The receptor tyrosine kinase MerTK activates phospholipase C γ2 during recognition of apoptotic thymocytes by murine macrophages

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Abstract: Apoptotic leukocytes must be cleared efficiently by macrophages (Mø). Apoptotic cell phagocytosis by Mø requires the receptor tyrosine kinase (RTK) MerTK (also known as c-Mer and Tyro12), the phosphatidylserine receptor (PS-R), and the classical protein kinase C (PKC) isoform βII, which translocates to Mø membrane and cytoskeletal fractions in a PS-R-dependent manner. How these molecules cooperate to induce phagocytosis is unknown. As the phosphatidylinositol-specific phospholipase (PI–PLC) γ2 is downstream of RTKs in some cell types and can activate classical PKCs, we hypothesized that MerTK signals via PLC γ2. To test this hypothesis, we examined the interaction of MerTK and PLC γ2 in resident, murine peritoneal (P)Mø and in the murine Mø cell line J774A.1 (J774) following exposure to apoptotic thymocytes. We found that as with PMø, J774 phagocytosis of apoptotic thymocytes was inhibited by antibody against MerTK. Western blotting and immunoprecipitation showed that exposure to apoptotic cells produced three time-dependent changes in PMø and J774: tyrosine phosphorylation of MerTK; association of PLC γ2 with MerTK; and tyrosine phosphorylation of PLC γ2. Cross-linking MerTK using antibody also induced phosphorylation of PLC γ2 and its association with MerTK. A PI–PLC appears to be required for phagocytosis of apoptotic cells, as the PI–PLC inhibitor Et-18-OCH3 and the PLC inhibitor U73122, but not the inactive control U73343, blocked phagocytosis without impairing adhesion. On apoptotic cell adhesion to Mø, MerTK signals at least in part via PLC γ2. *J. Leukoc. Biol.* 75: 705–713; 2004.

Key Words: *apoptosis · phagocytosis · signal transduction · protein kinases/phosphatases · mice · inbred strains*

INTRODUCTION

Apoptotic leukocytes must be cleared efficiently during resolving inflammation [1] to avoid tissue injury and the risk of autoimmunity as a result of inappropriate presentation of self-antigens [2–4]. Ingestion of apoptotic cells by macrophages (Mø) reduces inflammatory cytokine production by secretion of transforming growth factor-β and prostaglandin E₂ [5, 6], which hastens resolution of inflammation [1] but which may also impair host defenses [7, 8]. Understanding signaling pathways in apoptotic cell clearance could improve therapies of diseases that combine cell death and immunocompromise, such as acute lung injury, in which secondary infection is a major cause of mortality [9].

Specific recognition of apoptotic cells by Mø is initiated by at least two pathways. First, using a 70-kDa glycosylated type II transmembrane protein, the phosphatidylserine receptor (PS-R) [10], Mø recognize externalized PS, which translocates to the outer leaflet of the cell membrane early in apoptosis [11–15]. Recognition of externalized PS is necessary and sufficient to induce ingestion [11]. A monoclonal antibody against PS-R specifically blocks Mø phagocytosis of apoptotic thymocytes [10], and we have recently shown that this effect is not a result of inhibition of adhesion [16]. Second, a receptor tyrosine kinase (RTK) of the Tyro3 family, MerTK (also known as c-Mer and Tyro12), is crucial for apoptotic cell clearance by murine Mø in vivo and in vitro [4, 17, 18]. A host of other Mø cell-surface receptors (reviewed in ref. [19]) has been implicated in clearance of apoptotic cells, but most appear to be involved primarily in adhesion of the apoptotic cell [20, 21].

How signals from PS-R and MerTK trigger apoptotic cell phagocytosis remains incompletely defined. We and others have shown that inhibition of phosphatidylinositol 3-kinase (PI-3K) blocks apoptotic cell phagocytosis in vitro [22, 23]. However, as PI-3K inhibitors block phagosome closure, this effect might be a downstream event, as it appears to be in Fc receptor for immunoglobulin G (IgG; FcγR)-mediated phagocytosis [24]. Requirements for TKs [22, 23] and for protein kinase C (PKC) [23] have also been identified during apoptotic cell ingestion. We recently reported [16] that a single protein kinase C (PKC) isoform, PKC βII, is uniquely required for

phagocytosis of apoptotic thymocytes by murine tissue Mø and showed that an antibody against PS-R blocks translocation of PKC βII to membrane and cytoskeletal fractions in response to PS liposomes [16], a commonly used model of apoptotic cells. As classical PKC isoforms such as PKC βII require diacylglycerol (DAG) and calcium, we turned our attention to the PI-specific phospholipase C (PLC) family of enzymes as a possible means to link the actions of a RTK such as MerTK to activation of PKC βII.

Eukaryotic PI–PLC isozymes (reviewed in refs. [25, 26]) hydrolyze PI 4,5-bisphosphate (PIP_2) to produce DAG and inositol 1,4,5-trisphosphate (IP_3), a calcium-mobilizing, second messenger. Mammalian PI–PLCs comprise four subtypes, β, δ, γ, and ε [26]. All four subtypes contain pleckstrin homology (PH) domains in their NH_2-terminal region, which allow binding to specific polyphosphorylated PIs [26]. PLC γ isozymes are unique in that they also contain two Src homology (SH)2 domains and one SH3 domain, which allow interaction with proteins that contain phosphorylated tyrosine residues and proline-rich sequences, respectively [25]. PLC γ isozymes can be regulated by TKs in two ways. When cells are stimulated by growth factors that activate RTKs, PLC γ is recruited via its SH2 domains to autophosphorylated tyrosine residues of the RTK, leading to the tyrosine phosphorylation and activation of PLC γ [27]. Alternatively, in response to ligation of certain cell-surface receptors, which themselves lack RTK activity, PLC γ isozymes can be recruited into membrane signaling complexes and phosphorylated by cytoplasmic TKs [28–31]. Two PLC γ isoforms have been identified, PLC γ1 and PLC γ2. Expression of PLC γ2 is highest in cells of hematopoietic origin, and murine peritoneal Mø (PMø) have been reported to express PLC γ2 but not PLC γ1 [32, 33].

