Efficient clearance of apoptotic cells is essential for tissue homeostasis, allowing for cellular turnover without inflammatory consequences. The Mer (Nyk and c-Eyk) receptor tyrosine kinase (Mertk) is involved in two aspects of apoptotic cell clearance by acting as a receptor for Gas6, a γ-carboxylated phosphatidylserine-binding protein that bridges apoptotic and viable cells. First, Mertk acts in a bona fide engulfment pathway in concert with αvβ5 integrin by regulating cytoskeletal assemblies, and second, it acts as a negative regulator for inflammation by down-modulating pro-inflammatory signals mediated from bacterial lipopolysaccharide-Toll-like receptor 4 (TLR4) signaling, and hence recapitulating anti-inflammatory immune modulation by apoptotic cells. Here we describe Mertk post-receptor events that govern phagocytosis and cytoskeletal signaling are principally mediated by autophosphorylation site Tyr-867. Using the Mertk Y867F mutant and pharmacological inhibitors, we show that Tyr-867 is required for phosphatidylinositol 3-kinase and phospholipase Cγ2 activation; their activation in turn elicits protein kinase C-dependent signals that act on the actin cytoskeleton. Although MertkY867F blocked the tyrosine phosphorylation of FAK on Tyr-861 and p130Cas and also abrogated the phagocytosis of apoptotic cells, this mutant did not suppress lipopolysaccharide-inducible NF-κB transcription, nor was NF-κB activation dependent on the protein kinase C inhibitor, calphostin C. Finally, unlike the cytoskeletal events associated with Tyr-867 autophosphorylation, the trans-inhibition of NF-κB occurred in a postnuclear-dependent fashion independent of cytosolic IκB phosphorylation and p65/RelA sequestration. Taken together, these data suggest that Mertk has distinct and separable effects for phagocytosis and for resolving inflammation, providing a molecular rationale for how immune licensing and inflammation can be dissociated from phagocytosis in a single phagocytic receptor.

The Mer/Nyk receptor tyrosine kinase (Mertk) was first described as a tyrosine kinase retroviral oncogene (RPL30), called v-Ryk and later v-Eyk, that contained the retroviral envelope gp37-coding region fused to the intracellular domain of Eyk (1, 2). Overexpression of the v-eyk gene product by infectious virus caused transformation of chicken embryo fibroblasts and produced sarcomas and lymphomas in vivo. Cloning of the cellular form of v-Eyk, c-Eyk, revealed an unusual extra-cellular domain structure, consisting of novel repeating units containing two C2-type Ig domains and two type III FN-like domains that were truncated in the viral genome (3). Based on homology of the ectodomains, Mertk is part of a subfamily of RTKs together with Tyro-3/Sky and Axl that have atypical ectodomains resembling the adhesion molecules neural cell adhesion molecule and L1 (4, 5). Mertk, which is predominantly expressed in cells of myeloid, epithelial, and reproductive (Mer) origin, induces transformation when ectopically expressed in fibroblasts (6) but has also been shown to be overexpressed in neoplastic T and B cells (7, 8), as well as in mantle cell lymphomas (9). Although Axl and Mertk were first identified and associated with various human malignancies, it is now clear that they have complex and pleiotrophic functions depending on the cell type where they are expressed as well as in the context in which they are activated (10).

