

# A Role for Myristoylated Alanine Rich C-Kinase Substrate (MARCKS) in Neutrophil Outside-in Beta2-integrin Activation

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## Extended Methods for Cell Bio 2020 P555

**Neutrophil Isolation:** The human neutrophils utilized for this study were isolated from the peripheral blood of healthy, adult volunteers using the protocol approved by the Institutional Research Ethics Committee of NCSU (IRB approval #616). Human neutrophils were isolated from whole blood using Ficoll gradient centrifugation of dextran-sedimented leukocyte rich plasma. When needed, contaminating RBCs were removed by hypotonic lysis.

**Peptide Treatment:** Neutrophils were resuspended to appropriate concentration in HBSS<sup>++</sup> with 2% FCS and pretreated with indicated concentrations of MANS peptide, RNS control peptide, vehicle control, or left untreated for 30 minutes at 37°C.

**Flow Cytometry:** Cells were pretreated as specified, stimulated with 100 nM fMLP or vehicle control for 5 minutes, diluted with 1 volume ice cold PBS and placed on ice for 5 minutes, centrifuged at 1200 rpm for 5 minutes, resuspended in sterile PBS with 5% FBS and labeled with the specified primary antibodies. Flow cytometry was performed using Caliber FACScan.

**Microscopy:** Cells were pretreated as specified, plated on ICAM-1/Fc coated chambered coverslips and allowed to settle for 10 minutes, then appropriate wells were treated with 100 nM fMLP or vehicle control for 5 minutes. Cells were then fixed to slide, washed with PBS, blocked with rabbit serum, and labeled with anti-CD11b antibody before imaging with an Olympus IX83 Inverted Microscope.

**Adhesion:** 96-well plates were coated with 10 µg/mL Recombinant ICAM-1 or 5% FBS (for controls). Isolated human neutrophils were labeled with calcein AM (2 µg/mL) and primed with human GM-CSF at 5 ng/mL for 30 minutes. Cells were then centrifuged at 1000 rpm for 8 minutes and then resuspended in HBSS<sup>++</sup> + 2% FCS. MANS or RNS peptides, anti-CD18 F(ab)<sup>'</sup><sub>2</sub>, or isotype controls were added to cells and incubated for 30 minutes at 37°C. The ICAM-1 coated plate was washed once with 1X PBS before cells were added to individual wells. Cells were added to wells and allowed to settle for 10 minutes at 37°C. 0.5 mM Mn<sup>2+</sup> was added to individual wells. Plates were incubated for 10 minutes prior to initial fluorescence reading. Cells were gently dumped and washed with PBS, reading fluorescence (485 nm excitation, 535 nm emission) at each wash step. Fluorescence washing was divided by initial fluorescence and multiplied by 100 to calculate percent adhesion. The first was that demonstrated less than 10% adhesion in the non-stimulated cells (plated on 5% FBS coating) was considered the final results. Treatment groups were tested in triplicate.

**Respiratory Burst:** Cells were incubated with indicated treatments at 37°C for 30 minutes prior to each experiment and 100 µL of cells from indicated treatment groups were placed in individual wells of 5% FBS or insoluble immune complex (IIC) coated Immulon2HB plates. Plates were coated overnight at 4°C with BSA (100 µg/mL). To generate IIC substrate, BSA coated wells were washed three times and recoated with anti-BSA antibody (50 µg/well) and incubated for 2 hours at 37°C. Prior to the addition of cells, all wells were washed three times with sterile PBS. For PMA-stimulated respiratory burst, cells were allowed to settle for 10 minutes prior to the addition of dihydrorhodamine-123 (DHR-123) (10 µM final concentration) and PMA (100 ng/mL final concentration). In the case of IIC-mediated respiratory burst, DHR-123 was added immediately following addition of cells to the well. An fMax fluorescence plate reader was used to measure initial fluorescence (485 nm excitation, 530 nm emission) followed by a fluorescence reading every 15 minutes for 120 minutes. Results are reported as nm fluorescence. Treatment groups were tested in triplicate.