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PRAM-1 Is Required for Optimal Integrin-Dependent Neutrophil Function

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PML-retinoic acid receptor alpha (RARα) regulated adaptor molecule 1 (PRAM-1) is an intracellular adaptor molecule that is upregulated during the induced granulocytic differentiation of promyelocytic leukemic cells and during normal human myelopoiesis. This report describes the generation of PRAM-1-deficient mice and an analysis of the function of this adaptor in neutrophil differentiation and mature neutrophil function. We demonstrate here that neutrophil differentiation is not impaired in PRAM-1-deficient mice and that PRAM-1-deficient neutrophils function normally following engagement of Fcγ receptors. In contrast, mature PRAM-1-null neutrophils exhibit significant defects in adhesion-dependent reactive oxygen intermediate production and degranulation. Surprisingly, other integrin-dependent responses, such as cell spreading and activation of several signaling pathways, are normal. Together, these findings demonstrate the uncoupling of key integrin-dependent responses in the absence of PRAM-1 and show this adaptor to be critical for select integrin functions in neutrophils.

Neutrophils are critical for host defense against pathogens. Comprising a majority of leukocytes in humans, neutrophils are the first circulating cells to migrate to the site of infection, where they contain and eliminate invading microorganisms through phagocytosis, production of reactive oxygen intermediates (ROI), and release of cytotoxic granule contents (6). As the first responders of the innate immune system, neutrophils also release cytokines and chemokines that initiate and amplify the inflammatory response (6, 56). Neutrophil dysfunction underlies several human immunodeficiencies, underscoring the importance of their function in vivo. Patients with defects in neutrophil migration (leukocyte adhesion deficiency) or ROI production (chronic granulomatous disease) are unable to mount an appropriate immune response to pyogenic bacteria or fungi and consequently die at an early age as a result of recurrent infections (6, 28). Conversely, given the toxic nature of an activated neutrophil, inappropriately regulated neutrophil activity is similarly pathological and associated with conditions such as endotoxic shock (1, 31), inflammatory arthritis (58), and immune complex-mediated disease (10).

A myriad of cell-surface receptors, including tumor necrosis factor (TNF) family receptors, chemokine receptors, toll-like receptors, and tyrosine kinase-associated receptors, initiate signaling cascades that regulate neutrophil function. Thus, understanding the mechanisms underlying neutrophil activation requires knowledge of the signaling pathways that transduce information from these receptors to effector function. Adaptors are a class of molecules that modulate receptor signaling via assembly of protein complexes. Work with other hematopoietic lineages has demonstrated that adaptor molecules are essential for coupling receptor activation with downstream signaling events. Though comparatively little is known about the importance of adaptors in neutrophils, recent studies of several adaptors, including SLP-76 (43), Shc (12, 44, 47), and MyD88 (14), indicate that these molecules play critical roles in regulating neutrophil signaling pathways.

PML-retinoic acid receptor alpha (RARα) regulated adaptor molecule 1 (PRAM-1) is an intracellular adaptor that was discovered in a screen for molecules that are upregulated in promyelocytic leukemic cells upon all-*trans*-retinoic acid-induced granulocytic differentiation (41). Expression of human PRAM-1 is restricted to granulocytes in terminal stages of maturation (41). PRAM-1 is a 683-amino-acid protein with an apparent molecular weight of 97 kDa comprised of three structural domains: an N-terminal proline-rich region with several putative SH3 recognition motifs, a central SH2 binding site, and a C-terminal SH3-like domain. The organization of these domains demonstrates structural homology with the hematopoietic-specific adaptor adhesion and degranulation-promoting adaptor protein (ADAP), and indeed, PRAM-1 shares a number of binding partners with ADAP (5, 30, 36, 41, 48, 51). Coimmunoprecipitation studies have documented constitutive PRAM-1 association with SKAP55-hom and inducible binding to SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) and the Src family kinase lyn (41). Studies of T cells, mast cells, and platelets have shown that ADAP (19, 45), SKAP55-hom (3), SLP-76 (9, 20, 21, 43, 46), and the Src family kinases (34, 35, 38) function in signaling pathways

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linked to tyrosine kinase-associated receptors, including those which engage antigen (T-cell receptor and B-cell receptor), immune complexes (FcγR and FcεR), and integrin ligands ($\alpha_M\beta_2$ and $\alpha_{IIb}\beta_3$), suggesting that PRAM-1 may also be important for the signaling initiated by similar receptors in granulocytes.

To determine the importance of PRAM-1 in neutrophil differentiation and mature neutrophil function, we generated mice deficient in PRAM-1. In this report we show that neutrophil differentiation is not impaired in PRAM-1-deficient mice and that PRAM-1-deficient neutrophils appear to function normally following engagement of Fcγ receptors. In contrast, mature PRAM-1-null neutrophils exhibit significant defects in integrin- and adhesion-dependent ROI production and degranulation. Surprisingly, other integrin-dependent responses, such as cell spreading and activation of several signaling pathways, are normal in the absence of PRAM-1. Together, these findings demonstrate the uncoupling of several integrin-dependent responses in the absence of PRAM-1 and show this adaptor to be critical for select integrin functions.

MATERIALS AND METHODS

Cloning of murine PRAM-1. In order to isolate the murine homolog of PRAM-1, mRNA was isolated from murine bone marrow-derived macrophages, and primers derived from human PRAM-1 were used to amplify a 400-bp fragment of the 3′ end of the cDNA via reverse transcription (RT)-PCR. The 400-bp fragment was sequenced and used as a probe to search the National Center for Biotechnology Information murine expressed sequence tag database using the BLASTn program. Overlapping, aligned expressed sequence tags were used to design primers for amplification and sequencing of 1,100 bp of the 3′ end. The complete coding sequence was generated by 5′ rapid amplification of cDNA ends using a murine spleen cDNA library (Clontech; catalog no. ML5011t). The complete sequence was confirmed by amplification of full-length cDNA from murine bone marrow-derived mRNA.

Generation of PRAM-1-deficient mice. A 3-kb XbaI-HindIII genomic DNA fragment containing the PRAM-1 start codon and 1,000 bp of the second coding exon was replaced by a neomycin resistance gene cassette. The targeting construct was electroporated into 129/SVJ embryonic stem cells (R1; Genome Systems, St. Louis, Mo.) grown on feeder layers of murine embryonic fibroblasts. Targeted embryonic stem cells were positively selected with G418 (200 μg/ml) and negatively selected with ganciclovir. Specificity of targeting vector integration within the PRAM-1 locus was determined by PCR screening and Southern analysis of G418-resistant clones. Targeted clones were microinjected into blastocysts obtained from C57BL/6 mice, which were then transferred to pseudopregnant recipient mice. Two founder chimeric mice were mated with C57BL/6 mice, and heterozygous offspring were identified by Southern blotting of tail genomic DNA. Mice harboring the targeted allele were backcrossed to C57BL/6 four times; control mice were backcrossed and age-matched for all experiments. Mice were housed at the University of Pennsylvania Animal Care Facility under pathogen-free conditions and used in accordance with the National Institutes of Health and Institutional Animal Care and Use Committee guidelines.

