for both neutrophil migration and adhesion. More detailed genetic-based analyses of the effects of MANS on endogenous MARCKS function, F-actin and MARCKS distribution, and cell shape changes are underway to better understand the mechanism by which the aminoterminal of MARCKS regulates MARCKS function in cell adhesion and migration.

The transient pattern of PKC-mediated phosphorylation of MARCKS tightly corresponds to the polymerization of actin in chemoattractant-stimulated neutrophils. At 30 seconds after fMLF stimulation, the point of maximal F-actin content in the cell during polarization, MARCKS is maximally phosphorylated and redistributed to the cytosolic fraction (22). As F-actin content returns to baseline levels at 3 minutes after stimulation, MARCKS becomes dephosphorylated and returns to the membrane (22). MARCKS binds F-actin via the basic effector domain, and, through alteration of the basic charge of the effector domain, PKC-dependent phosphorylation negatively affects the F-actin binding and cross-linking activity of MARCKS (25, 26). MARCKS localizes to the leading edge lamellipodium and colocalizes with F-actin in spreading fibroblasts (30), and associates with PKC (37–39), suggesting that MARCKS is an effector of PKC in regulating the actin cytoskeleton in migration. Perhaps, in the immediate stages after chemoattractant stimulation when a globally pliable cell membrane is required to achieve a dynamic shape change, PKC-dependent phosphorylation of MARCKS serves to transiently decrease cross-linking and reduce the rigidity of the cell membrane to allow remodeling. As actin continues to polymerize and redistribute, MARCKS may return to the

membrane and resume cross-linking activity to complete and/or stabilize cell shape during migration.

Our data show that the MANS peptide reduced F-actin levels at 30 seconds after fMLF stimulation, suggesting that MANS treatment alters MARCKS-actin interactions very early after the onset of fMLF stimulation while cells are changing shape to polarize. However, this effect appears quite mild and MANS treatment did not affect F-actin content or distribution at later time points during polarization, indicating that MANS peptide alters MARCKS functions distinct from, or in addition to, those regulating the actin cytoskeleton. It has been suggested that the aminoterminal of MARCKS contains a motif that targets MARCKS to intracellular compartments by a mechanism that involves protein-protein interactions (40). It is possible that the MANS peptide disrupts appropriate MARCKS targeting during migration and adhesion by interfering with MARCKS interactions with protein partners. Our data demonstrating that MANS peptide dislocates MARCKS from cell membranes suggest that while MARCKS association with the cell membrane is not required for initial cell spreading and even polarization of the actin cytoskeleton, it may be required for subsequent firm adhesion and ultimately migration in neutrophils.

As circulating neutrophils encounter inflammatory mediators, surface L-selectin, responsible for the initial rolling and contact with endothelial cells, is shed and CD11b/CD18 is up-regulated through translocation to the plasma membrane from intracellular secretory vesicles (41). This increase in total $\beta 2$ integrin expression on the cell surface contributes to the overall avidity of adhesion in activated neutrophils. Although MANS inhibits the exocytic process of MPO release from neutrophils (36) and mucin release from airway epithelial cells (33), it does not seem to affect chemoattractant-induced up-regulation of surface CD18, and seems to inhibit adhesion through a different mechanism. Along with its role in the cross-linking and bundling of actin (26, 29), MARCKS has been implicated in stabilizing focal adhesions (42, 43). As highly motile cells, neutrophils have smaller areas of adhesion, called podosomes, where transient adhesion to the extracellular matrix provides the traction necessary for migration (44, 45). In general, $\beta 2$ integrins on the surface of resting leukocytes are constrained by the cytoskeleton and evenly distributed across the cell membrane. Stimulators of adhesion release $\beta 2$ integrins from their cytoskeletal constraints, increasing the lateral mobility necessary for integrin clustering and formation of new adhesion sites, such as those at the leading edge of migratory cells (46, 47). MARCKS localizes to adhesion sites in cultured human macrophages and fibrosarcoma cell lines (43, 48), suggesting a role in regulating integrin function in adhesion. We show here that MANS decreases $\beta 2$ integrin-dependent adhesion without altering the ability of cells to spread in response to PMA stimulation on a $\beta 2$ integrin substrate or to up-regulate surface CD18 expression. Perhaps MANS peptide inhibits adhesion by disrupting the ability of MARCKS to regulate integrin mobility in the membrane, integrin clustering, affinity activation, or the ability to form mature podosomes needed for firm adhesion. Our data provide supportive evidence for the stabilization of adhesion sites by MARCKS; MANS-induced disruption of MARCKS function reduces strength of adhesion and abolishes directed migration.