Based on this reported differential isozyme expression in Mø, the ability of PLC γ to produce DAG and IP_3, and its proven capacity to activate classical PKCs in some cell types [34–36], and as PLC γ has been shown to be phosphorylated in vitro by a chimeric protein containing the cytoplasmic portion of MerTK [37], we hypothesized that MerTK signals via PLC γ2 in response to apoptotic cells. To test this hypothesis, we examined the interactions of MerTK and PLC γ2 in resident, murine PMø and the murine Mø cell line J774A.1 (J774). Both of these Mø cell types avidly ingest apoptotic cells [38]. Using immunoprecipitation, Western blotting, immunohistochemistry, and functional assays of in vitro phagocytosis and adhesion incorporating enzyme inhibition, we provide evidence that MerTK signaling during apoptotic cell ingestion involves PLC γ2.

MATERIALS AND METHODS

Reagents

The following reagents were purchased from the indicated vendors: phosphate-buffered saline (PBS), RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HEPES, pyruvate, and penicillin/streptomycin from Invitrogen Life Technologies (Carlsbad, CA); dimethysulfoxide (DMSO), dexamethasone, 2-mercaptoethanol (2-ME), sodium deoxycholate, glycerol, NaCl, Tris HCl, Triton X-100, and phosphatase inhibitor cocktail II from Sigma Chemical Co. (St. Louis, MO); U73122, U73343, and 1-O-octa-decyl-2-O-methyl-sn-glycero-3-phosphorylcholine (Et-18-OCH3) from Biomol Research Laboratories (Plymouth Meeting, PA); rat anti-MerTK antibody and goat anti-MerTK antibody from R & D Systems (Minneapolis, MN); polyclonal rabbit anti-PLC γ1 antibody (sc-81), polyclonal rabbit anti-PLC γ2 antibody (sc-407), and blocking peptide (sc-407P), goat antiactin antibody, mouse IgG, goat IgG, protein L-agarose, protein A/G agarose (50% slurry), and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG from Santa Cruz Biotechnology (Santa Cruz, CA); anti-rat IgG [F(ab')$_2$ fragment-specific] antibody and anti-goat IgG [F(ab')$_2$ fragment-specific] antibody from Jackson ImmunoResearch Laboratories (West Grove, PA); complete miniprotease inhibitor tablets from Roche (Indianapolis, IN); sodium dodecyl sulfate (SDS), 0.2 μm polyvinylidene difluoride (PVDF) membrane, nonfat dry milk blocker, and 7.5% ready acrylamide gels from BioRad (Hercules, CA); Supersignal West Pico and Supersignal West Femto maximum sensitivity substrates from Pierce (Rockford, IL); and Kodak X-Omat AR film and eight-well Lab-Tek slides from Fisher Scientific (Chicago, IL).

Mice

All PMø were obtained from pathogen-free C57BL/6 female mice purchased from Charles River Laboratories (Wilmington, MA) at 7–8 weeks of age and were used at 8–14 weeks of age. Mice were housed in the Animal Care Facility at the Ann Arbor Veterans Affairs Medical Center (Michigan), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. This study complied with the 1996 National Academy of Sciences "Guide for the Care and Use of Laboratory Animals" (www.nap.edu/readingroom/books/labrats/) and followed a protocol approved by the Animal Care Subcommittee of the local Institutional Review Board.

Isolation and culture of PMø

Mice were killed by asphyxia in a high CO_2 environment. Resident PMø were harvested and cultured as described previously in detail [38]. PMø were isolated by adherence to sterile eight-well Lab-Tek slides (Nalge Nunc International, Naperville, IL; for phagocytosis and adhesion assays) or to tissue-culture plates (for immunoprecipitation or protein isolation). Cells were plated in 30-mm plates at a final density of $3–5 \times 10^6$ cells/plate in 1 ml complete medium (RPMI 1640 containing 10% heat-inactivated FBS, 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 100 units/ml penicillin/streptomycin, 55 μM 2-ME) and were incubated for 1.5–2 h at 37°C in 5% CO_2. Nonadherent cells were removed by gentle washing. PMø were serum-starved for at least 2 h before immunoprecipitation experiments.

Preparation of apoptotic thymocytes

Thymuses were harvested from normal mice and minced to yield a single-cell suspension. To induce apoptosis, thymocytes were resuspended in complete medium to a concentration of 1×10^6 cells/ml and incubated for 6 h in complete medium containing 1 μM dexamethasone. This treatment yields a population with a low degree of contamination by late apoptotic or necrotic cells [23, 38].

Cell line

The J774 cell line was obtained from American Type Culture Collection (Manassas, VA). These Mø were grown in 75 cm^2 plastic flasks in DMEM high-glucose medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 units/ml penicillin/streptomycin. The cells were incubated at 37°C in air (95%)–CO_2 (5%) in a humidified incubator. Cells were removed from the flasks by scraping, placed in 30 mm plates at $5–10 \times 10^6$ cells/plate (for immunoprecipitation) or chamber slides, and used for experiments (immunoprecipitation, phagocytosis, and adhesion assays) after 24 h adherence. J774 were serum-starved for at least 2 h before immunoprecipitation experiments.