Isolation of bone marrow neutrophils. Bone marrow-derived neutrophils were prepared as previously described (39). Briefly, bone marrow was flushed from the femur and tibia of 2- to 6-month-old mice with Hanks' balanced salt solution (HBSS) Prep (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137 mM NaCl, 5.6 mM dextrose, 20 mM HEPES, 0.5% fetal calf serum [FCS]). Red blood cells were lysed in hypotonic NaCl, followed by addition of an equal volume of hypertonic NaCl solution to restore normal tonicity. Cells were resuspended in 5 ml of HBSS Prep, layered over a 62.5% Percoll gradient, and centrifuged at 2,200 rpm for 30 min. Mature neutrophils were harvested from the bottom layer of the gradient. The purity of the neutrophil preparation was assessed using flow cytometry; standard preparations yielded neutrophils of >90% purity determined by surface expression of Gr-1 (Pharmingen, San Diego, Calif.).

Antibodies. Chicken antiserum recognizing a peptide (CZPQTELSEQPKK SSQSE) from the amino terminus of murine PRAM-1 was generated by Aves Labs Inc. (Tigard, Oreg.). Sheep antiserum directed against a glutathione *S*-transferase fusion protein containing the carboxyl-terminal 300 amino acids of murine PRAM-1 was generated by Elmira Biologicals Inc. (Iowa City, Iowa). The antiphosphotyrosine monoclonal 4G10 and anti-Vav antibodies were purchased from Upstate Biotechnologies Inc. (Waltham, Mass.). All other antibodies were purchased from Cell Signaling Inc. (Beverly, Mass.).

Immunoprecipitation and Western blotting. For poly-RGD stimulation, tissue culture plates were coated with 15 μg of poly-RGD (Sigma Aldrich, St. Louis, Mo.)/ml in phosphate-buffered saline (PBS) for 2 h and then washed three times with PBS. Neutrophils were plated on poly-RGD or left in suspension at 37°C. Cells were lysed at indicated time points using a radioimmunoprecipitation assay-based buffer (40); insoluble material was removed through centrifugation at 14,000 rpm for 10 min in a tabletop microfuge. For Western blotting of whole cell lysates, 4× sample buffer (0.5 M Tris-HCl [pH 6.8], 277 mM sodium dodecyl sulfate, 40% glycerol, 20% 2-mercaptoethanol, 1% bromophenol blue) was added, and samples were boiled for 5 min. For immunoprecipitation, lysates containing approximately 10 million cells were precleared with GammaBind G-Sepharose (Amersham Pharmacia Biotech, Piscataway, N.J.). Two micrograms of anti-Vav antibody or 3 μl of PRAM-1 antiserum was added to the cell extracts and rotated at 4°C for 2 h. Twenty-five microliters of GammaBind beads were then added, and samples in tubes were rotated for an additional 4 h. Samples were washed three times and then boiled for 5 min in 2× sample buffer. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, Calif.) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline–Tween 20 for 30 min and then probed overnight with the appropriate antibody diluted in Tris-buffered saline–Tween20–5% bovine serum albumin. Antibody binding was detected by horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and autoradiography.

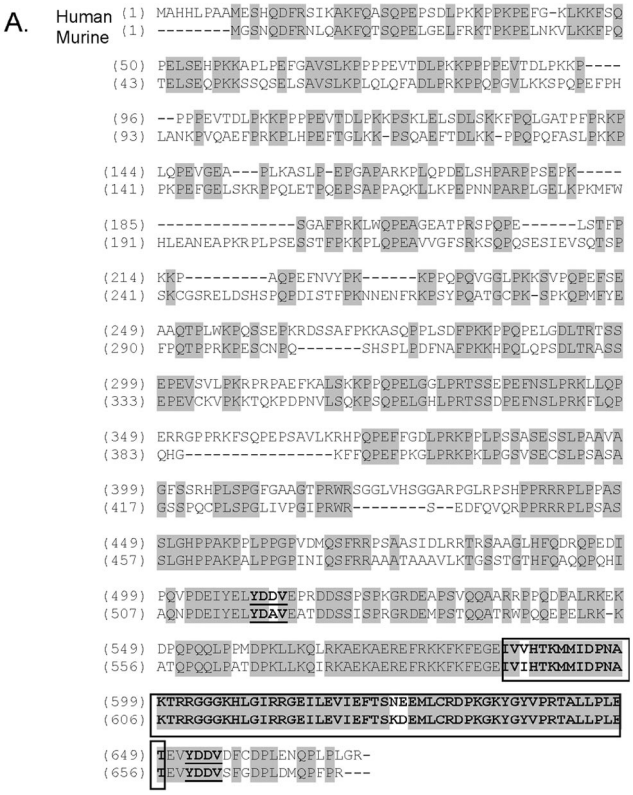
Complete blood count. Cardiac puncture was used to withdraw 250 μl of blood from carbon dioxide-asphyxiated mice. The blood was immediately transferred into EDTA-coated tubes (Becton-Dickinson, Franklin, N.J.). The complete blood count (CBC) was determined with a HEMA VET MASCOT 850 instrument (CDC Technologies Inc., Oxford, Conn.).

Measurement of FcγR-induced calcium flux and ROI production. For labeling with the calcium-sensitive fluorochrome Indo-1, neutrophils were resuspended at 10^7 cells/ml in HBSS Prep containing 3 μg of Indo-1 (Molecular Probes, Eugene, Oreg.)/ml and 4 mM probenecid and incubated at 37°C for 20 min. Cells were washed twice in PBS and then incubated with 2 μg of anti-FcγRII/III (Pharmingen)/ml in PBSg (125 mM sodium chloride, 8 mM sodium phosphate, 2 mM sodium phosphate monobasic, 5 mM potassium chloride, 5 mM glucose) for 15 min at 4°C. Following three washes in cold PBSg, cells were resuspended in ice-cold PBSg at 10^7 cells/ml until initiation of the assay. Two minutes prior to stimulation, cells were diluted to 0.5 million cells/ml in 37°C KRP buffer (PBSg, 1 mM calcium chloride, 1.5 mM magnesium chloride). Cell stimulation was initiated by adding 15 to 30 μg of goat anti-rat immunoglobulin G (FcγR; Pharmingen)/ml or 2.5 μM formyl-MetLeuPhe (fMLP). Calcium levels were analyzed on an LSR flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Simultaneous immune complex-induced calcium flux and ROI production were detected by using the immune complex reagent Fc OxyBurst green (Molecular Probes), and data were collected on a FACStar Plus (Becton Dickinson Immunocytometry Systems) cytometer while the cell suspension was maintained at 37°C with continuous stirring.

