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The podosome marker protein Tks5 regulates macrophage invasive behavior

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Abstract

Tks5 is a Src substrate and adaptor protein previously recognized for its regulation of cancer cell invasion through modulation of specialized adhesion structures called podosomes/invadopodia. Here we show for the first time that Tks5 localizes to the podosomes of primary macrophages, and that Tks5 protein levels increase concurrently with podosome deposition during the differentiation of monocytes into macrophages. Similar results are reported for model THP-1 cells, which differentiate into macrophages and form proteolytically-active podosomes in response to a PKC signaling agonist (PMA) and with sensitivity to a PKC inhibitor (bisindolylmaleimide). Genetic manipulation of Tks5 expression (silencing and overexpression) in stable THP-1 cell lines does not independently alter this macrophage differentiation process. Nor do these cells lose the ability to focalize F-actin and its accessory proteins into podosome-like structures following PMA treatment. However, Tks5 directly controls podosome-associated gelatin degradation and invasion through collective changes in adhesion, chemotaxis, and the expression/proteolytic activity of MMP9. The Src family kinase-dependent phosphorylation of Tks5 is also implicated in the regulation of THP-1 macrophage invasive behavior. These results therefore define a previously unappreciated function of Tks5 signaling specific to the functional attributes of the macrophage podosome in adhesion, motility, and extracellular matrix-remodeling.

Keywords

podosomes; invadopodia; adhesion; migration; invasion; Src

INTRODUCTION

Macrophages are part of the myeloid lineage of the hematopoietic system and differentiate from circulating monocyte precursors in response to pathogen exposure or tissue damage. As phagocytes, macrophages rid the body of harmful microorganisms or debris from damaged tissue. By secreting chemokines/cytokines, macrophages also perform an important function in the recruitment of other immune cells, and the activation of cell growth necessary for tissue repair. To perform these important functions, macrophages must pass from the vasculature to the interstitial tissue in a complex series of invasive activities

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involving permeation of the endothelial lining of blood vessels (transendothelial migration), the dense matrix barrier that comprises the basement membrane, and the interstitial matrix of the tissue itself (Nourshargh et al. 2010). Recent studies indicate that the type of barrier to which a macrophage is confronted dictates the invasive mechanism that is deployed (Friedl and Wolf 2010; Van Goethem et al. 2010). For example, loosely attached and highly contractile macrophages squeeze their way through a porous matrix by amoeboid-type invasion. Alternatively, macrophages may couple integrin-based adhesion with proteolysis to actively create channels through which the matrix may be successfully navigated.

First recognized by their punctate arrangement of filamentous actin (F-actin), podosomes have long been recognized as the primary matrix adhesion structures of macrophages (Lehto et al. 1982; Marchisio et al. 1987; Evans et al. 2003; Calle et al. 2006). Podosomes are defined microscopically as actin-rich protrusions of the ventral cell surface with co-localized marker proteins that facilitate the rapid turnover of these structures and their protease-mediated, matrix-remodeling activity (Gimona and Buccione 2006; Linder 2007; Gimona et al. 2008). This latter function, measured by an *in situ* zymography assay, is a distinguishing property of podosomes relative to classic focal adhesions, and likely contributes to the invasive properties of podosome-competent cell types (smooth muscle cells, endothelial cells, osteoclasts, dendritic cells), including macrophages (Chen 1989; Bowden et al. 2001; Block et al. 2008; Van Goethem et al. 2010). At present, our knowledge of macrophage podosome formation and activity is based on the study of constituent proteins capable of initiating podosome deposition (CSF-1, Src family tyrosine kinases, PKC agonists), facilitating actin polymerization (Arp2/3 complex, WASp), acting as scaffolds (WASp), or contributing directly to proteolysis (MMPs, lysosomal proteases) (Linder et al. 1999; Linder et al. 2000; Launay et al. 2003; Linder et al. 2003; Calle et al. 2006; Tsuboi 2006; Wheeler et al. 2006; Yamaguchi et al. 2006; Tsuboi 2007; Dovas et al. 2009; Cougoule et al. 2010; Van Goethem et al. 2010).

Originally identified in a search for novel Src substrates, the adaptor protein Tks5 is broadly recognized as a general marker for podosomes in normal cell types and for related structures called invadopodia in cancer cell lines (Lock et al. 1998; Courtneidge et al. 2005). The amino terminal Phox (PX) homology domain of Tks5 serves as a lipid-binding module for PtdIns(3)P and PtdIns(3,4)P₂ (Abram et al. 2003). In model Src-transformed NIH3T3 (Src3T3) cells these phosphoinositides appear to be an early signal for the membrane recruitment of Tks5 and the formation of invadopodia structures (Abram et al. 2003; Oikawa et al. 2008). This is consistent with the PX domain of Tks5 being both necessary and sufficient for invadopodia localization in these cells (Abram et al. 2003). The SH3 domains of Tks5 are protein-binding modules. Ongoing research suggests that Src-dependent Tks5 phosphorylation may lead to the recruitment of proteins that scaffold invadopodia machinery (Nck and Grb2), focalize actin polymerization (N-WASp), and potentially degrade matrix proteins (ADAMs) (Abram et al. 2003; Oikawa et al. 2008; Stylli et al. 2009). Indeed Tks5 silencing in Src3T3 cells leads to a deficiency in invadopodia, an impairment in invasion through a Matrigel matrix, and attenuated tumor growth and angiogenesis *in vivo* (Seals et al. 2005; Blouw et al. 2008). In human breast cancer and melanoma cell lines and tissue samples, Tks5 protein levels predict matrix-remodeling and invasive potential as well as tumor stage (Seals et al. 2005). It is possible that Tks5 could serve as a biomarker for later stages of tumor progression associated with invasion and metastasis.

There have been few studies of Tks5 in normal cell types, and with the exception of a putative role in neurotoxicity (Malinin et al. 2005), much of this research describes the podosome localization of Tks5 (osteoclasts, myoblasts, smooth muscle cells) (Courtneidge et al. 2005; Thompson et al. 2008; Crimaldi et al. 2009). These studies also suggest that interactions between Tks5 and dystroglycan, AFAP-110, p190RhoGAP, and cortactin may

be important for podosome formation, however podosome-associated matrix degradation and invasion were not investigated (Thompson et al. 2008; Crimaldi et al. 2009). Nor has the role of Tks5 in macrophages been studied in any context to date. Here we demonstrate that Tks5 is a marker protein for podosomes in both primary macrophages and in macrophages derived from model THP-1 cells. We further show that Tks5 protein accumulates during macrophage differentiation concurrently with podosome deposition. However, while genetic manipulation of Tks5 expression and activity impacts the functional attributes of podosomes associated with invasive behavior, the structural features associated with the focalization of F-actin remain relatively unaltered. These studies confirm the utility of THP-1 cells for podosome development studies, and the potentially selective contributions of the Tks5 adaptor protein to podosome functionality in this cell type.