Immunoprecipitation

For immunoprecipitation studies, PMø or J774 were exposed to apoptotic thymocytes in a 1:10 ratio for various times. For the cross-linking experiment, cells were preincubated with 8 μg/ml (for J774) or 30 μg/ml (for PMø) goat anti-MerTK (PMø), rat anti-MerTK (J774), or IgG for 30 min at 37°C and 5% CO_2 in serum-free medium. Following primary antibody treatment, PMø or

J774 were incubated with or without a secondary anti-goat or anti-rat $F(ab')_2$ antibody for various times to cross-link MerTK. For lysis, J774 or PMø were gently washed twice using cold PBS with protease inhibitors (complete mini-tablet) and phosphatase inhibitor cocktail II (1:100) and were then lysed for 30 min on ice using cold radioimmunoassay precipitation assay (RIPA) buffer (1.0% Triton X-100, 20 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 2 mM EDTA, and 10% glycerol with protease inhibitors and phosphatase inhibitors, as above) for phosphorylation studies or using cold Nonidet P-40 (NP-40) buffer (1% NP-40, 25 mM Tris HCl, pH 8.0, 150 mM NaCl, and 2 mM EDTA with protease and phosphatase inhibitors as above) for association studies. The lysed cells were centrifuged at 27,000 g for 5 min at 4°C. To preclear lysed cell supernatants, one of the following combinations was added: 1 µg mouse IgG and 50 µl protein A/G agarose (for phosphorylation studies), 1 µg goat IgG and 50 µl protein L-agarose (for PMø MerTK cross-linking studies), or 1 µg goat IgG and 50 µl protein A/G agarose (for PMø MerTK association studies). Cell lysates were rocked for 30 min at 4°C and then were centrifuged at 1137 g for 5 min at 4°C. The pellets were washed twice in RIPA or NP-40 buffer, and the supernatants of the washes were combined. The immunoprecipitating antibody (5 µg; 10 µg for immunoprecipitation with goat anti-MerTK) or IgG was added to the combined supernatants, and the mixture was rocked overnight at 4°C. Next, 50 µl protein A/G agarose or protein L agarose was added, and the mixture was rocked for 2 h at 4°C. Finally, the protein bound to the agarose conjugate was centrifuged at 1137 g for 5 min at 4°C, and the pellet was washed twice using RIPA or NP-40 buffer.

Western analysis

To run samples on SDS-polyacrylamide gel electrophoresis (PAGE), 10 µl 4× SDS-PAGE sample buffer and 5 µl 1 dithiothreitol were added to the pellet from immunoprecipitation, and samples were heated at 95°C for 4 min. The samples were centrifuged at 1137 g for 5 min at room temperature, and the supernatant was saved for SDS-PAGE. Protein from an equal number of cells (for immunoprecipitation) or an equal amount of protein (for expression of PLC γ2 or PLC γ1) was loaded onto 7.5% acrylamide-ready gels, run at 150 V, and transferred to 0.2 µm sequencing-grade PVDF membranes overnight at 30 V in 20% methanol, 25 mM Tris HCl, and 192 mM glycine. Blots were blocked in 5% milk, 0.1% Tween–PBS (for anti-MerTK, anti-PLC γ) without Ca/Mg or 5% bovine serum albumin (BSA), 0.1% Tween–PBS [for antiphosphotyrosine (anti-pTyr); blocker] for 45 min at room temperature. Primary antibody was added in optimal dilution in blocker and incubated overnight at 4°C. Blots were washed five times for 15 min each using Tween–PBS. Secondary antibody was added in blocker, incubated for 45 min at room temperature, and washed five times for 15 min each using PBS–Tween. Blots were stained for 5 min at room temperature using Pierce Supersignal West Pico or Supersignal West Femto detection systems. Control samples consisted of apoptotic thymocytes alone at one-tenth the amount added to PMø or J774 (which exceeds the amount calculated to adhere after 15 min, unpublished result) and PMø or J774 exposed to apoptotic thymocytes for 5 min, substituting nonspecific IgG for the immunoprecipitating antibody. Control blots stained with the secondary antibody alone showed no detectable bands.

Phagocytosis assay

Phagocytosis of apoptotic thymocytes in vitro was assayed by coincubation of $1.0–2.0 × 10^5$ adherent PMø or J774 with $2.0–4.0 × 10^6$ apoptotic thymocytes for 90 min (for PMø) or 130 min (for J774) at 37°C in 5% CO_2 as described previously [38]. Results are expressed as percentage of PMø or J774 containing at least one ingested thymocyte (percent phagocytosis) and as phagocytic index, which was generated by multiplying the percentage of phagocytosis by the mean number of ingested cells per Mø. Cell-permeable PLC or PI–PLC inhibitors were added 30 min before addition of apoptotic thymocytes at concentrations previously found to be inhibitory [39, 40].

Adhesion assay

Adherence of apoptotic thymocytes to PMø or J774 in vitro was assayed in the same manner as phagocytosis, except that $1–2 × 10^7$ apoptotic thymocytes were added to each well, yielding a thymocyte:Mø ratio of 100:1. The slides were incubated for 15 min at 37°C and then were washed in a standardized manner by dipping individual slides in each of two Wheaton jars filled with

ice-cold PBS, stained using hematoxylin-eosin Y (H & E; Richard-Allan Scientific, Kalamazoo, MI). These assay conditions have been found to be optimal (unpublished observation). Adhesion was evaluated by counting 200 PMø or J774 per well at 1000 × magnification under oil immersion and scoring for bound thymocytes. Results are expressed as percentage of PMø or J774 binding at least one thymocyte (percent adhesive Mø) and as adhesion index, which was generated by multiplying the percentage of adherence-positive Mø by the mean number of adherent thymocytes per Mø.

Statistical analysis

Data are expressed as mean ± SEM. Statistical calculations were performed using Statview 5.0 (SAS Institute, Cary, NC) on a Macintosh Power PC G4 computer. Continuous ratio scale data were evaluated by ANOVA with post-hoc analysis by the two-tailed Dunnett test, which specifically compares treatment groups with a control group [41]. Use of this parametric statistic was deemed appropriate, as phagocytosis of apoptotic thymocytes by PMø has been shown to follow a Gaussian distribution [42]. Significant differences were defined as $P < 0.05$.