Adhesion-dependent degranulation. Immulon 4 HBX plates (Thermo Labsystems, Helsinki, Finland) were coated overnight at room temperature with 150 μg of sheep fibrinogen (Sigma Aldrich)/ml in PBS. The wells were washed three times, and 10 μl of a 10 mM solution of $MgCl_2$ with or without TNF-α (500 ng/ml) or phorbol myristate acetate (PMA; 1 μM) was added to the appropriate wells (four wells per condition per time point). Prewarmed neutrophils (100 μl) suspended at 3×10^6 cells/ml in HBSS supplemented with 10 mM HEPES and 1 mM Ca^{2+} were added to each well. Wells were harvested after 0, 30, and 45 min, and cells were pelleted. Supernatant was diluted 4 to 20 times in carbonate buffer, and 100 μl per well was transferred to a fresh 96-well Immulon 4 HBX plate to allow lactoferrin binding overnight at 4°C. The enzyme-linked immunosorbent assay for lactoferrin was performed as previously described (38).

Expression of cell surface markers. Levels of receptor surface expression were measured by using flow cytometry. Cells were labeled with fluorescently conjugated antibodies (BD Pharmingen) for Ly6G (RB6-8C5), CD16/CD32 (2.4G2), CD18 (C71/16), Mac-1 (M1/70), and LFA-1 (M17/4). For the evaluation of upregulation of surface integrin expression following inflammatory stimulus, neutrophils were incubated with 50 ng of TNF-α (Peprotech, Princeton, N.J.)/ml for 30 min at 37°C.

Adherent respiratory burst. Ninety-six-well Immulon 4 HBX plates (Thermo Labsystems) were coated with 150 μg of fibrinogen (Sigma; F-9754)/ml in PBS, 15 μg of poly-RGD (Sigma; F-5022)/ml in PBS, or 20 μg of anti-CD18 (C71/16)



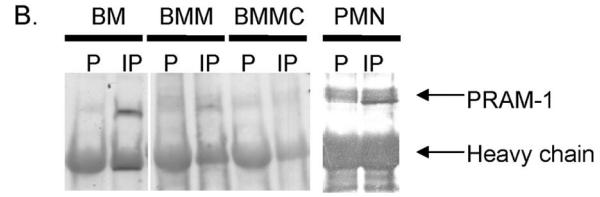


FIG. 1. Murine PRAM-1 sequence and expression pattern. (A) Alignment of human and murine PRAM-1 amino acid sequences. The amino acid sequences were aligned using VectorNTI DNA analysis software (Informax, Frederick, Md.). Identical residues are shaded. The SH3-like domain is boxed, and the tyrosine-based SH2 recognition sequences are underlined. (B) Murine PRAM-1 is expressed predominately in neutrophils. PRAM-1 was immunoprecipitated with PRAM-1 antiserum (IP) or with preimmune serum (P) from total bone marrow (BM), bone marrow-derived macrophages (BMM), bone marrow-derived mast cells (BMMC), or purified bone marrow neutrophils (PMN). Immunoprecipitates were blotted for PRAM-1.

or isotype control antibody (Pharmingen)/ml in carbonate buffer. Antibody-coated wells were blocked with 10% FCS for 30 min. Prewarmed neutrophils (100 μl) suspended at 4×10^6 cells/ml in HBSS–10 mM HEPES–100 mM ferricytochrome *c* (Sigma Aldrich)–1 mM Ca^{2+} were added to the wells. Adherent respiratory burst was measured through oxidation-induced changes in ferricytochrome C absorbance as previously described (34). When indicated, 50 ng of murine TNF-α/ml, 10 μM fMLP, or 100 nM PMA was added.

***Staphylococcus aureus* killing assay.** *S. aureus*, wood strain (American Type Culture Collection, Manassas, Va.), was grown overnight on brain heart infusion (BHI) agar (Difco, Sparks, Md.). The morning of the assay, five colonies were transferred into BHI broth and grown at 37°C with constant shaking. The bacterial concentration was measured by absorbance at 590 nm. Shaking was terminated when absorbance reached 0.1 to 0.4, and the bacterial concentration was calculated using an absorbance constant attained from previous serial dilutions of cultures. The *S. aureus* cells were opsonized with 1 μl of opsonizing antibody (Molecular Probes) in HBSS with 50% mouse serum for 15 min at 37°C and then washed twice with PBS. Isolated bone marrow-derived neutrophils suspended in HBSS–10 mM HEPES were warmed for 30 min at 37°C. Bacteria and neutrophils (20×10^6 cells/ml) were combined at a 1:2 ratio in HBSS containing 10% mouse serum and rotated at 37°C. At designated time points, three 40-μl aliquots were removed and combined with equal volumes of 2% NP-40 on ice to lyse neutrophils. Serial dilutions of the lysates were plated upon BHI agar and grown overnight. The reported percent survival is represented as the percentage of colonies remaining at each time point in comparison to the number at time zero for each condition.

Lipopolysaccharide-induced endotoxic shock. Cohorts of 8 to 10 age-, weight-, backcross-, and gender-matched mice were injected intraperitoneally with 0.5 or 1 mg of lipopolysaccharide (LPS; Sigma Aldrich) in sterile PBS. Mice were monitored for signs of endotoxic shock (diarrhea, eye and nose discharge, fur ruffling) every 6 h in the first 24 h and twice per day in the following days. Moribund mice were euthanized to minimize stress and suffering. All remaining mice were euthanized on day 7.