MATERIALS and METHODS

Cell Culture

The THP-1 cell line (ATCC, Manassas, VA) was routinely maintained as a suspension culture at a concentration between 2×10^5 and 1×10^6 cells/ml at 37°C and 5% CO₂. THP-1 cells were cultured in RPMI-1640 media (Thermo Scientific Hyclone, Logan, UT) formulated with 4.5g/L glucose, 1.5g/L sodium bicarbonate, 10mM Hepes, 110mg/L sodium pyruvate and supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 2mM L-glutamine (Hyclone), 1% MEM vitamins (Hyclone), 0.05mM 2-mercaptoethanol (EMD, Gibbstown, NJ), and 1% penicillin/streptomycin (Hyclone). THP-1 cells were differentiated into macrophages by supplementing 25nM PMA/0.1% DMSO (Sigma-Aldrich) or 100nM 1,25-(OH)₂D₃/0.1% ethanol (final concentrations) into the culture for a period of 4 to 72 hours in the presence or absence of either 10μM GM6001 (Enzo Life Sciences, Plymouth Meeting, PA)/DMSO, 6μM bisindolylmaleimide GF 109203X (Cell Signaling, Danvers, MA)/DMSO, or 10μM PP2 (EMD)/DMSO. Primary macrophages were differentiated from human peripheral blood monocytes (PBMCs) over a 6 day period. Briefly, whole blood was collected from healthy donors in accordance with an institutionally-approved IRB protocol, immediately diluted 1:1 in Hank's Balanced Salt Solution (Hyclone), overlaid on an equal volume of Histopaque (Sigma-Aldrich), and separated into a monocyte-enriched fraction by density gradient centrifugation at 900g for 30 minutes at room temperature. The interface was then collected, and washed thrice in 5 volumes of PBS to remove platelets, before the final pellet was suspended in RPMI-1640 media formulated with 2mM glutamine and supplemented with 10% FBS, 10mM Hepes, pH 7.4 (Lonza, Chicago, IL), 1% sodium pyruvate (Invitrogen, Carlsbad, CA), 1% non-essential amino acids (Lonza), and 1% penicillin/streptomycin. PBMCs were distributed at a range of 4×10^6 – 1×10^7 cells per 6cm cell culture dish, and cultured at 37°C and 5% CO₂ with an exchange of media every 2 days. Alternatively, CD14-positive human peripheral blood monocytes were purchased from Lonza and differentiated over a 6 day period as described above, but in the presence of 100ng/ml CSF-1 (Invitrogen).

Tks5-modified THP-1 Cell Lines

Murine cDNAs encoding Tks5 and Tks5ΔPX as well as shRNAs selectively targeting human Tks5 (shTks5#1 targets GCCAAAGCAAGGACGAGAT; shTks5#2 targets AAACCAGTGGCGACCTGAA) were subcloned into the retroviral expression vectors pBABEpuro (Cell Biolabs, San Diego, CA) and pSUPER.retro.puro (Oligoengine, Seattle, WA), respectively, and verified by sequencing. Each Tks5-modification vector, along with empty control vectors, were transfected into the Phoenix 293T amphotropic retrovirus packaging cell line (ATCC, Manassas, VA) using Lipofectamine 2000 (Invitrogen), and the virus-enriched conditioned media was collected at 24 or 48 hours. Parental THP-1 monocyte cultures were subsequently infected with each virus preparation, and placed under selection

with 0.5µg/ml puromycin, ultimately leading to the formation of puromycin-resistant, multiclonal cell lines that continually exhibited the desired phenotypic changes in Tks5 expression. Tks5-modified THP-1 cell lines were routinely maintained in THP-1 growth media containing 0.5µg/ml puromycin, with puromycin being excluded from all experimental studies.

Podosome Formation

Podosome formation was monitored in cells (primary macrophages, THP-1 cells) that were cultured directly on glass coverslips (Carolina Biological Supply, Burlington, NC) under described assay conditions. At the end of the experiment, the coverslips were fixed in 3% formaldehyde (Electron Microscopy Sciences, Hatfield, PA)/PBS for 10 minutes, permeabilized in 0.1% Triton X-100 (EMD)/PBS for 10 minutes, and extensively washed in PBS (5 × 5 minutes). Primary antibodies were used to detect WASp (1:50; #sc-8353; Santa Cruz Biotechnology, Santa Cruz, CA), cortactin (1:100; #05-180; Millipore, Billerica, MA), vinculin (1:100; #05-386; Millipore, Billerica, MA), phosphotyrosine (1:250; #05-321; Cell Signaling), Tks5 (1:250), and Src (1:200; cst.1) in 5% donkey serum (The Jackson Laboratory, Bar Harbor, ME)/PBS for 2 hours at room temperature or for overnight at 4°C. Tks5 antibodies to the first (D7771, polyclonal) and fourth (2F4G8D2, monoclonal) SH3 domains were generated in the laboratory of Dr. Sara Courtneidge (Sanford-Burnham Medical Research Institute). The cst.1 Src antibody was also provided by Dr. Courtneidge (Kypta et al. 1990). After primary antibody application and successive washes in PBS (5 × 5 minutes), coverslips were incubated for 1 hour at room temperature in 5% donkey serum/PBS containing an appropriate species-specific AlexaFluor488-conjugated secondary antibody (1:1000, Invitrogen) along with AlexaFluor594-conjugated phalloidin (1:200; Invitrogen) to visualize F-actin. After successive washes in PBS (1 × 5 minutes), Hoechst 33258 (1:10,000; Invitrogen)/PBS to visualize nuclei, and PBS (5 × 5 minutes), the coverslips were mounted onto glass slides with ProLong Gold anti-fade reagent (Invitrogen) and cured overnight at room temperature. Ten random images were captured with an Olympus IX70 inverted fluorescent microscope equipped with a Retiga 2000R digital color camera and using a UPlanFl 60X/1.25 objective (Olympus, Center Valley, PA). Images were analyzed with Image Pro Plus 5.1 software. The percentage of cells with focalized F-actin staining (podosome incidence) and the number of focalized F-actin structures/cell (podosome multiplicity) were determined in Tks5-modified THP-1 cell lines (minimum of 50 cells counted). Podosome incidence was normalized to vector control cells.

In Situ Zymography

Podosome-associated matrix degradation (*i.e.* gelatin degradation by *in situ* zymography) was monitored in parental and Tks5-modified THP-1 cell lines similar to previous published procedures (Bowden et al. 2001; Seals et al. 2005). Briefly, 100mg gelatin (300 bloom; Sigma-Aldrich) was conjugated to 1mg AlexaFluor488-TFP (Invitrogen) by dialysis in 100mM sodium bicarbonate (EMD), pH=9.0 for 2 hours at 37°C according to manufacturer instructions. Conjugated gelatin was then dialyzed into PBS for several days at 37°C, supplemented with 20mg/ml sucrose (Sigma-Aldrich), and stored for long-term use at 4°C. A thin layer of AlexaFluor488-conjugated gelatin was coated onto glass coverslips, and immediately cross-linked with ice-cold 0.8% glutaraldehyde (Electron Microscopy Sciences)/PBS for 15 minutes at 4°C and for 30 minutes at room temperature. Coverslips were then successively washed in PBS (3 × 5 minutes), 5mg/ml sodium borohydride (Sigma-Aldrich)/PBS (1 × 3 minutes), PBS (3 × 5 minutes), and 70% ethanol (1 × 10 minutes). Coverslips were quenched in RPMI-1640 media for 1 hour prior to cell seeding. Matrix degradation was monitored in THP-1 cells that were cultured on AlexaFluor488-gelatin-coated coverslips under described assay conditions. Coverslips were processed essentially as described for podosome formation, except that staining was limited to F-actin

and nuclei. Images were captured with an LPlanFI 20X/0.40 objective (Olympus) and processed with Image Pro Plus 5.1 and ImageJ 1.43u software. Data were plotted as the area of degradation per cell (minimum of 75 cells) from no fewer than 5 random images), and normalized to untreated or vector control cells.