RESULTS

Antibody against MerTK significantly reduces J774 phagocytosis of apoptotic thymocytes

The importance of MerTK for phagocytosis of apoptotic cells by murine PMø has been shown by a genetic approach in vivo and in vitro [17], and we have recently found that antibody against MerTK inhibits phagocytosis (but not adhesion) of apoptotic cells by murine PMø [43]. To verify the importance of MerTK in the phagocytosis of apoptotic thymocytes by the J774 cell line, we measured phagocytosis in the presence of goat anti-MerTK or IgG control. Pretreatment with anti-MerTK significantly inhibited phagocytosis of apoptotic thymocytes by J774 (**Fig. 1, A** and **B**). By contrast, MerTK inhibition had no effect on adhesion by J774 (Fig. 1, C and D). Thus, both Mø cell types used in this study use MerTK in apoptotic cell uptake.
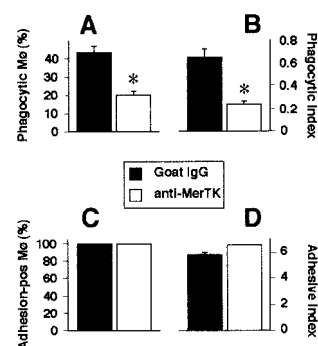

Fig. 1. Blocking MerTK reduces phagocytosis but not adhesion of apoptotic thymocytes by J774. The Mø cell line J774 was preincubated for 30 min in medium containing goat IgG (solid bars) or goat anti-MerTK (open bars; 50 µg/ml concentration) and without washing, were then incubated with apoptotic thymocytes and assayed for phagocytosis (A, B) or adhesion (C, D), as determined by examining H & E-stained slides under oil immersion. (A) Percentage of phagocytic J774 ingesting at least one apoptotic thymocyte. (B) Phagocytic index. Data are mean ± SEM of three to six replicates in each of two independent experiments. *, $P < 0.05$, compared with control; ANOVA with post-hoc testing by the two-tailed Dunnett test. (C) Percentage of phagocytic J774 binding at least one apoptotic thymocyte. (D) Adhesion index. Data are mean ± SEM of three replicates per condition in a single experiment. Note difference in scales between phagocytosis and adhesion data.

A
Cell: PMo
IP: anti-pTyr
IB: anti-MerTK

C'   5'   15'   30'   60'

215 kDa →   ← MerTK

B
Cell: J774
IP: anti-pTyr
IB: anti-MerTK

C'   5'   10'   15'   20'   Thy

207 kDa →   ← MerTK

C
Cell: PMo
IP: anti-MerTK
IB: anti-pTyr
reblot: anti-MerTK

C'   5'   15'   30'   60'   Thy

233 kDa →   ← MerTK
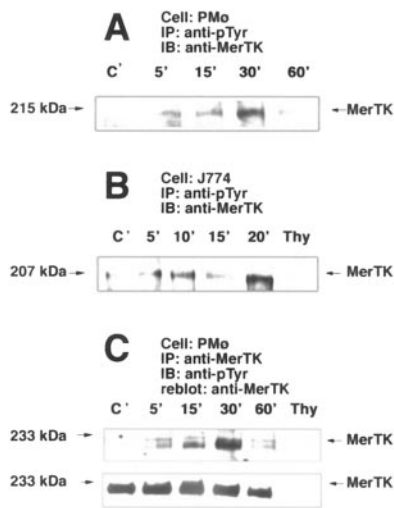
233 kDa →   ← MerTK

Fig. 2. Exposure to apoptotic thymocytes induces transient tyrosine phosphorylation of MerTK. (A, B) Immunoprecipitation using anti-pTyr antibody. Equal numbers of PMø (A) or J774 (B) were incubated with a tenfold greater number of apoptotic thymocytes for the indicated time (in minutes), washed extensively, and then lysed in RIPA buffer. After preclearing, Mø lysates were immunoprecipitated (IP) using anti-pTyr antibody and then immunoblotted (IB) using goat anti-MerTK. After incubation with HRP-conjugated donkey anti-goat IgG in Tween–PBS containing 5% milk, signal was detected by chemiluminescence. C′, Control; Thy, thymocytes (10^7) alone. Similar results were seen in an independent experiment of this design using each Mø cell type. (C) Immunoprecipitation using goat antibody against MerTK. PMø were incubated with apoptotic thymocytes for the indicated time, washed extensively, and then lysed in RIPA buffer. After preclearing, Mø lysates were immunoprecipitated using goat antibody against MerTK and then were immunoblotted using anti-pTyr antibody. After incubation with HRP-conjugated donkey anti-rabbit IgG in BSA, signal was detected by chemiluminescence. The membrane was then stripped and reblotted using goat antibody against MerTK to demonstrate loading. Similar results were seen in four independent experiments of this design using PMø.

MerTK is phosphorylated in response to apoptotic thymocytes

Formation of docking sites capable of binding the SH2 domains of signaling proteins requires tyrosine phosphorylation of the RTK itself [44, 45]. Therefore, we next used two different approaches to examine phosphorylation of MerTK in response to apoptotic thymocytes. Immunoprecipitation with anti-pTyr and Western blotting with goat anti-MerTK showed no detectable phospho-MerTK at baseline but rapid induction that was maximal at 30 min for PMø and 20 min for J774 (**Fig. 2, A** and **B**). The alternate approach, immunoprecipitating with anti-MerTK and Western blotting with anti-pTyr, gave similar results (Fig. 2C). The specificity of this approach was tested in control samples of PMø incubated for 5 min with apoptotic cells and using goat IgG as the immunoprecipitating antibody and anti-pTyr as the immunoblotting antibody. This analysis demonstrated no bands at 205 kDa, the approximate molecular weight (MW) of MerTK (unpublished result). Nor did apoptotic thymocytes themselves have detectable MerTK (unpublished result), consistent with previous results in normal thymocytes [46]. Thus, on exposure of murine PMø or J774 to apoptotic cells, MerTK rapidly and specifically undergoes tyrosine phosphorylation, potentially forming sites for docking of SH2 domain-containing proteins.