RESULTS

Murine PRAM-1 is expressed predominantly in neutrophils. Previous studies of human tissue and myeloid leukemic cell lines demonstrated that human PRAM-1 is expressed during myeloid differentiation and is upregulated during granulocytic (retinoid-induced) differentiation of the promyelocytic cell line NB4 (41). To develop a murine model system, we first isolated cDNA for murine PRAM-1 (GenBank AY665714). Analysis of the full-length amino acid sequence revealed that murine PRAM-1 is 53% identical and 57% similar to human PRAM-1 (Fig. 1A), with greatest homology in the carboxy-terminal SH3-like domain (98% identical). Human PRAM-1 also contains two tyrosine-based motifs that conform to the consensus sequence recognized by group I SH2 domains (52). One of these motifs is wholly conserved in murine PRAM-1, while the other contains a single amino acid substitution at position Y + 2 (D→A), which likely alters its SH2 domain binding affinity. A BLAST search with murine PRAM-1 identified two mRNA sequences generated by Riken (Kanagawa, Japan). The first sequence was derived using automated computational analysis (GenBank XM128537). The murine PRAM-1 sequence generated by RT-PCR in our laboratory is identical to this derived sequence except for a 45-amino-acid region (residues 525 to 662) near the carboxy terminus, which is not present in the Riken sequence. Interestingly, the PRAM-1 homolog ADAP exhibits 120- and 130-kDa forms that result from alternative splicing of 138 bp (46 amino acids), also within the carboxy terminus (57). Analysis of the murine PRAM-1 locus on chromosome 17 revealed that exons 3 and 4 encode the inserted 45 amino acids, suggesting that the Riken sequence may represent a splice variant of PRAM-1. However, multiple independent derivations of PRAM-1 cDNA by RT-PCR from murine bone marrow yielded only the insert-containing sequence (data not shown). The second Riken sequence was derived using a cDNA library from mouse neonatal skin (GenBank AK076291). This sequence includes the 45 amino acids representing exons 3 and 4 but differs from both our sequence and the computationally derived sequence at several nucleotide positions.

Like human PRAM-1, the murine ortholog is found predominately in murine bone marrow and in purified mature neutrophils. Although PRAM-1 mRNA was detectable by RT-PCR in bone marrow-derived macrophages and mast cells (data not shown), immunoprecipitation and Western blotting

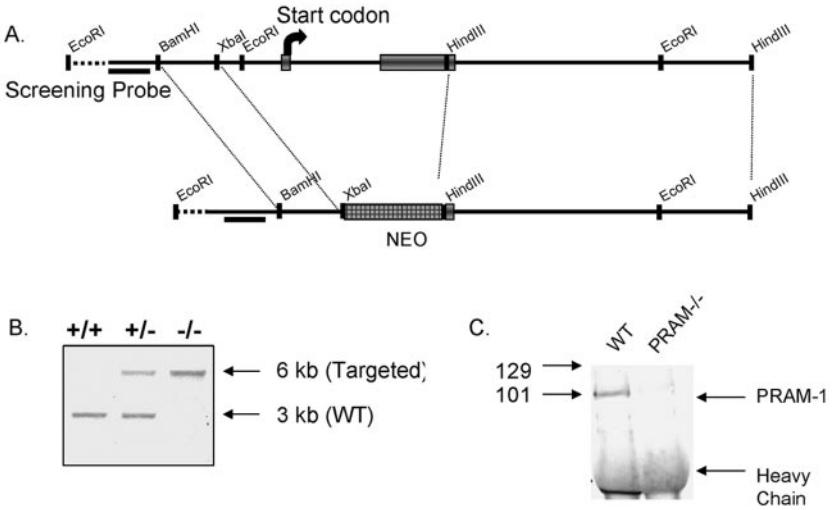


FIG. 2. PRAM-1 genetic targeting. (A) Genetic targeting scheme showing the neomycin resistance cassette (NEO) that was inserted by homologous recombination, replacing the bulk of the first and second exons (filled bars). Also indicated is the 5′ probe for Southern blot screening. (B) Genomic tail DNA was analyzed by Southern blotting, confirming the presence of the targeted band in heterozygote and knockout mice. (C) Western analysis of anti-PRAM-1 immunoprecipitates from bone marrow derived from WT or PRAM-1$^{-/-}$ mice.

for PRAM-1 protein indicated only very weak expression in macrophages and undetectable levels in mast cells (Fig. 1B). Together, these data identify murine PRAM-1 as a largely neutrophil-specific adaptor protein.

Genetic targeting of PRAM-1. To evaluate the importance of PRAM-1 for neutrophil differentiation and function, we generated mice with targeted disruption of the PRAM-1 gene. A neomycin resistance cassette was inserted into the PRAM-1 locus by homologous recombination, replacing the first exon and most of the second exon (Fig. 2A). Locus-specific integration was confirmed by Southern analysis of EcoRI-digested genomic DNA (Fig. 2B). Immunoprecipitation and Western analysis of bone marrow cell lysates confirmed that PRAM-1 protein is not expressed in mice homozygous for the disrupted gene (Fig. 2C). PRAM-1-deficient mice are viable, fertile, and born at the expected frequency from heterozygote-by-heterozygote matings.

PRAM-1$^{-/-}$ mice have normal numbers of neutrophils. The expression pattern and regulation of PRAM-1 are similar to that of the transcription factor CCAAT/enhancer binding protein epsilon (C/EBPε). Both proteins are predominantly expressed in myeloid tissue and are transcriptionally repressed by the oncogenic fusion protein PML-RARα in human acute promyelocytic leukemia blasts (26, 41, 60, 61). C/EBPε is required for granulocytic differentiation in both mice and humans (27); therefore, we asked if PRAM-1 might facilitate signaling pathways regulating neutrophil differentiation or maturation. However, analysis of PRAM-1-deficient mice revealed no gross abnormalities in the neutrophil compartment or any other hematopoietic lineage. As measured by CBC, wild-type (WT) and PRAM-1$^{-/-}$ mice had comparable numbers of peripheral blood neutrophils as well as total leukocytes (Fig. 3A). Microscopically, PRAM-1$^{-/-}$ bone marrow was indistinguishable from that of the wild type, demonstrating normal cellularity and morphology of developing neutrophils and other hematopoietic precursors (Fig. 3B, upper panel). Bone marrow-purified PRAM-1$^{-/-}$ neutrophils also demonstrate normal nuclear morphology and stain for chloroacetate esterase, a neutrophil-specific marker (2, 29) (Fig. 3B, lower panels). Additionally, cell surface expressions of the granulocyte markers Gr-1, FcγRII/III, and β_2 (CD18) and β_3 (CD61) integrins were equivalent in the WT and PRAM-1$^{-/-}$ neutrophils (Fig. 3C). Collectively, these data indicate that PRAM-1 is not essential for normal neutrophil differentiation or maturation.