Immunoblotting

Lysates were prepared from cells (primary macrophages, THP-1 cells) grown on cell culture dishes under described assay conditions. Dishes containing cells were laid on a bed of crushed ice, washed twice in ice-cold PBS containing 100 μ M sodium orthovanadate (Sigma-Aldrich), and then overlaid with ice-cold lysis buffer composed of 20mM Hepes (pH=7.0), 140mM NaCl (EMD), 40mM NaF (EMD), 1% NP-40 (Roche, Indianapolis, IN), 2mM DTT (IBI Scientific, Peosta, IA), 100 μ M sodium orthovanadate, and the following protease inhibitors: 10 μ g/ml aprotinin (EMD)/PBS, 1mM benzamidine (Alfa Aesar, Ward Hill, MA)/PBS, 10 μ g/ml leupeptin (Thermo Fisher Scientific, Pittsburgh, PA)/PBS, and 10 μ g/ml pepstatin A (MP Biomedicals)/DMSO, and 1mM PMSF (Thermo Fisher Scientific)/DMSO. Cells were scraped from the dishes, transferred to ice-cold microfuge tubes, briefly vortexed, and then incubated on ice for 10 minutes. Lysates were cleared of debris by a 10 minute centrifugation at 10,000g at 4°C. Supernatants were transferred to fresh ice-cold microfuge tubes and either assayed directly for protein concentration or flash-frozen in liquid nitrogen and stored at -80°C for future use. Protein concentrations were determined with a Lowry-based detergent compatible stain (Bio-Rad, Hercules, CA) according to manufacturer instructions using BSA (Fraction V, Thermo Fisher Scientific) as a standard. Lysates were diluted into SDS-containing loading buffer and heated to 95°C prior to protein separation by SDS-PAGE (Bio-Rad). Proteins were transferred from acrylamide gels to nitrocellulose membranes (Bio-Rad) using a semi-dry blotting apparatus (Bio-Rad) at 3mA/cm² for 35 minutes. Each membrane was incubated in blocking buffer containing 5% powdered milk in PBS/0.1% Tween-20 (EMD) (PBST) prior to antibody applications, except for phosphotyrosine antibody applications where the blocking buffer contained 5% BSA/PBST. Immunoblotting antibodies used in this study include cortactin (1:1000; #3502; Cell Signaling), WASp (1:1000; #sc-8353; Santa Cruz), GAPDH (1:1000; #sc-25778; Santa Cruz), MMP9 (1:500; #MS-816-PO; Thermo Fisher Scientific), MT1-MMP (1:500; #AB6004; Millipore), phosphotyrosine (1:1000), Src (1:1000; #2108; Cell Signaling), and appropriate species-specific peroxidase-conjugated secondary antibodies (1:2500; GE Healthcare, Piscataway, NJ) applied to each membrane in 0.5% milk/PBST for a minimum of 1 hour at room temperature. Tks5 immunoblotting antibodies were to the C-terminal region (1:500; #sc-9945; Santa Cruz) or the fourth SH3 domain (1:1000; 1736.9) (Lock et al. 1998). Blots were developed in a chemiluminescence system (ECL Plus, GE Healthcare) according to manufacturer guidelines, exposed to film (Phenix Research Products, Candler, NC), and developed with a processor. ImageJ 1.43u was used to perform densitometric analysis of Tks5 protein levels relative to untreated cells, and was normalized to GAPDH protein levels.

Immunoprecipitation

Lysates (200–500 μ g) were diluted to 500 μ l with ice-cold lysis buffer and a Tks5 antibody (200ng antibody/100 μ g lysate; #sc-9945; Santa Cruz) and mixed by rotation at 4°C for 1 hour. Antibody-protein complexes were separated from unbound proteins by adding 10 μ l protein A sepharose beads (Zymed; #10-1042; San Francisco, CA) and mixing by rotation at 4°C for an additional 2 hours. Beads were washed thrice in 500 μ l ice-cold lysis buffer by sedimentation at 1000g for 1 minute at 4°C, gentle removal of the supernatant by vacuum suction, and the addition of fresh lysis buffer. Complexes were eluted from the beads with 100 μ l SDS-containing loading buffer at 95°C for 5 minutes, and proteins separated by SDS-PAGE as described above.

Adhesion Assay

Parental or Tks5-modified THP-1 cell lines were washed in PBS, counted on a haemocytometer in a 1:1 dilution of Trypan Blue (Hyclone), and 4×10^4 cells were transferred in triplicate to individual wells of a 96-well plate in the presence of 25nM PMA/DMSO at 37°C. Bisindolylmaleimide or PP2 were also included in the assay where described. At 4, 24, and/or 72 hours, plates were gently washed twice with PBS to remove unattached cells, fixed and stained with 0.09% Crystal Violet/10% ethanol for 20 minutes at room temperature, and washed thrice with PBS to remove residual stain. Plates were allowed to dry overnight before eluting with 1% SDS (Thermo Fisher Scientific) and measuring the absorbance on a plate reader (SpectraMax 340PC; Molecular Devices, Sunnyvale, CA) at 490nm. Adhesion was normalized to the results from untreated or vector control cells.

CD11b Expression/Flow Cytometry

Cell surface CD11b expression was monitored in parental THP-1 cells by FACS analysis under described assay conditions. First, THP-1 cells were washed in PBS, counted, and 1.6×10^6 cells were distributed into 6cm cell culture dishes in THP-1 growth media containing 25nM PMA/DMSO with or without 6μM bisindolylmaleimide/DMSO for 72 hours. Cells were washed in PBS, detached with Accutase (Innovative Cell Technologies, San Diego, CA) at room temperature for 30 minutes, and then washed successively in PBS and 10% FBS/PBS. The detached and washed cells were then counted such that 4×10^5 cells could then be stained with an allophycocyanin-conjugated CD11b antibody (1:50; #550019; BD Biosciences) in 10% FBS/PBS for 30 minutes on ice. Flow cytometry was performed on 1×10^4 cells with a FACSCalibur (BD Biosciences), and the resulting data were analyzed with CellQuest Pro software. CD11b protein levels were normalized to the results from untreated cells.

Migration and Invasion Assays

Tks5-modified THP-1 cell lines were washed in PBS, counted, and 1×10^5 cells were transferred in triplicate to the upper well of rehydrated, trans-well motility (5μ; Corning, Lowell, MA) or Matrigel-coated invasion (8μ; BD Biosciences, San Jose, CA) chambers in RPMI-1640 media containing 0.5% FBS and 25nM PMA/DMSO in a final volume of either 100μl (motility) or 500μl (invasion). Lower wells contained either 600μl (motility) or 750μl (invasion) of RPMI-1640 media supplemented with 10% FBS and 50ng/ml MCP-1 (PeproTech, Rocky Hill, NJ). Motility assays proceeded at 37°C for 4 hours, while invasion assays were at 37°C for 24 hours. Trans-well chambers were gently washed twice in PBS, and either fixed with DiffQuick (IMEB, San Marcos, CA) or with 0.09% Crystal Violet (Sigma-Aldrich)/10% ethanol before drying overnight at room temperature. Migration and invasion were based on the average number of cells on the underside of the membrane in 10 random images generated at 200X magnification per chamber, and was normalized to the results from vector control cells.

Zymography

Tks5-modified THP-1 cell lines were washed in PBS, counted, and 1.6×10^6 cells were transferred to 6-well plate in the presence of 25nM PMA/DMSO at 37°C for 72 hours. Cells were washed twice in PBS, and then incubated an additional 24 hours at 37°C with 2ml serum-free THP-1 growth media. The conditioned media was collected, filtered at 0.2μm, and diluted with zymogram sample buffer (Bio-Rad). Sample proteins were separated on 10% acrylamide/0.127% gelatin gels, and the gels were processed sequentially with renaturation buffer (Bio-Rad) for 1 hour, development buffer (Bio-Rad) for overnight at

37°C, Coomassie Brilliant Blue R-250 staining solution (Bio-Rad) for 30 minutes, and 50% methanol/10% acetic acid until proteolytic activity could be resolved.