In a separate, control experiment using PMø, we confirmed that cross-linking MerTK using anti-MerTK antibody followed by anti-goat IgG Fc fragment-induced MerTK phosphorylation (unpublished result). This result implies that MerTK undergoes autotransphosphorylation in resident, murine PMø, as has been shown previously in green monkey kidney CV-1 cells transiently overexpressing a constitutively active form of MerTK [44].

PLC γ2 is expressed strongly by PMø and J774 and weakly by apoptotic thymocytes

To determine the relative expression of PLC γ isoforms in PMø, J774, and apoptotic thymocytes, we used Western blot analysis of equal amounts of protein from each cell type. We confirmed that PLC γ2 was strongly expressed in PMø (**Fig. 3A**), in agreement with previously published results [32], as well as in J774 (Fig. 3C). Apoptotic thymocytes themselves expressed PLC γ2 only weakly (Fig. 3, A and C), making it unlikely that any residual, adherent thymocytes remaining after washing would contribute to our analysis of Mø PLC γ2. By contrast, PLC γ1 was barely detectable in murine PMø and J774 (Fig. 3, B and D), which allowing for our use of a very sensitive detection system, is consistent with previous observations [32, 33]. These data led us to focus on the PLC γ2 isozyme in subsequent studies.

MerTK and PLC γ2 physically associate in response to apoptotic thymocytes

To confirm that MerTK phosphorylation forms functional docking sites, we used immunoprecipitation with anti-MerTK and Western blotting with anti-PLC γ2 to measure the possible association of MerTK and PLC γ2 in response to exposure to apoptotic thymocytes. We found that there was slight association of MerTK and PLC γ2 in PMø and J774 without apoptotic thymocytes, but that association increased after apoptotic thymocyte addition at 5, 15, and 30 min for PMø (**Fig. 4A**) and at

A
Anti-PLC γ2   Anti-PLC γ2 plus blocker

Thy PMø   Thy PMø

207 kDa →
129 kDa →   ← PLC γ2

39.7 kDa →   ← Actin

B
Thy PMø

207 kDa →
129 kDa →   ← PLC γ1

39.7 kDa →   ← Actin

C
Anti-PLC γ2   Anti-PLC γ2 plus blocker

Thy J774   Thy J774

207 kDa →
129 kDa →   ← PLC γ2

39.7 kDa →   ← Actin

D
Thy J774

207 kDa →
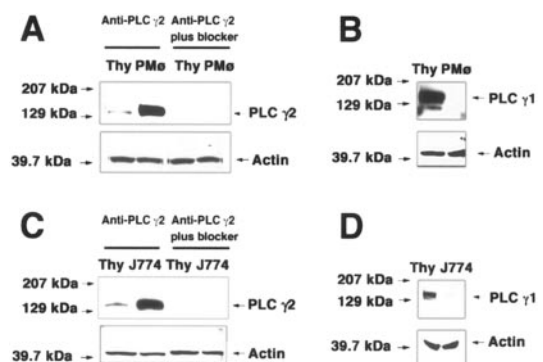129 kDa →   ← PLC γ1

39.7 kDa →   ← Actin

Fig. 3. Expression of PLC γ isoforms. Resident PMø and apoptotic thymocytes (Thy) from normal mice and J774 were lysed, and equal amounts of protein were run on a 7.5% acrylamide gel under reducing conditions and transferred to PVDF membranes. Blots were probed first with specific anti-PLC γ2 or anti-PLC γ2 plus blocking PLC γ2 peptide (A, C, upper row) or with anti-PLC γ1 (B, D, upper row). Blots were then washed for 15 min using Tween–PBS and incubated with HRP-conjugated donkey anti-rabbit IgG diluted in Tween–PBS containing 5% milk, and signal was detected by chemiluminescence. Blots were then stripped and reprobed using antiactin antibody (A–D, lower row) to demonstrate protein loading.
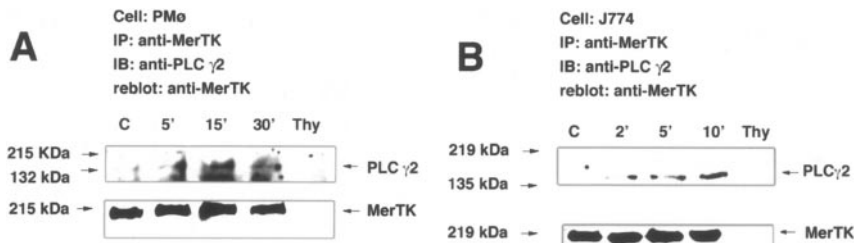
Fig. 4. Exposure of PMø or J774 to apoptotic thymocytes (Thy) induces physical association of PLC γ2 and MerTK. Equal numbers of resident PMø (A) or J774 (B) were incubated with a tenfold greater number of apoptotic thymocytes for various times as indicated, washed extensively, and then lysed in NP-40 buffer. After preclearing, Mø lysates were immunoprecipitated (IP) using goat anti-MerTK and protein A/G agarose, run on a 7.5% acrylamide gel under reducing conditions, and transferred to PVDF membranes. Blots were then probed (IB) using anti-PLC γ2 antibody (A, B, upper row), developed as described in the legend to Figure 2, and then stripped and reprobed using goat anti-MerTK antibody (A, B, lower row) to demonstrate protein loading. Similar results were seen in an additional, independent experiment. C, Control.

10 min for J774 (Fig. 4B). As expected, there was no association of MerTK and PLC γ2 in preparations of apoptotic thymocyte alone, as that cell type lacks MerTK. Control samples of Mø and apoptotic cells at 5 min with goat IgG as the immunoprecipitating antibody and anti-PLC γ2 as the immunoblotting antibody demonstrated no bands at 140 kDa, the MW of PLC γ2 (unpublished result). Therefore, exposure to apoptotic thymocytes induces the association of MerTK and PLC γ2 in PMø, potentially bringing the latter enzyme into a position in which it could act on membrane lipids.