PRAM-1$^{-/-}$ neutrophils flux calcium and produce ROI after FcγR stimulation. The adaptor SLP-76, a binding partner of PRAM-1 (41), modulates signaling pathways downstream of tyrosine kinase-associated receptors in several hematopoietic lineages, including the T-cell receptor in T cells (9), FcεR in mast cells (46), and GPVI-collagen receptor and $\alpha_{IIb}\beta_3$ integrin in platelets (8, 24). Recently, studies of neutrophils demonstrated that SLP-76 is also required for signaling through Fcγ receptors and integrins (43). As PRAM-1 can associate with SLP-76 in myeloid cell lines, we hypothesized that PRAM-1 might also modulate the signaling cascades of these receptors in neutrophils. In response to cross-linking of FcγR, WT neutrophils mobilize calcium and produce ROI (11, 25). To test the ability of PRAM-1-deficient neutrophils to generate a calcium flux, Indo-1-labeled PRAM-1$^{-/-}$ and WT neutrophils were incubated with antibodies to FcγRII/III. When stimulated by hyper-cross-linking, the FcγR PRAM-1$^{-/-}$ neutrophils produced a calcium flux equivalent to that of WT neutrophils (Fig. 4A). To assess FcγR-induced ROI production, neutrophils were incubated with Fc OxyBurst, a synthetic immune complex reagent conjugated to the oxidation-sensitive compound dichlorodihydrofluorescein diacetate (DCHF). When Fc OxyBurst is phagocytosed and oxidized, DCHF fluorescence can be quantified by flow cytometry (17, 23). Figure 4B shows ROI production by WT and PRAM-1$^{-/-}$ neutrophils following immune complex internalization. Though in this experiment PRAM-1$^{-/-}$ neutrophils produce slightly fewer ROI, this difference is not reproducible. Together, these studies sug-

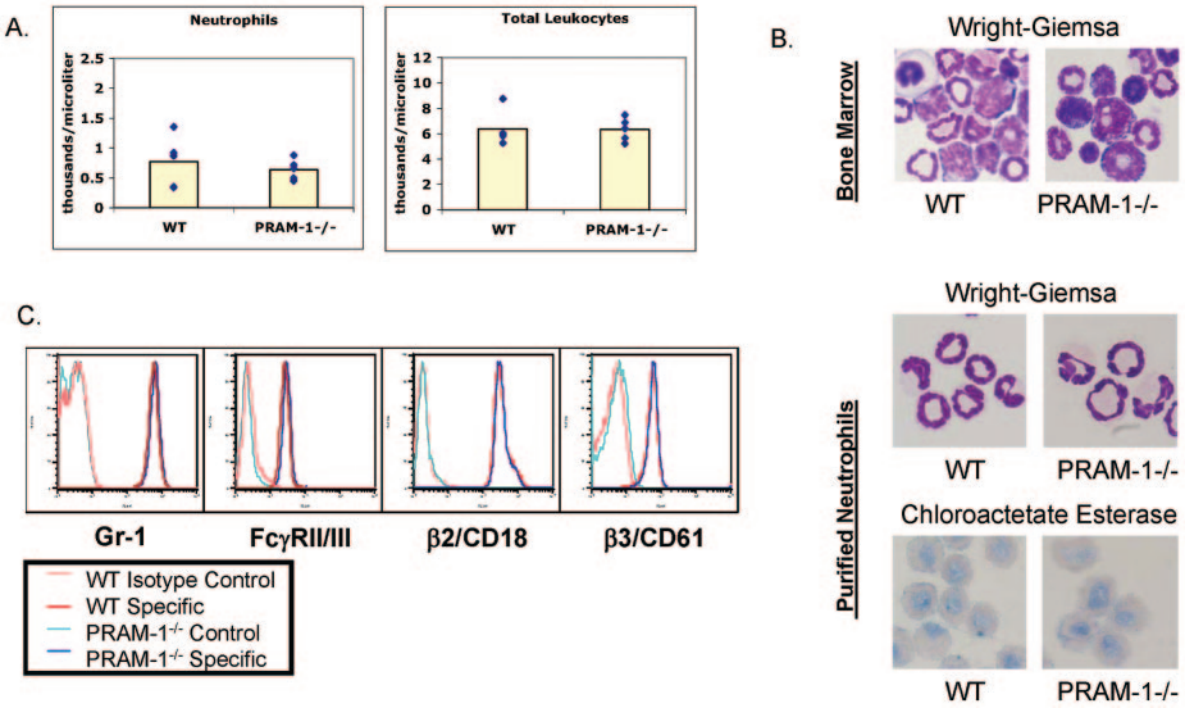


FIG. 3. PRAM-1$^{-/-}$ mice show normal neutrophil development. (A) Neutrophil (left) and total leukocyte (right) numbers were quantified by CBCs of peripheral blood. (B) Bone marrow cells and purified neutrophil smears were stained with Wright-Giemsa stain. Staining with chloroacetate esterase (lower panels), a neutrophil-specific marker, was also normal. (C) Surface phenotype of purified neutrophils was assessed by staining with antibodies to the indicated cell surface markers or with an isotype control antibody and flow cytometric analysis.

gest that FcγR-dependent signaling remains intact in the absence of PRAM-1.

PRAM-1$^{-/-}$ neutrophils have defects in integrin-mediated responses. In neutrophils, integrins are essential for several functions, including adhesion, spreading, migration, and adhesion-dependent ROI production and degranulation (4, 32). The PRAM-1 homolog ADAP has been shown to be required for integrin-mediated T-cell adhesion (19, 45). We therefore assessed each of these responses following integrin stimulation of PRAM-1$^{-/-}$ neutrophils. Mature, unstimulated neutrophils do not spread on fibrinogen-coated surfaces; however, stimulants such as TNF-α activate integrins, allowing for cell spreading. During activation with TNF-α, both the percentage of cells that spread (WT, 29.8% ± 8.8%; knockout, 34.1% ± 4.4%) and the area of the spread cells (WT, 800.6 ± 58 pixels; knockout, 745.5 ± 40.2 pixels) were equivalent in WT and PRAM-

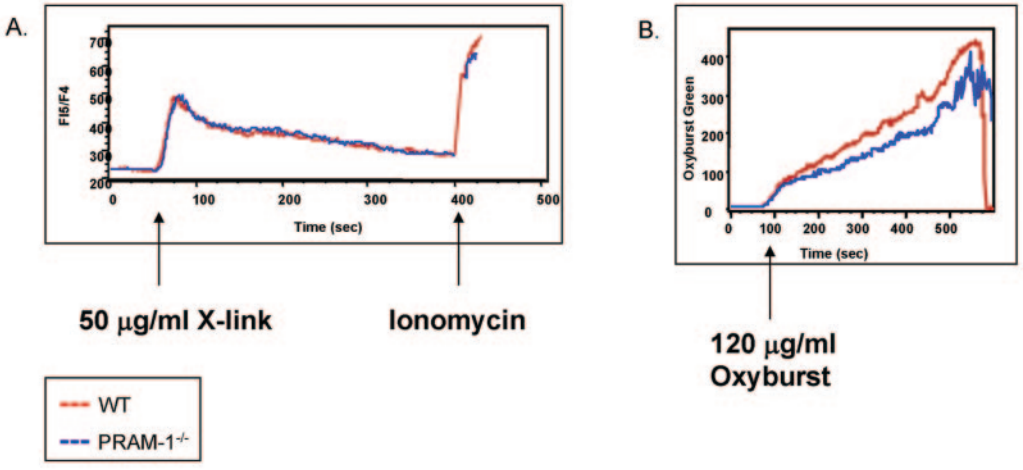


FIG. 4. PRAM-1$^{-/-}$ neutrophils mobilize calcium and produce ROI following FcγR stimulation. (A) Neutrophils were labeled with Indo-1 and incubated with an antibody against FcγR. Calcium flux was measured by flow cytometry following cross-linking of the receptors with anti-rat immunoglobulin G (first arrow) and ionomycin (second arrow). (B) ROI production and phagocytosis were measured by flow cytometry following stimulation with Fc OxyBurst reagent, an immune complex linked to an oxidation-sensitive dye.