Recent *in vivo* studies in mice show that intratracheal injection of MANS peptide potently inhibits ozone-induced neutrophil migration and chemokine and cytokine expression in the airways (49). Our data and these results suggest that inhibition of MARCKS offers a potential novel molecular-based approach to treat inflammatory diseases. Characterization of the association of MARCKS protein with neutrophil adhesion sites and its effects on integrin mobility and affinity will help us understand the

mechanisms of MARCKS regulation of neutrophil migration and provide substantiation for further development of MARCKS-targeted anti-inflammatory agents.

Conflict of Interest Statement: K.B.A. serves on the scientific advisory board of Sepracor, Inc. and received \$5,000 for this service in 2005, \$5,000 in 2006, and \$5,000 in 2007, and served as a consultant to Immunogen and received \$1,500 in 2004. He received \$68,000 in 2004–2005 as a research grant from AstraZeneca and approximately \$140,000 in research grants from Sepracor since 2002. He holds 150,000 founders shares of a start-up biotech company, BioMarck, and serves as a scientific consultant and member of the scientific advisory board without monetary compensation. S.L.J. received \$180 from Gerson Lehman Group for consultations in 2008 and 2007, and also received \$31,000 from BioMarck Pharmaceuticals in 2006 as a research grant. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

- Glogauer M, Hartwig J, Stossel T. Two pathways through Cdc42 couple the N-formyl receptor to actin nucleation in permeabilized human neutrophils. *J Cell Biol* 2000;150:785–796.
- Cicchetti G, Allen PG, Glogauer M. Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. *Crit Rev Oral Biol Med* 2002;13:220–228.
- Defilippi P, Olivo C, Venturino M, Dolce L, Silengo L, Tarone G. Actin cytoskeleton organization in response to integrin-mediated adhesion. *Microsc Res Tech* 1999;47:67–78.
- Jones SL, Sharief Y. Asymmetrical protein kinase A activity establishes neutrophil cytoskeletal polarity and enables chemotaxis. *J Leukoc Biol* 2005;78:248–258.
- Jones SL. Protein kinase A regulates $\beta 2$ integrin avidity in neutrophils. *J Leukoc Biol* 2002;71:1042–1048.
- Eckert RE, Jones SL. Regulation of VASP serine 157 phosphorylation in human neutrophils after stimulation by a chemoattractant. *J Leukoc Biol* 2007;82:1311–1321.
- Jones SL, Knaus UG, Bokoch GM, Brown EJ. Two signaling mechanisms for activation of α 4 β 2 avidity in polymorphonuclear neutrophils. *J Biol Chem* 1998;273:10556–10566.
- Sheterline P, Rickard JE, Boothroyd B, Richards RC. Phorbol ester induces rapid actin assembly in neutrophil leukocytes independently of changes in $[Ca^{2+}]_i$ and pHi. *J Muscle Res Cell Motil* 1986;7:405–412.
- Howard TH, Wang D. Calcium ionophore, phorbol ester, and chemotactic peptide-induced cytoskeleton reorganization in human neutrophils. *J Clin Invest* 1987;79:1359–1364.
- Roos FJ, Zimmermann A, Keller HU. Effect of phorbol myristate acetate and the chemotactic peptide fMLP on shape and movement of human neutrophils. *J Cell Sci* 1987;88:399–406.
- Downey GP, Chan CK, Lea P, Takai A, Grinstein S. Phorbol ester-induced actin assembly in neutrophils: role of protein kinase C. *J Cell Biol* 1992;116:695–706.
- White JR, Huang CK, Hill JM Jr, Naccache PH, Becker EL, Sha'afi RI. Effect of phorbol 12-myristate 13-acetate and its analogue 4 α -phorbol 12,13-didecanoate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils. *J Biol Chem* 1984;259:8605–8611.
- Waki K, Inanami O, Yamamori T, Nagahata H, Kuwabara M. Involvement of protein kinase C δ in the activation of NADPH oxidase and the phagocytosis of neutrophils. *Free Radic Res* 2006;40:359–367.
- Wrann CD, Winter SW, Barkhausen T, Hildebrand F, Krettek C, Riedemann NC. Distinct involvement of p38-, ERK1/2 and PKC signaling pathways in C5a-mediated priming of oxidative burst in phagocytic cells. *Cell Immunol* 2007;245:63–69.
- Merritt JE, Moores KE, Evans AT, Sharma P, Evans FJ, MacPhee CH. Involvement of calcium in modulation of neutrophil function by phorbol esters that activate protein kinase C isotypes and related enzymes. *Biochem J* 1993;289:919–926.
- Patarroyo M, Yogeewaran G, Biberfeld P, Klein E, Klein G. Morphological changes, cell aggregation and cell membrane alterations caused by phorbol 12,13-dibutyrate in human blood lymphocytes. *Int J Cancer* 1982;30:707–717.
- Laudanna C, Mochly-Rosen D, Liron T, Constantin G, Butcher EC. Evidence of zeta protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *J Biol Chem* 1998;273:30306–30315.