Statistical Methods

Graphical representation and descriptive statistics were used to describe the measurements discussed in this paper. A Student's t-test is used to test the fold change between groups for most of the outcomes in the paper. To compare differences in the podosome multiplicity between cell lines, a linear mixed model was used. Podosome counts were log transformed to meet the normality assumption of a linear model. To account for the differences between the three independent experiments, a random experiment effect was added to the model. Least square means and corresponding 95% confidence intervals were produced and back transformed to provide a summary of the results. The differences between the adjusted means were tested to determine which cell lines showed a significant difference from the other cell lines. This analysis was done using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

THP-1 cells model macrophage podosome development

The phorbol ester PMA supports a differentiation program that converts THP-1 suspension cells into an adherent population with a macrophage-like phenotype (see Figure 3 and Supplementary Figure 3) (Auwerx 1991). Previous studies have demonstrated that these model macrophages are replete with punctate actin structures highly reminiscent of podosome adhesions (Tsuboi 2007). We have also observed this focalized arrangement of F-actin, and that the extent of their development increased with the time of PMA exposure such that by 72 hours they were present in approximately two-thirds of the adherent cells. To further test for podosomes, THP-1 cells were treated with 25nM PMA for 72 hours, then fixed and stained with phalloidin to monitor F-actin morphology as well as with antibodies to other podosome marker proteins. First, we confirmed a previous report that the cytoskeletal regulatory protein WASp co-localized with F-actin puncta in PMA-treated THP1 cells (Figure 1A) (Tsuboi 2007). A similar result was observed for cortactin which, like WASp, primarily localized to the core of focalized F-actin structures (Figure 1B). In contrast, the actin accessory and podosome marker protein vinculin was localized just peripheral to the F-actin cores in a ring-like configuration consistent with its distribution in this and other podosome-competent cell types (Figure 1C) (Linder et al. 1999; Launay et al. 2003; Moreau et al. 2006; Webb et al. 2006; Nusblat et al. 2011). Since these and other podosome-associated proteins can be phosphorylated by Src family tyrosine kinases, podosomes can also be visualized with a phosphotyrosine antibody; this was also true of THP-1 macrophages (Supplementary Figure 1) (Tarone et al. 1985; Pfaff and Jurdic 2001; Linder and Aepfelbacher 2003; Bowden et al. 2006).

We further addressed whether these podosome-like structures were functionally-active by culturing THP-1 cells on fluorescent-conjugated gelatin films in the presence of PMA for 24 hours. Podosome-competent cells are capable of degrading the gelatin and forming holes in the fluorescent monolayer. As demonstrated in Figure 1D, THP-1 cells treated with PMA formed large holes in the gelatin matrix that often encompassed much of the area occupied by the cell. This ability to degrade gelatin was seen in approximately one-third of the adherent cells, and was sensitive to the broad spectrum protease inhibitor GM6001 suggesting that degradation was a proteolytic process (Figure 1E). In contrast, while some vehicle control-treated THP-1 cells adhered to the gelatin matrix, no degradation was observed (Figure 1F). Thus, treatment of THP-1 cells with PMA leads to the formation of punctate actin structures that based on their morphological, compositional, and proteolytic features represent bona fide podosomes.

Tks5 is a podosome marker protein that accumulates during macrophage differentiation

To characterize Tks5 in THP-1 macrophages we first tested whether it too co-localized with F-actin upon PMA treatment. Using antibodies specific to a region spanning its fourth SH3 domain, we found that Tks5 co-localized with F-actin in punctate podosome structures consistent with past observations of Tks5 localization in other podosome-competent cell types (Figure 2A) (Abram et al. 2003; Courtneidge et al. 2005; Thompson et al. 2008; Crimaldi et al. 2009). A similar result was observed using a different antibody recognizing the first SH3 domain of Tks5 (Supplementary Figure 2). To verify the podosome localization of Tks5, we also analyzed primary human macrophages. Monocytes, isolated from the peripheral blood, were subsequently differentiated in culture over a period of 6 days, and the resulting macrophages fixed and stained with phalloidin and a Tks5 antibody. As with differentiated THP-1 macrophages, Tks5 co-localized with F-actin in the podosomes of primary macrophages as well (Figure 2B; Supplementary Figure 2). Together, these data support the conclusion that Tks5 is a marker protein for podosomes in this cell type.

Because both macrophage differentiation and podosome formation are inducible in THP-1 cells, we next determined the relative expression of Tks5 and other podosome-associated proteins as a function of PMA treatment. While Tks5 protein was detectable in undifferentiated THP-1 monocytes, it also increased during macrophage differentiation in a time- and PMA concentration-dependent manner (Figures 2C and 2D). In response to PMA, Tks5 protein increased within 24 hours and this continued up to at least 96 hours (Figure 2C). After exposure to PMA for 72 hours (the time of podosome morphology assessment in Figures 1A–1C and 2A), Tks5 protein levels had consistently increased ~4.5-fold (Figure 2D). A similar substantial increase in Tks5 protein occurred during the differentiation of peripheral blood monocytes into macrophages (Figure 2E).

In the THP-1 macrophage model, we also observed a time-dependent increase in cortactin upon PMA treatment (Figures 2C). Other podosome markers, however, either did not show appreciable changes in protein levels (vinculin) or in the case of WASp was slightly reduced in response to PMA (Figures 2C and data not shown). This induction in cortactin and reduction in WASp was also seen during the differentiation of primary macrophages (Figure 2E), and has been previously reported by others (Launay et al. 2003). Thus, there is differential expression of podosome-associated proteins during macrophage differentiation; some like Tks5 and cortactin are induced, while WASp protein levels decline.

Podosome development and Tks5 protein accumulation are dependent on PKC signaling

PMA is structurally similar to diacylglycerol and therefore a prominent agonist for members of the protein kinase C (PKC) family (Brose and Rosenmund 2002). To address whether PKC signaling is necessary for macrophage differentiation in general, and podosome development and Tks5 protein accumulation in particular, THP-1 cells were treated with PMA for 4 to 72 hours in the presence or absence of the PKC inhibitor bisindolylmaleimide. General inspection of phase contrast images indicated that THP-1 cells grew as a dispersed suspension, but exhibited marked cell adhesion upon exposure to PMA (compare Figures 3A and 3B). Such adhesion could be observed as early as 4 hours after PMA addition with evident cell spreading by 24 hours (Figures 3B and 3D). However, when THP-1 cells were co-treated with PMA and bisindolylmaleimide, these features of macrophage differentiation were strongly inhibited (Figure 3C). Quantitative analysis revealed that bisindolylmaleimide decreased adhesion at all time points, including statistically significant reductions at 24 and 72 hours (Figure 3D). Moreover, FACS analysis indicated that this PKC inhibitor also blocked the accumulation of cell surface CD11b, a component of the integrin receptor Mac-1 and a primary marker of adhesion during macrophage differentiation (Supplementary

Figure 3) (Schwende et al. 1996). Bisindolylmaleimide therefore strongly inhibits the adhesion phenotype that accompanies PMA-associated THP-1 macrophage differentiation.