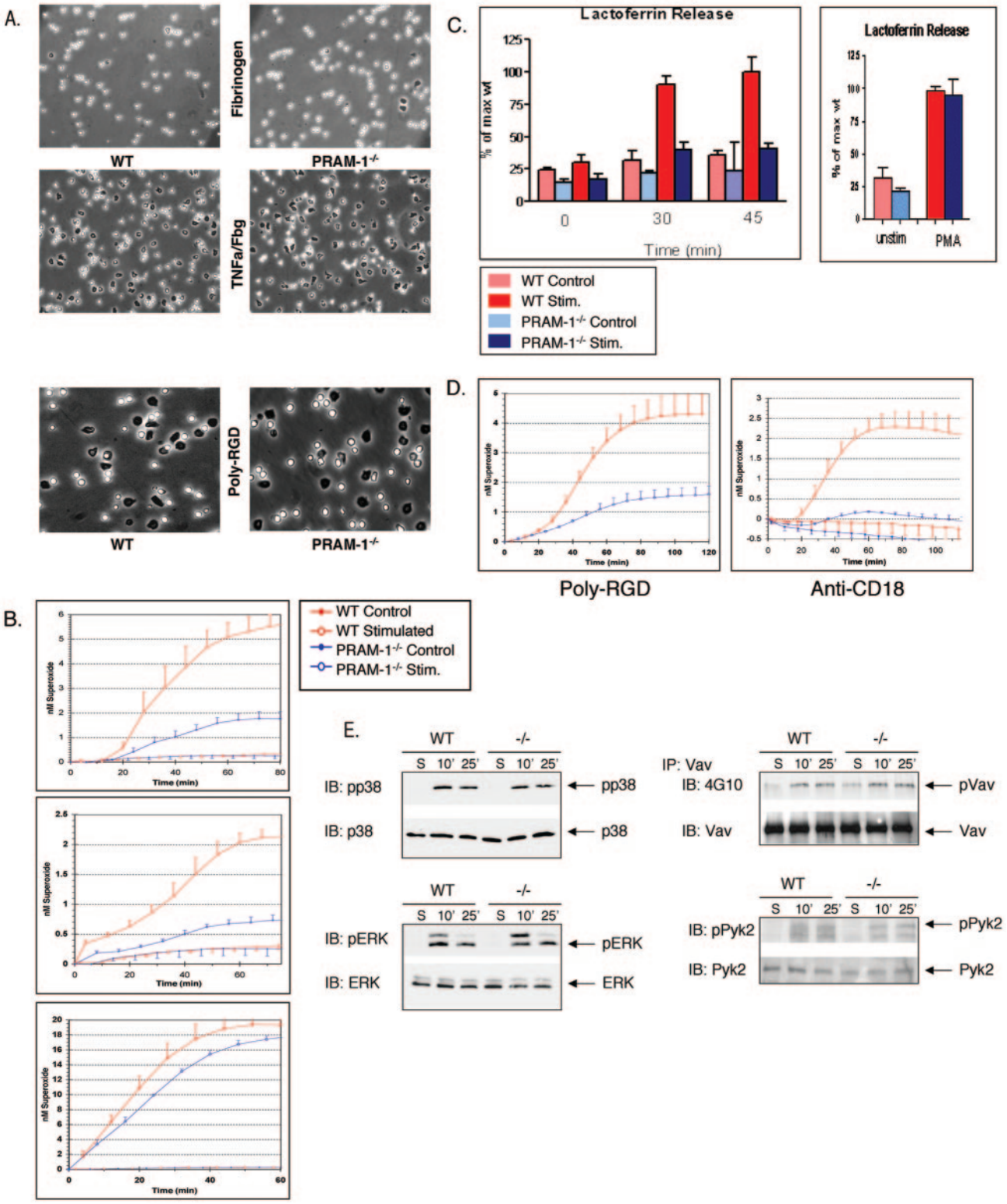


FIG. 5. PRAM-1 neutrophils display selective defects in integrin-dependent responses. (A) Neutrophils were placed in chamber slides coated with fibrinogen (integrin-ligand) with or without 50 nM TNF or 15 μg of poly-RGD/ml (lower panels) for 30 min at 37°C and were viewed by phase contrast microscopy. Spread cells (dark, irregular cells) were distinguished from nonspread cells (round, bright cells) by morphology and were quantified using immunoprecipitation lab image analysis software. (B) Adhesion-dependent ROI production. Purified neutrophils were plated in wells coated with fibrinogen in the presence of 50 nM TNF (top), 10 μM fMLP (middle), or 1 μM PMA (bottom), and ROI production was measured by ferricytochrome *c* oxidation. (C) Adhesion-dependent degranulation. Neutrophils were stimulated in wells coated with fibrinogen with or without TNF (left) or PMA (right). Lactoferrin in supernatants was measured by enzyme-linked immunosorbent assay. Results are

$1^{-/-}$ neutrophils (Fig. 5A, upper panels). Spreading of PRAM-$1^{-/-}$ and WT neutrophils was also equivalent on slides coated with poly-RGD (Fig. 5A, lower panels), an engineered polymer of the fibronectin-binding motif (Arg-Glu-Asp) that directly stimulates integrins without an additional activation signal.

Studies suggest that integrin-induced spreading is a prerequisite for adhesion-dependent inflammatory responses, such as ROI production and degranulation (42). However, despite normal spreading in response to integrin ligands, we observed that adhesion-dependent ROI production in the PRAM-$1^{-/-}$ neutrophils was significantly decreased following stimulation with fibrinogen in concert with the inflammatory agent TNF-α or the bacterial peptide fMLP (Fig. 5B). Adhesion-dependent degranulation, as measured by release of the granule enzyme lactoferrin, was also significantly decreased in the PRAM-$1^{-/-}$ neutrophils (Fig. 5C). The defects in both ROI production and degranulation were rescued by addition of PMA, demonstrating that the machinery required for each of these processes is functional in PRAM-$1^{-/-}$ neutrophils. To ensure that the observed defects are restricted to integrin-dependent signaling events, we next examined the effect of stimulating WT and PRAM-1-deficient neutrophils with fMLP on plates coated with FCS. These experiments were performed in magnesium-free medium to ensure that integrin ligands present within the FCS would not interact with their receptors. While ROI production in response to fMLP alone is quantitatively less robust than the adhesion-dependent response, both WT and PRAM-1-deficient neutrophils generate ROI under these conditions, confirming that the phenotype of the PRAM-1-null cells is restricted to the adhesion-dependent response (data not shown).