18. Zimmermann A, Gehr P, Keller HU. Diacylglycerol-induced shape changes, movements and altered F-actin distribution in human neutrophils. *J Cell Sci* 1988;90:657–666.
19. Keller HU, Niggli V. The PKC-inhibitor Ro 31-8220 selectively suppresses PMA- and diacylglycerol-induced fluid pinocytosis and actin polymerization in PMNs. *Biochem Biophys Res Commun* 1993;194:1111–1116.
20. Niggli V, Keller H. Inhibition of chemotactic peptide-induced development of cell polarity and locomotion by the protein kinase C inhibitor CGP 41 251 in human neutrophils correlates with inhibition of protein phosphorylation. *Exp Cell Res* 1993;204:346–355.
21. Kent JD, Sergeant S, Burns DJ, McPhail LC. Identification and regulation of protein kinase C- δ in human neutrophils. *J Immunol* 1996;157:4641–4647.
22. Thelen M, Rosen A, Nairn AC, Aderem A. Regulation by phosphorylation of reversible association of a myristoylated protein kinase C substrate with the plasma membrane. *Nature* 1991;351:320–322.
23. Verploegen S, van Leeuwen CM, van Deutekom HW, Lammers JW, Koenderman L, Coffey PJ. Role of Ca^{2+} /calmodulin regulated signaling pathways in chemoattractant induced neutrophil effector functions. Comparison with the role of phosphatidylinositol-3 kinase. *Eur J Biochem* 2002;269:4625–4634.
24. Arbuzova A, Schmitz AA, Vergeres G. Cross-talk unfolded: MARCKS proteins. *Biochem J* 2002;362:1–12.
25. Hartwig JH, Thelen M, Rosen A, Janmey PA, Nairn AC, Aderem A. MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* 1992;356:618–622.
26. Yarmola EG, Edison AS, Lenox RH, Bubb MR. Actin filament cross-linking by MARCKS: characterization of two actin-binding sites within the phosphorylation site domain. *J Biol Chem* 2001;276:22351–22358.
27. Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. *J Biol Chem* 2001;276:40982–40990.
28. Wohnsland F, Schmitz AA, Steinmetz MO, Aebi U, Vergeres G. Influence of the effector peptide of MARCKS-related protein on actin polymerization: a kinetic analysis. *Biophys Chem* 2000;85:169–177.
29. Tang JX, Janmey PA. The polyelectrolyte nature of F-actin and the mechanism of actin bundle formation. *J Biol Chem* 1996;271:8556–8563.
30. Myat MM, Anderson S, Allen LA, Aderem A. MARCKS regulates membrane ruffling and cell spreading. *Curr Biol* 1997;7:611–614.
31. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LS, Iida K, Talle MA, Westberg EF, Goldstein G, Silverstein SC. Identification of the C3b β receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc Natl Acad Sci USA* 1983;80:5699–5703.
32. Kajstura J, Cigola E, Malhotra A, Li P, Cheng W, Meggs LG, Anversa P. Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J Mol Cell Cardiol* 1997;29:859–870.
33. Singer M, Martin LD, Vargaftig BB, Park J, Gruber AD, Li Y, Adler KB. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. *Nat Med* 2004;10:193–196.