The biological ligands for Axl, Tyro-3/Sky, and Mertk are two homologous vitamin K-modified proteins, called growth...
arrest-specific factor-6 (Gas6) and Protein S (11, 12), the latter also being a negative regulator of blood coagulation (12). Both proteins contain signal sequences that promote their secretion, followed by an N-terminal segment that is post-translationally modified by vitamin K to produce γ-carboxyglutamic acid residues (Gla domain). γ-Carboxylation of Gas6 is responsible for calcium-dependent interaction with PS and bridging of the apoptotic cell to the phagocyte (13). To mediate Mertk binding and autophosphorylation, Gas6 has central EGF-like repeats, followed by a C-terminal sex hormone binding globulin-like domain consisting of tandem laminin G-like domains (14). Recent studies have elucidated the structure of a Gas6-Axl ectodomain complex, which provides an eloquent model for ligand-dependent dimerization, and subsequent autophosphorylation and productive trans-membrane signaling (15, 16). A novel aspect of Mertk biology has been identified in recent years based on intriguing and unexpected phenotypes from Axl-, Tyro-3/Sky-, and Mertk-deficient mice (17) and independent studies from a Mertk mutant transgene that carries a mutation in the intracellular kinase domain (18–20). Mutant mice that lack Tyro-3/Sky, Axl, and Mertk develop a severe lymphoproliferative disorder accompanied by broad spectrum autoimmunity (17). Similarly, mice that express mutant Mertk (termed MerKDP), when back-crossed onto the C57/Bl/6 background, show multiple defects in monocyte function and develop an age-dependent autoimmune disease characterized by the loss of the ability to ingest apoptotic cells (20). Current theory holds that persistent apoptotic debris results in the accumulation of pro-inflammatory mediators in tissues and represents a source of chronic inflammation and availability of intracellular antigens that can lead to autoimmune disease. MertkKD transgenic mouse show increased circulating TNF-α, age-dependent glomerulonephritis, increased susceptibility to endotoxin, and lupus-like pathology with auto-antibodies to self components (20). Interestingly, a seemingly unrelated retinal dystrophy lesion resulting in blindness has been observed in the Royal College of Surgeons (RCS) rat strain (21, 22), associated with a loss of function of Mertk in retinal pigmented epithelial cells. However, the RCS phenotype results from an inability to clear PS-opsonized shed rod outer segments in an analogous pathway as described for apoptotic cells (23, 24). Although these effects on apoptotic cell clearance were first ascribed to a function intrinsic to Mertk, recent studies now suggest that Axl and Sky also regulate phagocytosis, but are expressed in different cell types and therefore have cell-type specific phagocytic functions in cells other than macrophages (25).

Together, studies from the knockout mice and kinase-dead MertkKD transgenic mice indicate that Mertk regulates two aspects of apoptotic cell clearance. First, it is involved in a bona fide cytoskeletal signaling pathway that involves bidirectional tyrosine phosphorylation-dependent cross-talk with αβ5 integrin, and culminates in the activation of Rac1 and actin-mediated phagocytosis (26–28). Second, Mertk is implicated in the resolution of inflammation and modulation of immune function. One aspect of this pathway is illustrated by the ability of Mertk to decrease LPS/TLR4-inducible NF-κB transcriptional activation and the ensuing production of inflammatory cytokines that include interleukin-1β, TNF-α, and interleukin-12 (18, 29). Although both cytoskeletal signals and those signals that down-modulate pro-inflammatory signals rely on the activation of Mertk activity and tyrosine phosphorylation, it is not yet clear what interplay there is between common initiating signals, or whether engulfment is required for down-modulating inflammation.

In the present study, we found that the tyrosine phosphorylation of Akt, PLCγ2, and FAK, signals that impinge on cytoskeletal reorganization and engulfment, are mediated by a single docking Tyr-867 autophosphorylation site in Mertk. Through mutagenesis and pharmacological inhibitors, we identified a direct link between Tyr-867 and activation of cytoskeletal assemblages leading to phagocytosis. Phosphorylation of Tyr-867 results in the activation of both Akt and PLCγ2, which further leads to the activation of PKC, and its ensuing effects on the actin cytoskeleton that include tyrosine phosphorylation of FAK and p130Cas. In contrast, we found no evidence for cross-talk between the Tyr-867 pathway and down-modulation of LPS-TLR4-mediated NF-κB transcriptional activation. Moreover, Gas6-Mertk-inducible suppression of LPS signaling appears to be a postnuclear event, because transcriptional suppression was accompanied by decreased reporter activity, but not decreased IκB phosphorylation or inhibition of translocation of p65 Rel/A into the nucleus. Taken together, our data suggest that Mertk has distinct and separable effects for phagocytosis and resolving inflammation, providing a molecular rationale for how engulfment and immune licensing can be dissociated within a single phagocytic receptor.

**Materials and Methods**

**Antibodies, Plasmids, and Reagents**—Monoclonal antibodies (mAB) were purchased from the following vendors: anti-FAK and p130Cas were from BD Biosciences; anti-phosphotyrosine from Santa Cruz Biotechnology; polyclonal antibody against PLCγ2 was from Santa Cruz Biotechnology; polyclonal antibody specific for AKT was from Cell Signaling. Polyclonal antibody against Mertk (anti-Mertk) was from FabGennix (MKT-101AP). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were obtained from Jackson Laboratories. The phospho-antibodies directed to FAK (Tyr-861) and AKT (Ser-473) were from BIOSOURCE. IκB and phospho-IκB were purchased from Cell Signaling Technology. The Luciferase kit was purchased from Promega (Madison, WI). Lipopolysaccharide from Escherichia coli (0111:B4) was purchased from Sigma-Aldrich. Lipofectamine 2000 was purchased from Invitrogen. FuGENE was purchased from Roche Pharmaceuticals; calphostin C was from Calbiochem-Novabiochem International; myristoylated PKC inhibitory peptide (mer-RFARKGALRQKNV) and other reagents were obtained from Sigma unless otherwise specified.