Adhesion-dependent neutrophil functions require two stimuli. First, an "inside-out" proinflammatory stimulus serves to activate the integrins by increasing receptor affinity (conformational change), avidity (membrane relocalization), and level of surface expression. Second, an "outside-in" signal is initiated by binding of the activated integrin to its ligand, yielding adhesion-dependent effector functions. To determine whether the adhesion-dependent deficiencies in PRAM-$1^{-/-}$ neutrophils resulted from a defect in inside-out or outside-in signaling, we assayed each of these pathways in isolation. Stimulation of suspended cells with chemokines or cytokines induces several inside-out responses, including upregulation of integrin surface expression via membrane fusion of primary granules (32). We found that upregulation of Mac-1 ($\alpha_M\beta_2$) surface expression in response to TNF-α was equivalent in PRAM-$1^{-/-}$ and WT neutrophils. In addition, activation of cells with the chemoattractant fMLP produced a normal calcium flux and adhesion-independent ROI production in PRAM-$1^{-/-}$ neutrophils (data not shown). Collectively, these data suggest that these inside-out signals are intact in the absence of PRAM-1.

We assessed outside-in signaling by measuring ROI production following direct integrin stimulation with either plate-bound anti-CD18 (β_2 integrin) antibody or poly-RGD. Both of these stimuli cross-link integrins and initiate integrin signaling without prior inside-out integrin activation. ROI production following either anti-CD18 or poly-RGD stimulation was decreased by 60 to 80% in the PRAM-$1^{-/-}$ neutrophils (Fig. 5D). Together these data suggest that PRAM-1 is necessary for the integrin-mediated outside-in signal but not for cytokine-induced integrin activation.

To address the mechanism behind the defect in adhesion-dependent ROI production and degranulation, we undertook a biochemical survey of several signaling molecules known to be active in integrin signaling pathways. Vav is a guanine nucleotide exchange factor that regulates actin reorganization following integrin ligation (13, 37, 49, 63). Pyk2 is a member of the FAK family kinases, and a recent study has implicated this molecule in adhesion-dependent ROI production in neutrophils (18, 22). The mitogen-activated protein (MAP) kinases ERK2 and p38 are also activated following integrin stimulation and are thought to be necessary for activation of effector functions, although their precise role in neutrophils is not well defined (15, 32). Vav, MAPK, and Pyk2 family members all undergo phosphorylation following integrin stimulation. As shown in Fig. 5E, PRAM-$1^{-/-}$ and WT neutrophils demonstrate equivalent phosphorylation of these molecules in response to the stimulus poly-RGD. These results suggest that PRAM-1 is not required for key phosphorylation events initiated by integrin stimulation.

PRAM$^{-/-}$ neutrophils kill bacteria in vitro, and PRAM$^{-/-}$ mice show normal susceptibility to LPS-induced endotoxic shock. ROI production and the release of toxic granule contents are essential processes that enable neutrophils to kill invading pathogens, yet integrin-dependent production of these substances has also been implicated in tissue damage associated with endotoxic shock (7, 33). Thus, we asked next whether the observed reduction in ROI production and degranulation impairs the ability of PRAM-$1^{-/-}$ neutrophils to kill bacteria or, conversely, attenuates neutrophil-dependent pathology in an in vivo model of endotoxic shock. To measure bacterial killing, purified neutrophils were incubated with *S. aureus* cells, and bacterial survival was assessed at several time points. In this assay, WT and PRAM-1-deficient neutrophils killed bacteria with similar efficiencies (Fig. 6A). In addition, no survival benefit was conferred by loss of PRAM-1 in LPS-induced endotoxic shock, suggesting that residual integrin-dependent ROI generation and degranulation capabilities observed in PRAM-1 mutant mice are sufficient to mediate tissue damage leading to lethality (Fig. 6B).

DISCUSSION

Our initial studies described human PRAM-1 as a novel adaptor that is expressed early during myeloid differentiation and in mature granulocytes. In this report we have expanded

represented as the percentage of maximal degranulation by the wild-type neutrophils. (D) ROI production in response to direct integrin stimulation. Neutrophils were placed in wells coated with poly-RGD or with anti-CD18 (β_2) antibody, and ROI production was measured as described for panel B. As a control for antibody stimulation, cells were placed in wells coated with isotype control antibody. (E) Integrin-mediated biochemical events. Neutrophils were stimulated on plates coated with poly-RGD or left in suspension (S) and then lysed and immunoprecipitated (IP) and/or immunoblotted (IB) with the indicated antibodies. Data in this figure are representative of results from at least four independent experiments.

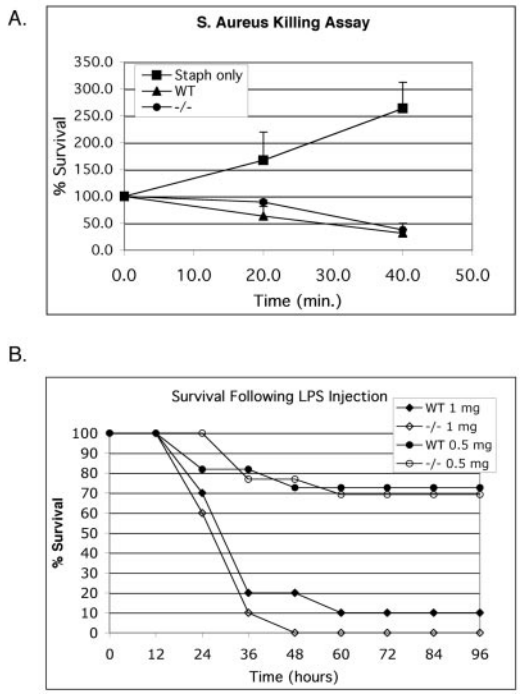


FIG. 6. PRAM$^{-/-}$ neutrophils kill bacteria in vitro and show normal susceptibility to LPS-induced endotoxic shock. (A) Fresh cultures of *S. aureus* cells in log-phase growth were incubated alone or with wild-type or PRAM-1$^{-/-}$ neutrophils. Aliquots were removed at the indicated time points, diluted, and plated on BHI agar plates. Bacterial colonies were counted 12 h later. (B) Mice were injected intraperitoneally with 0.5 mg of LPS and monitored for 7 days. Data are representative of results from three independent experiments.

this work to characterize murine PRAM-1, which is 57% similar to its human counterpart and is also expressed predominantly in neutrophils. To explore the biology of PRAM-1, we generated mice with a targeted deficiency in the PRAM-1 locus. These mice develop normal numbers of neutrophils, which exhibit normal morphology and surface phenotype, demonstrating that PRAM-1 is not required for neutrophil differentiation. Functional studies of mature neutrophils revealed that while PRAM-1 is not required for FcγR-induced ROI production or calcium flux, select integrin-dependent functions, including degranulation and production of reactive oxygen species, were markedly impaired in the absence of PRAM-1. Surprisingly, other integrin-dependent functions, including cell spreading and activation of key biochemical pathways, were normal. Thus, PRAM-1 appears to play a selective role in the signaling pathways leading to integrin-dependent functions.