34. Hickstein DD, Ozols J, Williams SA, Baenziger JU, Locksley RM, Roth GJ. Isolation and characterization of the receptor on human neutrophils that mediates cellular adherence. *J Biol Chem* 1987;262:5576–5580.
35. von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte β 2 integrins in vivo. *Proc Natl Acad Sci USA* 1991;88:7538–7542.
36. Takashi S, Park J, Fang S, Koyama S, Parikh I, Adler KB. A peptide against the N-terminus of myristoylated alanine-rich C kinase substrate inhibits degranulation of human leukocytes in vitro. *Am J Respir Cell Mol Biol* 2006;34:647–652.
37. Fujise A, Mizuno K, Ueda Y, Osada S, Hirai S, Takayanagi A, Shimizu N, Owada MK, Nakajima H, Ohno S. Specificity of the high affinity interaction of protein kinase C with a physiological substrate, myristoylated alanine-rich protein kinase C substrate. *J Biol Chem* 1994;269:31642–31648.
38. Poussard S, Dulong S, Aragon B, Jacques BJ, Veschambre P, Ducastaing A, Cottin P. Evidence for a MARCKS-PKC α complex in skeletal muscle. *Int J Biochem Cell Biol* 2001;33:711–721.
39. Allen LH, Aderem A. A role for MARCKS, the α isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. *J Exp Med* 1995;182:829–840.
40. Seykora JT, Myat MM, Allen LA, Ravetch JV, Aderem A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. *J Biol Chem* 1996;271:18797–18802.
41. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 2003;5:1317–1327.
42. Gatlin JC, Estrada-Bernal A, Sanford SD, Pfenninger KH. Myristoylated, alanine-rich C-kinase substrate phosphorylation regulates growth cone adhesion and pathfinding. *Mol Biol Cell* 2006;17:5115–5130.
43. Berditchevski F, Odintsova E. Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling. *J Cell Biol* 1999;146:477–492.
44. Jones SL, Brown EJ. Fc γ R2-mediated adhesion and phagocytosis induce L-plastin phosphorylation in human neutrophils. *J Biol Chem* 1996;271:14623–14630.
45. Gimona M, Buccione R, Courtneidge SA, Linder S. Assembly and biological role of podosomes and invadopodia. *Curr Opin Cell Biol* 2008;20:235–241.
46. van Kooyk Y, van Vliet SJ, Figdor CG. The actin cytoskeleton regulates LFA-1 ligand binding through avidity rather than affinity changes. *J Biol Chem* 1999;274:26869–26877.
47. Kucik DF, Dustin ML, Miller JM, Brown EJ. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J Clin Invest* 1996;97:2139–2144.
48. Rosen A, Keenan KF, Thelen M, Nairn AC, Aderem A. Activation of protein kinase C results in the displacement of its myristoylated, alanine-rich substrate from punctate structures in macrophage filopodia. *J Exp Med* 1990;172:1211–1215.
49. Jester WF, Jiang H, Zhao H, Liang J, Parikh I, Murphy T, Adler KB, Panettieri RA. Inhibition of Myristoylated Alanine-Rich C Kinase Substrate protein markedly inhibits ozone-induced airway inflammation. *Chest* 2008;132:522a.