Since podosomes are the adhesion structures of macrophages, we similarly addressed the sensitivity of podosome development and the accumulation of Tks5 to PKC inhibition. Despite a small increase in the number of adherent cells on a gelatin matrix relative to uncoated coverslips, we could not readily detect podosome structures in PMA-treated THP-1 cells in the presence of bisindolylmaleimide (data not shown), and there appeared to be negligible matrix-remodeling podosome activity as well (compare Figures 3E and 3F). Indeed, when normalized to the number of cells in captured images, bisindolylmaleimide significantly inhibited gelatin degradation in THP-1 macrophages (Figure 3G). Bisindolylmaleimide was also an effective inhibitor of the PMA-dependent induction of Tks5 protein seen in THP-1 cells (Figure 3H). These results thus indicate an important role for PKC signaling in many features of PMA-dependent, THP-1 macrophage differentiation, and suggest a general correlation between Tks5 expression and podosome development in this cell type.

It is noteworthy that PMA-mediated differentiation was distinguishable from a more natural differentiation program driven by the steroid hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃). The latter did not generally support adhesion and there was therefore no podosome-associated degradation of gelatin (Supplementary Figure 4). And while Tks5 protein levels increased in response to 1,25-(OH)₂D₃, the extent of that increase was lower than that induced by PMA and it was not antagonized by bisindolylmaleimide treatment (Supplementary Figure 4). These modest differentiation effects by 1,25-(OH)₂D₃ had been recognized before (Schwende et al. 1996). Since only PMA lead to podosome development, we continued to focus on PMA-related events in this study.

Tks5 regulates podosome-associated matrix degradation and invasion in THP-1 macrophages

Given that Tks5 localized to THP-1 macrophage podosomes, accumulated during the time of podosome deposition, and exhibited similar regulation by PKC signaling, we surmised that Tks5 may regulate podosome development in this cell line. To test this hypothesis, we generated viral vectors that confer stable expression of Tks5-specific shRNAs that would be expected to block the induction of Tks5 protein in response to PMA. Two different shRNAs were used to silence Tks5 expression, and both effectively blocked the PMA-dependent increase in Tks5 protein levels seen in the vector control THP-1 cell line (Figure 4A). Based on our previous studies in Src3T3 cells, we predicted that Tks5 silencing would inhibit podosome development (Seals et al. 2005). Indeed, Tks5-silenced THP-1 cell lines exhibited nearly a 5-fold reduction in gelatin-degrading podosome activity relative to vector control cells following a 24 hour treatment with PMA (compare Figures 4B–4D; Figure 4H). Moreover, this decreased gelatin degradation correlated with significant reductions in the ability to invade through Matrigel when under the influence of a chemotactic gradient comprised of serum and MCP-1 (Figure 4H). However to our surprise, there were no differences in the percentage of cells with focalized F-actin structures (podosome incidence) following PMA treatment nor were there obvious changes in F-actin morphology (compare Figures 4E–4G; Figure 4H). Wondering whether these punctate structures had lost some of the gross compositional features of podosomes, we also assessed the extent to which WASp, cortactin, and vinculin co-localized with F-actin following a 72 hour treatment with PMA. Yet despite differences in invasive behavior, there were no apparent changes in the distribution of these podosome marker proteins in each Tks5-silenced THP-1 cell line (Supplementary Figure 5).

As a complementary approach to studies of Tks5 silencing, we also determined whether Tks5 overexpression impacted podosome development and invasion in THP-1 cells. Again, we generated stable THP-1 cell lines, but this time with viral vectors that enable constitutive expression of either wild-type Tks5 or a mutant form of Tks5 lacking its PX domain. The Tks5 Δ PX construct had previously been shown to be disabled in its ability to associate with podosomes in other model systems (Abram et al. 2003; Oikawa et al. 2008). In Figure 5A we demonstrated the overexpression of Tks5 and Tks5 Δ PX protein in THP-1 cells with or without PMA treatment. We initially made note that both wild-type and mutant Tks5-expressing THP-1 cell lines were unable to adhere to uncoated surfaces or make podosomes independent of PMA treatment (data not shown). And like Tks5-silenced THP-1 cells, there were no obvious differences in podosome morphology, incidence, or composition (i.e. co-localization of WASp, cortactin, and vinculin with F-actin) when these cell lines were treated with PMA (compare Figures 5E-5G; Figure 5H; Supplementary Figure 6). We conclude then that, as with Tks5 silencing, Tks5 overexpression alone does not alter podosome incidence during PMA-dependent THP-1 macrophage differentiation. However, these results were in contrast to the 3–4-fold increase in gelatin-degrading podosome activity seen in wild-type Tks5-expressing THP-1 macrophages (compare Figures 5B and 5C; Figure 5H) as well as the 3–4-fold increase in invasion through Matrigel (Figure 5H). The extent of this increased invasive behavior by Tks5 overexpression was further noted by the occasional evidence of gelatin degradation in cells independent of PMA treatment (data not shown). Interestingly, this gain-of-function did not occur in THP-1 cells overexpressing Tks5 Δ PX. Indeed, we observed that overexpression of Tks5 Δ PX led to a slight decrease in invasion relative to the vector control cells suggesting a possible dominant-negative effect of this mutant construct (compare Figures 5B and 5D; Figure 5H).

So despite no significant impact on macrophage differentiation and apparent podosome incidence, Tks5 expression still regulates the invasive properties of PMA-differentiated THP-1 macrophages by limiting protease-associated, matrix-remodeling podosome activity.

Tks5-regulated macrophage invasive behavior is associated with changes in adhesion, motility, and MMP9 activity

The most direct explanation for how Tks5 could influence the invasive behavior of macrophages would be to regulate the expression and/or activity of matrix metalloproteinases, particularly MMP2 and MMP9. Studies in Src3T3 cells have previously shown that Tks5 silencing little impacts the activity of these secreted enzymes (Seals et al. 2005); we wanted to determine if this was similarly true in THP-1 cells. Using a standard gelatin zymography assay of conditioned media supernatants, we first confirmed previous reports regarding the upregulation of secreted MMP9 proteolytic activity in PMA-stimulated THP-1 cells (Supplementary Figure 7) (Kintscher et al. 2001; Worley et al. 2003; Zhou et al. 2005). MMP2 was also detected under these conditions, but its level of activity was much less than that of MMP9 also as previously reported (Supplementary Figure 7) (Zhou et al. 2005). When Tks5-silenced THP-1 cells lines were assessed by this technique, we noticed that the functional levels of secreted MMP9 changed in direct accordance with Tks5 protein levels; that is, reduction of Tks5 decreased MMP9 proteolytic activity in the conditioned media (Figure 6A). This decrease in MMP9 activity further correlated with a decrease in MMP9 protein levels in total cell lysates from the same cells (Figure 6B). These effects also appeared to be selective for MMP9 as little change was observed in MMP2 activity in any of the Tks5-silenced THP-1 cell lines (Figures 6A). There was also no apparent change in MT1-MMP expression in total cell lysates from these cells (Figure 6B). This positive relationship between Tks5 and MMP9 was also seen in THP-1 cells overexpressing Tks5, both in terms of secreted proteolytic activity and total protein expression (Supplementary Figure 7).

To discover other potential mechanisms for Tks5 regulation of macrophage invasive behavior, we next determined whether Tks5 protein levels influence the invasion-related functions of adhesion and motility (Evans et al. 2003). When PMA-differentiated THP-1 cells were placed in uncoated motility chambers under the influence of a chemotactic gradient of serum and MCP-1, we noted substantial changes in the number of migrating cells over a 4 hour period. In particular, both Tks5-silenced THP-1 cell lines showed nearly a 3-fold reduction in chemotaxis relative to vector control cells (Figure 6C). We also determined that Tks5-silenced THP-1 cell lines showed a significant deficiency in adhesion to uncoated plastic dishes after a 4 hour exposure to PMA (Figure 6C). This compromise of adhesion and motility seen in cells with reduced Tks5 expression at an early phase of PMA-mediated differentiation (4 hours) could also explain, along with the loss of MMP9 expression, the defect in gelatin degradation and invasion seen at later time points (24 hours).