In neutrophils, integrins are critical for cell adhesion, cell spreading, ROI production, degranulation, and phagocytosis (4, 32). However, understanding of the biochemical pathways underlying these effector functions is limited. Several studies have identified key signaling molecules that are essential for the integrin signaling cascade in neutrophils. Initiation of integrin signaling relies on the Src and Syk family protein tyrosine kinases, and cells deficient in multiple Src kinases or Syk are unable to spread, produce ROI, or degranulate in response to integrin stimulation (25, 33–35, 38, 40, 53, 62). A number of genetic and biochemical studies have suggested that adaptors, such as Shc (12, 44, 47) and MyD88 (14), are important in neutrophil biology. To date, few studies have documented a role for adaptors in neutrophil integrin signaling. However, a recent report from our lab found that the adaptor SLP-76 is essential for integrin-dependent cell spreading and ROI production in neutrophils (43).

In this report we show that the adaptor PRAM-1 is also required for optimal integrin signaling in neutrophils. However, unlike the broad defects in integrin-mediated function exhibited by neutrophils deficient in Syk, SLP-76, or multiple Src family kinases, PRAM-1 deficiency affects only select responses. While adhesion-dependent ROI production and degranulation are significantly impaired, spreading is intact in the absence of PRAM-1. These selective defects are intriguing because to date, few signaling molecules that allow separation of these integrin responses have been identified. Thus, our studies of PRAM-1 suggest a point at which integrin-dependent signaling pathways diverge, resulting in a multiplicity of adhesion-dependent responses.

Several models could potentially explain the selective impact of PRAM-1 deficiency on some, but not other, integrin-dependent responses. First, it is possible that cell spreading is simply a less sensitive assay integrin function than are ROI production or degranulation and that PRAM-1-deficient neutrophils may generate a signal that is sufficient to initiate spreading but below the threshold for maximal ROI and granule release. Alternatively, PRAM-1 may participate in a signaling pathway that is required for ROI production and degranulation but completely independent of cell spreading and actin cytoskeletal rearrangement. This option is perhaps less likely, as shown by the fact that several studies have demonstrated that pharmacologic disruption of actin reorganization also inhibits ROI production and degranulation (42, 54). A third possibility is that PRAM-1 participates in a signaling pathway downstream of and dependent upon cytoskeletal rearrangements, linking actin remodeling to assembly of the NADPH oxidase complex (required for ROI production) and exocytosis of granules. This notion is supported by a recent study of the actin cross-linking protein L-plastin (L-PL), which demonstrated that L-PL-deficient neutrophils adhere and spread similarly to WT neutrophils but do not produce detectable levels of reactive oxygen species following integrin stimulation (7). The authors of that study propose that the actin cross-linking activity of L-PL is necessary for assembly of the integrin signaling complex within a cytoskeletal scaffold. The phenotype of L-PL-deficient mice is similar in some regards to that of PRAM-1-deficient animals and suggests that PRAM-1, like L-PL, may play a role in the assembly of actin-associated signaling complexes.

Analysis of the phosphorylation state of several signaling molecules did not reveal a defect that would suggest a mechanism underlying the selective integrin defects in PRAM-1-deficient neutrophils. However, although phosphorylation is an important mechanism of signal modulation, the activity of signaling mediators is also regulated by other posttranslational modifications (acylation and ubiquitination), by cellular localization and sequestration, and by the level of transcription. Regulation of PRAM-1 function in integrin-mediated signal transduction may be complex and utilize several of these mechanisms. By facilitating multiple protein-protein interactions,

adaptors are thought to create molecular scaffolds that assure optimal spatiotemporal organization of effectors and substrates within a signaling cascade (59). Thus, PRAM-1 deficiency may not disturb the posttranslational modification of key signaling molecules but rather may disturb their localization. Future studies will therefore address PRAM-1 localization in normal neutrophils and determine how the absence of PRAM-1 affects and/or impairs the movement of relevant signaling molecules. Additionally, it is possible that PRAM-1 deficiency impairs the function of other, as yet unidentified, key signaling intermediates. As future studies yield greater insight into the molecular basis for integrin-dependent events, it will be possible to investigate the impact of PRAM-1 deficiency on additional signaling mediators.

Although PRAM-1-deficient neutrophils are significantly impaired in their ability to produce ROI and release granule enzymes, these defects did not hinder ex vivo bacterial killing or ameliorate neutrophil-mediated toxicity in LPS-induced endotoxic shock. It is well established that integrin-mediated phagocytosis and ROI production are required for killing of *S. aureus* (16, 50). However, many other receptors on the neutrophil surface also interact with bacteria. It is plausible that these interactions, in combination with the residual integrin-induced ROI production and degranulation, are sufficient for bacterial killing in this ex vivo model. Similarly, in vivo neutrophil function during endotoxic shock is complex. Although several groups have demonstrated with genetically targeted mice that integrin signaling molecules are critical for the neutrophil-mediated pathology in this model (33, 55), it is possible that the residual ROI and degranulation responses in PRAM-1-deficient neutrophils are sufficient to initiate and amplify an inflammatory response and resultant tissue damage.

Unlike in SLP-76- and Syk-deficient neutrophils, adhesion-dependent ROI production and degranulation are decreased but not absent in PRAM-$1^{-/-}$ neutrophils. One possible explanation for the residual integrin-dependent function in PRAM-1-deficient neutrophils is compensation by one or more homologous molecules. PRAM-1 does indeed have a structural homolog, ADAP, that is expressed in mature neutrophils (unpublished data). Preliminary analysis of ADAP-deficient neutrophils revealed a phenotype similar to PRAM-1-deficient neutrophils, with normal FcγR-induced calcium flux but adhesion-dependent ROI production that is only 40 to 60% of normal (data not shown). To address the possible functional redundancy of PRAM-1 and ADAP, we generated mice deficient in both adaptors. Interestingly, however, study of these animals has not revealed a compound phenotype (unpublished data). These findings suggest the possibility that additional adaptors or alternative signaling pathways support adhesion-dependent responses following integrin ligation in neutrophils.

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