PLC γ2 is phosphorylated in PMø and J774 in response to apoptotic thymocytes

We next examined whether Mø PLC γ2 becomes phosphorylated in response to apoptotic thymocytes, using immunoprecipitation with anti-pTyr and Western blotting with anti-PLC γ2. Baseline phosphorylation of PLC γ2 in PMø or J774 lysates was detectable without exposure to apoptotic thymocytes, but phosphorylation increased at 5 and 10 min after apoptotic thymocyte addition (**Fig. 5, A** and **B**). Apoptotic thymocytes alone showed undetectable phosphorylation of PLC γ2 (Fig. 5, A and B), which together with the small amount of total PLC γ2 present in apoptotic thymocytes (Fig. 2), implies that this is a

result of induced phosphorylation of PLC γ2 in PMø and not to immunoprecipitation of phosphorylated thymocyte PLC γ2.

However, to establish this point more rigorously, in control experiments, we showed that the small amount of PLC γ2 found in apoptotic thymocytes themselves did not become phosphorylated when exposed to PMø and then removed by washing (unpublished result). Additional control experiments were performed in which PMø were incubated with apoptotic cells for 5 min, and then mouse IgG and anti-PLC γ2 were used as the immunoprecipitating and immunoblotting antibodies, respectively; this procedure yielded no bands around 140 kDa, the MW of PLC γ2 (unpublished result). Collectively, these data indicate that PLC γ2 is phosphorylated in PMø or J774 in response to apoptotic thymocytes.

PLC γ2 is phosphorylated and associates with MerTK upon cross-linking of MerTK

To determine whether MerTK activation was sufficient to induce PLC γ2 phosphorylation in the absence of other signals emanating from apoptotic cell recognition, we next cross-linked MerTK in the absence of apoptotic thymocytes and measured PLC γ2 phosphorylation, which was minimal without MerTK cross-linking but increased at 5 and 10 min after cross-linker addition to PMø (**Fig. 6A**) or J774 (Fig. 6, B and C). In the control experiment using IgG as a substitute for anti-MerTK pretreatment, minimal increase in PLC γ2 phosphorylation was observed. In separate, control experiments, we showed that adding anti-MerTK without cross-linking did not induce PLC γ2 phosphorylation (unpublished result). We also measured MerTK and PLC γ2 association in J774 cells after cross-linking MerTK and found an increase in association at 5 and 10 min after cross-linking with minimal increase using IgG as a substitute for anti-MerTK (Fig. 6C). Thus, direct activation of MerTK induces phosphorylation of PLC γ2 and its association with MerTK even in the absence of apoptotic cells.

PLC and PI–PLC inhibitors significantly reduce Mø phagocytosis of apoptotic thymocytes

Finally, as it has not previously been determined whether any PLC is involved in Mø phagocytosis of apoptotic cells, we measured the effect of the nonspecific PLC inhibitor U73122 on phagocytosis and adhesion of apoptotic thymocytes. Pretreatment with U73122 significantly inhibited phagocytosis of apoptotic thymocytes by PMø (**Fig. 7, A** and **B**) or J774 (Fig. 7, C and D) at concentrations previously found to be inhibitory for PLC [39], whereas the inactive control reagent U73343 had
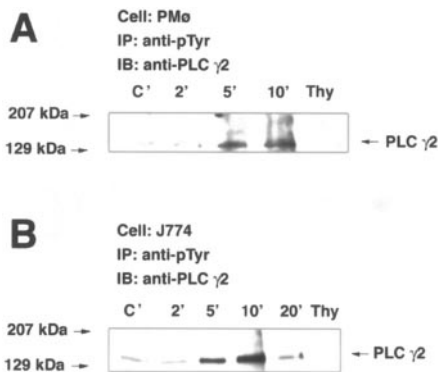

Fig. 5. Exposure of PMø or J774 to apoptotic thymocytes induces PLC γ2 phosphorylation. Equal numbers of resident PMø (A) or J774 (B) were stimulated in vitro with a tenfold greater number of apoptotic thymocytes for various times as indicated, washed extensively, and then lysed in RIPA buffer. After preclearing, Mø lysates were immunoprecipitated (IP) using anti-pTyr antibody and protein A/G agarose, run on a 7.5% acrylamide gel under reducing conditions, and transferred to PVDF membranes. Blots were then probed (IB) using anti-PLC γ2 antibody and developed as described in the legend to Figure 1. C′, Control; Thy, Thymocytes alone. Similar results were seen in an additional, independent experiment.
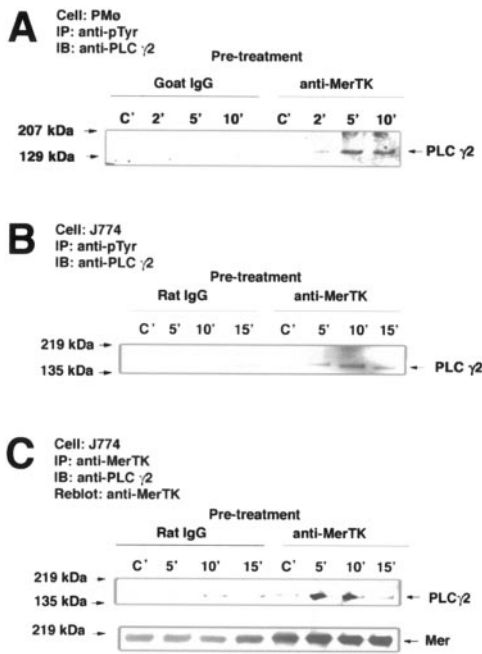
Fig. 6. Cross-linking MerTK induces PLC γ2 phosphorylation and its association with MerTK. Equal numbers of PMø (A) or J774 (B, C) were incubated with 30 µg/ml goat anti-MerTK (PMø), 8 µg/ml rat anti-MerTK (J774), or the appropriate control IgG in serum-free medium for 30 min at 37°C and 5% CO_2 as indicated. After washing, PMø or J774 were incubated for various times in serum-free medium containing a secondary anti-goat or anti-rat $F(ab')_2$ (30 or 8 µg/ml, respectively) to cross-link MerTK. PMø or J774 were next lysed in RIPA buffer (A) or NP-40 buffer (B, C), and lysates were immunoprecipitated (IP) using anti-pTyr (A, B) or goat anti-MerTK (C), run on a 7.5% acrylamide gel under reducing conditions, and transferred to PVDF membranes. Blots were then probed (IB) using anti-PLC γ2 antibody and developed as described in the legend to Figure 2. (C) The blot was then stripped and reprobed using goat anti-MerTK antibody to demonstrate protein loading. C′, Control.