Since podosomes are considered the adhesion structures of macrophages, we were initially surprised that Tks5-silenced THP-1 cells were less adherent than vector control cells when podosome incidence and morphology were little changed among these cell lines (Figure 4). So we also measured the number of podosomes in THP-1 macrophages. THP-1 macrophages exhibit a very wide range of podosome multiplicities ranging from as few as 3 to as high as 415 per cell. Based on their adhesion defect (and reduced invasive behavior), we initially hypothesized that the number of podosomes per cell might also be reduced in Tks5-silenced THP-1 cell lines, but the opposite was actually true in that there were small, but statistically significant increases in podosome number between vector control (adjusted mean of 34.4) and each Tks5-silenced cell line (adjusted means of 39.6 and 52.8) (Figure 6D). Thus, a stable reduction in Tks5 protein levels in THP-1 macrophages increases podosome multiplicity, though podosome functionality remains compromised.

Tks5 phosphorylation by Src family kinases drives THP-1 macrophage invasive behavior

Because Tks5 is a substrate of Src tyrosine kinase, we also wished to address whether there was any role for Tks5 phosphorylation in the regulation of macrophage invasive behavior. First, we noted that, like Tks5, Src was expressed in THP-1 cells, was induced by PMA treatment (Figure 7A), and co-localized with F-actin in podosomes (Figure 7B). This suggested that Src signaling might be upregulated during THP-1 macrophage differentiation, and, if so, that a Src inhibitor might inhibit this process much like we observed previously with the PKC inhibitor bisindolylmaleimide (Figure 3). To test this hypothesis, THP-1 cells were treated with PMA in the presence or absence of the Src family kinase inhibitor PP2 for 24 to 72 hours, then assayed for podosome formation and gelatin degradation as before. Similar to bisindolylmaleimide, PP2 antagonized podosome development in PMA-differentiated THP-1 cells. Few podosome structures could be visualized in THP-1 macrophages following PMA treatment (compare Figures 7C and 7D; Figure 7G), and gelatin degradation was significantly reduced as well (compare Figures 7E and 7F; Figure 7G). However unlike bisindolylmaleimide treatment, PP2 only moderately affected adhesion and only at the 72 hour time point (Figure 7H). And Tks5 protein levels actually seemed to increase in THP-1 cells after a 72 hour treatment with PP2 (Figure 7I). Thus, PP2 treatment uncovered a novel condition in THP-1 macrophages whereby Tks5 expression and podosome-associated matrix degradation were no longer correlated. We hypothesized that, as a Src inhibitor, PP2 might decrease Tks5 phosphorylation and this might correlate with the loss in podosome functionality. In addressing this hypothesis, we were initially unsuccessful in our attempt to detect Tks5 tyrosine phosphorylation using parental THP-1 cells under any condition. However, we did observe increased Tks5 tyrosine phosphorylation in the Tks5-overexpressing THP-1 cell line following PMA treatment (Figure 7J). This phosphorylation was detected as early as 24hrs after PMA application (the time matrix-remodeling podosome activity was assayed) and was sustained for as long as

72hrs (the time podosome morphology was assayed) (Figure 7J and data not shown). Moreover, the increase in Tks5 phosphorylation was not observed when these cells were co-treated with the Src inhibitor PP2 (Figure 7J). Thus, the phosphorylation of Tks5 accompanies PMA-dependent THP-1 macrophage differentiation and correlates with the extent of matrix-remodeling podosome activity in these cells.

DISCUSSION

Podosomes have long been recognized as the adhesion structures of macrophages (Lehto et al. 1982; Marchisio et al. 1987; DeFife et al. 1999; Linder et al. 1999; Evans et al. 2003; Cougoule et al. 2005; Calle et al. 2006; Wheeler et al. 2006; Yamaguchi et al. 2006; Perri et al. 2007; Cougoule et al. 2010). For the most part these structures form naturally during differentiation, though the presence of CSF-1 has been shown to activate podosome formation in bone marrow-derived macrophages (Wheeler et al. 2006; Yamaguchi et al. 2006). Podosomes have also been studied in differentiated macrophage cell lines (Marchisio et al. 1988a; Evans et al. 2003; Launay et al. 2003; Poincloux et al. 2006; Tsuboi 2006; Yamaguchi et al. 2006; Dovas et al. 2009). The THP-1 monocytic leukemia cell line has previously been used to study macrophage differentiation, innate immune reactions, inflammatory responses, and the function of tumor-associated macrophages (Auwerx 1991; Tjui et al. 2009). Recently, this cell line was used to demonstrate the regulatory role of WASp and associated proteins during the formation of podosome-like structures in response to the phorbol ester PMA (Tsuboi 2006; Spano et al. 2007; Tsuboi 2007; Tsuboi et al. 2009). Here we have expanded upon these studies by showing that numerous podosome markers (cortactin, vinculin, phosphotyrosine) co-localize with WASp and F-actin in these punctate structures, and that they degrade matrix proteins in a protease-dependent manner, all consistent with the formation of functionally-active podosomes.

Tks5 is an adaptor protein initially identified in a screen for novel substrates of Src tyrosine kinase (Lock et al. 1998). Clues to its function were initially based on its localization to the invadopodia of model Src3T3 cells (Abram et al. 2003; Seals et al. 2005). With similar localization to the invadopodia of cancer cell lines and the podosomes of osteoclasts, myoblasts, and smooth muscle cells, combined with its notable absence from the focal adhesions of fibroblasts, Tks5 has become a benchmark for confirming the existence of podosome/invadopod adhesions in newly studied cell types (Courtneidge et al. 2005; Seals et al. 2005; Thompson et al. 2008; Crimaldi et al. 2009). In this report we show that Tks5 is a marker of macrophage podosomes, is induced concurrently with podosome deposition, and regulates macrophage invasive behavior through modulation of adhesion, motility, and matrix-remodeling podosome activity.

To our knowledge, the macrophage represents the first model system where there is developmental regulation of Tks5 expression. This occurs naturally during peripheral blood monocyte differentiation and in response to PMA, 1,25-(OH)₂D₃, and cancer cell conditioned media in THP-1 cells (Burger and Seals, unpublished data), thus implying that multiple signaling pathways converge on the upregulation of Tks5. By mimicking the second messenger diacylglycerol, phorbol esters like PMA activate C1 domain-containing proteins of which the PKC family is most prominent (Brose and Rosenmund 2002). Indeed, PKC inhibitors commonly block podosome development, and both PKC α and PKC ϵ have been implicated in podosome formation in smooth muscle cells (Hai et al. 2002; Quintavalle et al. 2010). In our own studies, we have noted a broad antagonism of macrophage differentiation (adhesion, CD11b expression, podosome formation/activity, Tks5 protein accumulation) in response to the PKC inhibitor bisindolylmaleimide. The specific PKC isoform(s) involved in Tks5 upregulation are unknown, but PKC α , β , δ , and ϵ are all induced and activated in PMA-treated THP-1 cells and PKC ζ is localized to THP-1 podosomes

(Martiny-Baron et al. 1993; Schwende et al. 1996; Dieter and Schwende 2000; Traore et al. 2005; Tani et al. 2007; Qiao and May 2009). We also have preliminary evidence that the MAP kinase signaling pathway may be involved since the induction of Tks5 by PMA is sensitive to UO126 (Burger and Seals, unpublished data).

Tks5 tyrosine phosphorylation by Src family kinases may also be important for podosome development and macrophage invasion. Src is a common regulator of podosomes/invadopodia. In fact, many of the components of macrophage podosomes (cortactin, vinculin, α -actinin, talin, fimbrin, gelsolin, WASp, Tks5) are Src substrates. This explains the utility of using kinase inhibitors to antagonize podosome formation, phosphatase inhibitors to activate podosome formation, and phosphotyrosine antibodies to visualize them in the cell (Marchisio et al. 1988b; Linder et al. 2000; Cory et al. 2002; Poincloux et al. 2007). Src is the namesake member of a small family of nonreceptor tyrosine kinases of which Hck, Fgr, and Lyn are restricted to the myeloid lineage of hematopoietic stem cells (Korade-Mirnics and Corey 2000; Lowell 2004). Recently it was shown that macrophages from *Hck*^{-/-} mice had a significant reduction in the number and size of podosome rosettes, and this was adequate to inhibit their matrix-remodeling and invasive properties (Cougoule et al. 2010). The case for Hck as a central regulator of macrophage podosome formation is consistent with its localization to macrophage podosomes, and that its kinase activity enables podosome formation in NIH3T3 cells in a manner similar to Src (Poincloux et al. 2006; Poincloux et al. 2007; Cougoule et al. 2010). We know from past studies that Tks5 is a Src substrate (Lock et al. 1998), and in cancer cells, Tks5 phosphorylation is necessary for invadopodia development (Stylli et al. 2009). In the Tks5-overexpressing THP-1 cell line, we now have evidence of Src induction and Tks5 tyrosine phosphorylation following PMA treatment. And consistent with previous reports, macrophage podosome formation is sensitive to the Src family kinase inhibitor PP2, and importantly correlates with reduced Tks5 phosphorylation (Linder et al. 2000; Poincloux et al. 2007). Further studies will be necessary to discern how such Tks5 phosphorylation impacts these processes.

There are distinctions in how Tks5 regulates podosome development in macrophages relative to previously studied model systems. In Src3T3 cells, Tks5-silencing leads to a loss of invadopodia and an associated reduction in invasive behavior. This presumably occurs via alterations in the focalized distribution of proteases (Seals et al. 2005). Protease expression and chemotaxis, however, were unaltered (Seals et al. 2005). In THP-1 macrophages, we see that Tks5 expression influences invasive behavior by collectively modulating podosome-associated adhesion and chemotaxis, as well as MMP9 expression/activity (gelatin degradation). MMP9 alone might be able to control macrophage invasion. There is evidence, for example, that MMP9 can regulate plasminogen-induced macrophage invasion through Matrigel and into the peritoneal cavity of thioglycolate-injected mice (Gong et al. 2008). THP-1 macrophage invasion can also be regulated by MMP9 (Zhou et al. 2005). Other studies, however, bring the role of MMP9 into question. *Hck*^{-/-} macrophages, for example, exhibit reduced matrix degradation without any changes in MMP2, MMP9, and MT1-MMP expression suggesting that other types of proteases could be involved (Cougoule et al. 2010; Van Goethem et al. 2010). We also cannot rule out the possible role of protease recruitment to podosomes, both by Tks5 (e.g. ADAMs family metalloproteinases) or by other Tks5-associated scaffolding proteins (e.g. Nck, Grb2, etc.) (Seals et al. 2005; Oikawa et al. 2008; Stylli et al. 2009).

The other distinction with regard to Tks5 and macrophages from this study is the inability of Tks5 silencing to significantly modulate podosome incidence, but yet increase the number podosomes per cell. Past studies of Tks5 have demonstrated that the loss of Tks5 (in Src3T3 cells) or the mis-targeting of Tks5 to the mitochondrion (in smooth muscle cells) inhibits podosome/invadopod formation (Seals et al. 2005; Crimaldi et al. 2009). In THP-1 cells, it

remains possible that podosome formation is supported by a threshold of Tks5 unaffected by silencing. Alternatively, there may be redundant signals that substitute for Tks5 function after long-term culture of stable cell lines. One candidate is Tks4. In model Src3T3 cells, silencing of either Tks4 or Tks5 disrupts podosomes, however long-term maintenance of cell lines lacking Tks4 eventually recovered the ability to form podosomes, and this correlated with increased Tks5 expression (Buschman et al. 2009). While our own studies of Tks4 have been thus far inconclusive, it remains possible that Tks proteins compensate each other for podosome formation, while retaining independent, and non-compensatory, control of matrix degradation (Buschman et al. 2009). Indeed a similar observation has recently been made of the non-redundant functions of WASp and N-WASp on podosome functionality in macrophages (Nusblat et al. 2011). While it is not immediately clear why podosome multiplicity increases in Tks5-silenced THP-1 cells, it is possible that the inability to properly 'sample' the extracellular matrix shuts down a negative feedback loop that would normally limit new podosome assembly.

CONCLUSIONS

In sum, it is evident from these studies that the Src substrate and adaptor protein Tks5 is a marker of macrophage podosomes with distinguishable effects on macrophage invasive behavior relative to other known podosome markers. Tks5 further controls such invasive behavior through the collective regulation of podosome-associated adhesion, motility, and matrix remodeling, while still maintaining podosome assembly functions. The importance of Tks5 induction to podosome functionality in general suggests that this protein is a component of the core machinery that enables extracellular matrix and tissue permeation for which this cell type is well known.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Differentiated THP-1 macrophages form proteolytically active podosome adhesions. (A–C) THP-1 cells were treated with 25nM PMA for 72 hours, then fixed and stained with phalloidin to visualize F-actin (red) and with antibodies to either WASp (A), cortactin (B), or vinculin (C) (green). Shown are larger composite images; the demarcation of which depicts the results for each individual detection agent in the inset images. (D–F) THP-1 cells were cultured on fluorescently-labeled gelatin for 24 hours in the presence of 25nM PMA (A), PMA plus 10µM GM6001 (B), or DMSO as a negative control (C). Cells were fixed and stained with Hoechst 33258 to visualize nuclei (blue). Holes (dark regions) within the fluorescent gelatin monolayer (green) are indicative of gelatin degradation (arrows).

Figure 2.

Tks5 is a podosome marker protein that accumulates during macrophage differentiation. (A,B) THP-1 cells (A) were differentiated with 25nM PMA for 72 hours, while peripheral blood monocytes (B) were differentiated in the presence of 10% FBS for 6 days. All cells were fixed and stained with phalloidin to visualize F-actin (red) and with a Tks5 antibody (green). Shown are larger composite images; the demarcation of which depicts the results for each individual detection agent in the inset images. (C–E) THP-1 cells were treated with or without 25nM PMA for the indicated times (C) or for 72 hours with the indicated PMA concentrations (D). Protein levels for Tks5, cortactin, WASp, and GAPDH (loading control) were determined from total cell lysates by immunoblot analysis with specific antibodies. Densitometry in D was used to determine the fold change in Tks5 protein levels relative to untreated (0nM) cells. (E) Human peripheral blood monocytes differentiated for the indicated times were analyzed for Tks5, cortactin, WASp, and GAPDH (loading control) protein levels by immunoblot analysis of total cell lysates.

Figure 3.