MerTK, physical association of PLC γ2 with MerTK, and tyrosine phosphorylation of PLC γ2. We also showed that antibody cross-linking of MerTK leads to phosphorylation of MerTK and PLC γ2 and their physical association, indicating that activated MerTK is sufficient to induce these events even in the absence of apoptotic cells. Furthermore, we show that phagocytosis (but not adhesion) of apoptotic thymocytes was blocked by nonspecific and PI–PLC-specific inhibitors. These novel findings significantly advance the understanding of signal transduction during Mø phagocytosis of apoptotic cells.

The finding by coimmunoprecipitation that MerTK physically associates with PLC γ2, the predominant Mø isoform of PLC γ, is the first demonstration of an intracellular signaling protein recruited to wild-type MerTK on exposure to apoptotic cells. Ling and Kung [37] previously showed that overexpression of a chimeric RTK containing the transmembrane and cytoplasmic domains of human MerTK in NIH 3T3 fibroblast cells induced PLC γ phosphorylation when the extracellular portion of the receptor (Fms domain) was stimulated with its appropriate ligand (colony-stimulating factor type 1). It is
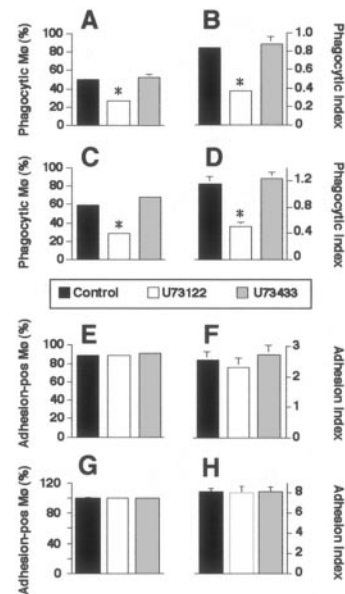

Fig. 7. PLC inhibitors reduce PMø and J774 phagocytosis but not adhesion of apoptotic thymocytes. (A–D) Phagocytosis assay. Resident PMø (A, B) or J774 (C, D) were plated on eight-well chamber slides in media containing 10% FBS at $1–2 \times 10^5$ cells/well. Mø were preincubated for 30 min at 37°C and 5% CO_2 in medium containing U73122 (open bars) or U73433 (shaded bars), each at a final concentration of 1.0 µM, or the appropriate amount of DMSO (solid bars). Next, without washing, apoptotic thymocytes were added ($1–2\times10^6$ for 90 min for PMø; $2–4\times10^6$ for 130 min for J774). Phagocytosis was determined by examining H & E-stained slides under oil immersion. (A, C) Percentage of phagocytic Mø; (B, D) phagocytic index. Data are mean ± SEM of three to six replicates per condition in a single experiment. *, $P < 0.05$, compared with DMSO control, ANOVA with post-hoc testing by the two-tailed Dunnett test. (E–H) Adhesion assay. Resident PMø (E, F) or J774 (G, H) were pretreated as above with U73122 (open bars), U73433 (shaded bars), or the appropriate amount of DMSO only (solid bars), and then apoptotic thymocytes ($1–2\times10^7$ cells/well) were added for 15 min. Mø adhesion of apoptotic thymocytes was determined by examining H & E-stained slides under oil immersion. (E, G) Percentage of adhesive Mø; (F, H) adhesion index. Note the difference in the scales between the two Mø cell types. Data are mean ± SEM of three replicates per condition in a single experiment.

no effect. By contrast, PLC inhibition had no effect on adhesion by PMø (Fig. 7, E and F) or J774 (Fig. 7, G and H). Moreover, at a concentration previously shown to be inhibitory for PI–PLC [40], Et-18-OCH3 significantly reduced phagocytosis of apoptotic cells by PMø (relative to untreated control, the percent phagocytic PMø was inhibited by 72.0±2.1%, and the phagocytic index was inhibited by 79.2±8.3%, $P<0.05$, unpaired t-test) and by J774 (relative to untreated control, the percent phagocytic J774 was inhibited by 71.3±2.8%, and the phagocytic index was inhibited by 81.4±1.8%, $P<0.05$, unpaired t-test). Et-18-OCH3 had no effect on adhesion of apoptotic cells to either type of Mø (unpublished result). These results indicate that a PI–PLC isozyme is necessary for Mø phagocytosis of apoptotic cells at a step distal to adhesion.

DISCUSSION

The principal findings of this study indicate that signaling by the RTK MerTK during recognition of apoptotic cells by murine Mø involves PLC γ2. This conclusion was verified using primary resident tissue Mø and a Mø cell line and is based on the induction of three time-dependent changes upon exposure of PMø or J774 to apoptotic cells: tyrosine phosphorylation of

important that our results extend this mechanism to a physiologic system, wild-type MerTK in a primary murine Mø cell responding to apoptotic cells. This point is significant, as previous studies using chimeras have shown that the identity of the extracellular domain used can drastically alter the intracellular response of members of the Tyro3 family of RTKs [47–49]. It is interesting that Mahajan and Earp [50] have recently identified another signaling partner of MerTK. Using human monocytes, they showed that MerTK sequesters the guanine exchange factor Vav1 under unstimulated conditions and can release it to hydrolyze RhoA family members upon MerTK activation by Gas6, a serum factor that binds apoptotic cells and MerTK [50]. Vav1 regulates actin cytoskeletal rearrangement via Rac during FcγR-mediated phagocytosis by Mø [51]. As Vav1 and PLC γ2 associate with MerTK via their SH2 domains (Vav1, unusually, in the absence of MerTK phosphorylation; ref. [50]), an intriguing possibility is that Vav1 release forms part of the signals that recruit PLC γ2 to activated MerTK. Studies in gene-targeted mice have shown that members of the Vav family are required to activate PLC optimally in T, B, and mast cells [52–54] but not in platelets [55]. Thus, our results together with those of Mahajan and Earp [50] suggest a means by which MerTK activation focuses a series of sequentially acting signaling intermediaries at the nascent phagosome.