PKC signaling regulates Tks5 accumulation and podosome development in THP-1 cells. (A–C) Phase contrast images of THP-1 cells treated 24 hours with DMSO (A), 25nM PMA (B), or PMA plus 6μM bisindolylmaleimide (PMA/Bis) (C). Evidence of cell spreading is indicated (arrows). (D) THP-1 cells were allowed to adhere to uncoated plastic for 4, 24, and 72 hours with or without PMA and bisindolylmaleimide. After extensive washing the adherent cells were stained with Crystal Violet, and the eluted stain was measured at 490nm by spectrophotometry. Data are plotted as the fold change in adhesion relative to untreated (DMSO) cells at 4 hours, and are the averages and standard deviations from four, independent experiments. (E,F) PMA-treated THP-1 cells were cultured on fluorescently-labeled gelatin for 24 hours in the absence (E) or presence (F) of bisindolylmaleimide. Cells were fixed and stained with Hoechst 33258 to visualize nuclei (blue). Holes depict regions of gelatin degradation. (G) Matrix-remodeling podosome activity was determined by the area of gelatin degradation/cell/image relative to cells treated with PMA alone. Data are the averages and standard deviations from three, independent experiments. (H) THP-1 cells, treated for 72 hours as described in A–C, were analyzed for Tks5 and GAPDH (loading control) protein levels in total cell lysates by immunoblot analysis. Statistical comparisons for adhesion at each time point and for degradation between PMA alone and PMA/Bis were done using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$.

Figure 4. Tks5 silencing impairs podosome-associated matrix degradation and invasion in THP-1 macrophages. (A) The indicated vector control (pSUPER; pS) and Tks5-silenced (shTks5#1 and shTks5#2) THP-1 cell lines were treated with 25nM PMA for 72 hours, then assayed for Tks5 and GAPDH (loading control) protein levels from total cell lysates by immunoblot analysis. (B–G) Representative fluorescent images of gelatin degradation at 24 hours (B–D) and F-actin at 72 hours (E–G) after vector control and Tks5-silenced THP-1 cell lines were treated with 25nM PMA. Nuclei (blue) in B, C, and D are stained with Hoechst 33258. (H) Quantitation of podosome incidence (the percentage of cells with focalized F-actin staining), gelatin degradation/cell, and invasion in vector control and Tks5-silenced THP-1 cell lines (#1 and #2). For invasion, cells were seeded in triplicate into the upper well of a Matrigel-coated, trans-well chamber in the presence of 25nM PMA and 0.5% FBS, and exposed to a chemotactic gradient composed of 10% FBS and 50ng/ml MCP-1. Invasion assays proceeded for 24 hours, after which the cells were fixed and stained with Crystal Violet, and counted as the average number of invaded cells/10 random images/chamber. All data are plotted as the fold change relative to vector control cells, and are the averages and standard deviations from three-five, independent experiments. Statistical comparisons between vector control and Tks5-silenced cells were done using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$.

Figure 5.

Tks5, but not Tks5ΔPX, overexpression increases podosome-associated gelatin degradation and invasion in THP-1 macrophages. (A) The indicated vector control (pB; pB) and Tks5-overexpressing (Tks5 and Tks5ΔPX) THP-1 cell lines were treated with or without 25nM PMA for 72 hours, then assayed for Tks5 (wild-type and Tks5ΔPX) and GAPDH (loading control) protein levels from total cell lysates by immunoblot analysis. (B–G) Representative fluorescent images of gelatin degradation at 24 hours (B–D) and F-actin at 72 hours (E–G) after vector control and Tks5-overexpressing THP-1 cell lines were treated with 25nM PMA. Nuclei (blue) in B, C, and D are stained with Hoechst 33258. (H) Quantitation of podosome incidence, gelatin degradation/cell, and invasion in vector control and Tks5-overexpressing THP-1 cell lines. Data are plotted as the fold change relative to vector control cells, and are the averages and standard deviations of three-six, independent experiments. Statistical comparisons between vector control and Tks5-overexpressing cells were done using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$

Figure 6.

Tks5 silencing impairs THP-1 adhesion, motility, and MMP9 expression and activity, while podosome multiplicity increases. (A,B) Vector control (pSUPER; pS) and Tks5-silenced THP-1 cell lines (shTks5#1 and shTks5#2) were treated with 25nM PMA for 72 hours. Cells were then washed in PBS and placed in low serum media for another 24 hours. (A) MMP2 and MMP9 gelatinase activity within conditioned media supernatants were normalized to the protein concentration of corresponding total cell lysates and detected by gelatin zymography. (B) Protein levels for MMP9, MT1-MMP, and GAPDH (loading control) were determined from total cell lysates by immunoblot analysis with specific antibodies. (C) For motility, vector control and Tks5-silenced THP-1 cell lines were seeded into the upper well of an uncoated, trans-well chamber in the presence of 25nM PMA and 0.5% FBS, and exposed to a chemotactic gradient composed of 10% FBS and 50ng/ml MCP-1. Motility assays proceeded for 4 hours, after which the cells were fixed and stained with Crystal Violet, and counted as described in Figure 4H. For adhesion, cells were treated with 25nM PMA and monitored for retention to uncoated plastic at 4 hours as described in Figure 3D. Data are plotted as the fold change relative to vector control cells, and are the averages and standard deviations from four-nine, independent experiments. Statistical comparisons between vector control and Tks5-silenced cells were done using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$ (D) Vector control and Tks5-silenced THP-1 cell lines were monitored for podosome multiplicity (the number of focalized F-actin structures per cell). Data are tabulated from four, independent experiments and plotted as the percentage of cells in each indicated categorical range of multiplicities (10 images/group, 50 cells minimum). Shown

for each cell line are the adjusted means (and corresponding 95% confidence intervals from the linear mixed model) that estimate podosome multiplicity.

Figure 7.

Src-Tks5 signaling correlates with podosome-associated gelatin degradation in THP-1 macrophages. (A) THP-1 cells were treated with or without 25nM PMA for 72 hours. Protein levels of Src and activated GAPDH were determined from total cell lysates by immunoblot analysis with specific antibodies. (B) THP-1 cells were treated with 25nM PMA for 72 hours then fixed and stained with phalloidin to visualize F-actin (red) and a Src antibody (green). Shown are larger composite images; the demarcation of which depicts the results for each individual detection agent in the inset images. (C–F) Representative fluorescent images of F-actin at 72 hours (C,D) and gelatin degradation at 24 hours (D,E) after THP-1 cells were treated with 25nM PMA treatment in the presence or absence of 10 μ M PP2. (G) Quantitation of podosome incidence and gelatin degradation/cell in response to PP2 treatment. Data are plotted as the fold change relative to untreated cells, and are the averages and standard deviations of three, independent experiments. (H) THP-1 cells were allowed to adhere to uncoated plastic for 4, 24, and 72 hours with or without PMA and PP2. Data are plotted as the fold change in adhesion relative to untreated (DMSO) cells at 4 hours, and are the averages and standard deviations from three, independent experiments. (I) THP-1 cells, treated with or without PMA and PP2 for 72 hours, were analyzed for Tks5 and GAPDH (loading control) protein levels in total cell lysates by immunoblot analysis. (J) Total cell lysates from Tks5-overexpressing THP1 cells, treated with or without PMA and PP2 for 72 hours, were immunoprecipitated with a Tks5 antibody and immunoblotted with a phosphotyrosine antibody in order to monitor Tks5 tyrosine phosphorylation. Tks5 protein levels were analyzed in total cell lysates by immunoblot analysis. Statistical comparisons for adhesion at each time point and for podosome incidence and gelatin degradation between PMA alone and PMA/PP2 were done using a Student's t-test. *, $p < 0.05$; **, $p < 0.01$