The finding that exposure to apoptotic cells induces PLC γ2 tyrosine phosphorylation is significant, as this modification is obligatory for its enzymatic action in vivo [56], which in turn, is required to produce the DAG and Ca^{++} transient needed to localize PKC βII to the cell membrane. Hence, these results provide evidence that PLC γ2 participates in a pathway linking stimulation of MerTK on the Mø surface with mobilization of PKC βII to the nascent phagosome. This pathway undoubtedly comprises multiple, additional components, likely including PI-3K, which also contributes to PLC regulation [32]. It is important to stress that we do not claim PLC γ2 to be the only PI–PLC involved in this putative pathway or in apoptotic cell phagocytosis itself. The incomplete specificity of available pharmacologic inhibitors of PLC precludes conclusive testing of that possibility, which will require transfection of dominant-negative and constitutively active PLC isoforms. Support for a possible role of other PLC isoforms comes from analysis of gene-targeted mice lacking PLC γ2 for FcγR-mediated phagocytosis, a process in many ways similar to apoptotic cell phagocytosis. Although these mice have not yet been studied for apoptotic cell clearance, their Mø unexpectedly show intact FcγR-induced phagocytosis, despite an absence of Ca^{++} flux [33]. These findings could indicate that FcγR-induced phagocytosis does not depend on PLC γ2 or that the process has some redundancy in its signaling pathway. Redundancy in the pathways mediating apoptotic cell clearance has been noted previously [4], and with the notable exception of mice lacking C1q [57], gene-targeting of receptors clearly involved in apoptotic cell recognition does not invariably lead to apoptotic cell accumulation [58]. MerTK-deficient mice themselves exhibit no accumulation of apoptotic bodies in the thymus [17], likely a result of compensation by other Tyro3 family members [18]. Hence, even if PLC γ2 knockout mice do prove to have normal apoptotic cell clearance, it would not negate the significance of the role we show for that molecule in MerTK signaling in Mø of wild-type mice.

Results of our inhibitor studies imply that a necessary, early step in Mø recognition of apoptotic cells is PLC-mediated hydrolysis of PIP_2, as has been suggested previously in FcγR-mediated phagocytosis [59]. Hydrolysis of PIP_2 by PLC to yield DAG and IP_3 is an early step in a very large number of signaling pathways [26]. This hydrolysis reaction has been postulated to be essential not only to produce these two crucial second messengers but also because elimination of PIP_2 removes attachment sites for many PH domain-containing molecules (although not for PLC γ2 itself) and thereby can control the actin cytoskeleton [60, 61]. One of the most common means of activating PLC γ is for it to be tyrosine-phosphorylated directly by growth factor receptors that are RTKs [56], and our data support a similar role for MerTK. In hematopoietic cell types other than Mø, PLC γ2 can also be phosphorylated and thus activated by nonreceptor Tks [62, 63]. For example, signaling through the B cell antigen receptor leads to phosphorylation of SLP-65 by Syk; PLC γ2 and the Tec family kinase Bruton's TK (BTK) then bind to pTyr on SLP-65, where BTK phosphorylates PLC γ2 [64]. Similarly, kinases from the Src, Syk, and Tec family have been implicated in glycoprotein VI-dependent phosphorylation of PLC γ2 in platelets [56]. However, as we have shown previously that the potent Src inhibitor PP2 does not inhibit apoptotic cell phagocytosis by PMø [23], we think it unlikely that Src family kinases are necessary for PLC γ phosphorylation in this system. Additionally, we have recently shown that murine PMø do not phosphorylate Syk during apoptotic cell ingestion [65], arguing strongly against its participation. Thus, we currently favor direct phosphorylation of PLC γ2 by MerTK itself in response to apoptotic cell binding, although definitive proof of that possibility will require further studies. Finally, PLC γ can be activated without tyrosine phosphorylation in some systems [26]. Phosphatidic acid, an immediate product of PC hydrolysis by PLD, has been shown to activate tyrosine-phosphorylated and unphosphorylated forms of PLC γ1 by increasing their affinity for substrate vesicles [66, 67]. Arachidonic acid also stimulates PLC γ activity independently of tyrosine phosphorylation in the presence of the neuronal microtubule-associated protein tau (126) or of a 680-kDa molecule, AHNAK, in non-neuronal cells [68]. Whether some degree of phosphorylation-independent PLC γ2 activation occurs during Mø ingestion of apoptotic cells will also require additional study.

Combined with our previous demonstration that the translocation of PKC βII induced by PS liposomes is inhibited by antibody against PS-R [16], the current results imply that upon apoptotic cell adhesion to Mø, signals from PS-R and from MerTK converge to recruit and activate PKC βII. The molecular basis for the essential role of PKC βII apoptotic cell ingestion is unknown. In separate studies, we and others have previously found that another type of resident Mø, the pulmonary alveolar Mø (AMø), expresses markedly reduced amounts of this particular PKC isoform [23, 69]. This reduction partially explains why resident AMø ingest apoptotic cells so poorly [38, 70, 71], although reduced adhesion also contributes [43]. Quantitative differences in expression of MerTK between AMø and other Mø do not appear to explain this relative reduction in

apoptotic cell phagocytosis (unpublished result). We are actively investigating the possible connection in signal-transduction pathways from PS-R and from MerTK. Understanding the signaling pathways controlling apoptotic cell clearance is a first step to manipulating the process therapeutically.

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