The retroviral expression vectors pLXSN expressing CD8-Mertk, a chimeric receptor generated from the intracellular part of Mertk (amino acids 521–994), and the extracellular and transmembrane domains of the human CD8ε (amino acids 1 to 209), and its kinase-negative mutant CD8-MertkKD (K614M mutates the ATP binding site), as well as four Tyr to Phe mutants (Y544F, Y825F, Y867F, and Y924F) were generated as
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previously described (30). Mutations in the ITIM motif at Y671F, Y680F, and Y689F in CD8-Mertk were generated by site-directed mutagenesis. The primers designed are as follows: ITIM-1 (Y671-F): forward, CCC TTC ATG AAA TTC GGA GAC CTC CAC ACC; reverse, GGT GTG GAG GTG TCC GAA GAA TTT CAT GAA GGG; ITIM-2 (Y680-F): forward, CAC ACC TTC CTG TTA TTC CGA TTA AAC ACA; reverse, TGT GTT TAA TGC GGA GAA TAA CAG GAA GGT GTG; and ITIM-3 (Y689-F): forward, ACA GGA CCC AAG TTC ATT CAC CTT GAC ACA; reverse, TGT CTG CAG GTG AAT GAA CTT GGG TCC TGT. The full-length Mertk (994 amino acids) was cloned from a 16-day-old mouse embryo cDNA library, and subcloned into pIRE2-EGFP at the sites of EcoRI and BamHI. The mutant of Mertk, Y687F, was generated by site-directed mutagenesis using PCR with the following primers; forward, 5′-GAATCCATCATCTTCAT-CAATACCAG-3′ and reverse, 5′-CTGGTATTTGGAAA- GATGATGGATC-3′. Plasmid pIRE2-EGFP-Mertk was used as template, and PCR amplifications were performed for 30 cycles with the proofreading DNA polymerase (Stratagene). The nucleotide sequence following mutagenesis was determined to ensure that the expected mutation was present and that no additional mutations were introduced by PCR.

Cell Culture and Transfection—Human embryonic kidney HEK-293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. Transient overexpression in HEK-293T cells was achieved using the Lipofectamine 2000 reagent or Lipo2000 reagent (Invitrogen) as indicated by the manufacturer. RAW 264.7 cells were maintained in RPMI supplemented with 10% FBS and 2 mM L-glutamine containing penicillin/streptomycin. Cell transient overexpression in HEK-293T cells was achieved using the Lipofectamine 2000 reagent or Lipo2000 reagent (Invitrogen) as indicated by the manufacturer. RAW 264.7 cells were maintained in RPMI supplemented with 10% FBS and 2 mM L-glutamine, and were transfected with FuGENE (Roche Applied Science) reagent that gave much better efficiency in these cells compared with Lipofectamine.

Immunoprecipitation and Western Blotting—HEK-293T cells were lysed in 1% HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM sodium vanadate, and 20 μg/ml aprotinin, and kept on ice for 30 min. All the subsequent steps were performed at 4 °C. The lysates were centrifuged at 15,000 × g for 10 min. Total protein was measured by the Bradford method. For immunoprecipitation, supernatants with equal amounts of proteins were incubated with primary antibody for 2 h at 4 °C, followed by incubation with protein A-Sepharose for 1 h at 4 °C. The beads were washed three times in low detergent (0.1% Triton X-100) HNTG lysis buffer, prior to the addition of Laemmli sample buffer. The samples were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% milk for 1 h, after which the blots were incubated with primary antibodies for 2 h at room temperature or overnight at 4 °C and washed in Tris-buffered saline containing 0.05% Tween 20. Antibody binding was detected by using horseradish peroxidase-conjugated secondary antibodies diluted at 1:5000 and was visualized with enhanced chemiluminescence reaction reagent (Western Lightening; PerkinElmer Life Sciences). For re-probing with other antibodies, the anti-body bound to polyvinylidene difluoride membranes was removed with stripping buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 100 μM 2-mercaptoethanol) at 50 °C for 20 min. After washing, the membranes were blocked with 5% milk and reprobed with the indicated antibodies.

Phagocytosis Assay—The phagocytosis assay was performed as previously described (26). Briefly, for apoptosis, CEM human T lymphoid leukemia cells were labeled with a red fluorescent cell linker PKH26-GL (Sigma) and induced to undergo apoptosis by UV-B irradiation at 25 ml/cm2 for 6–8 h. PS-positive cells were verified by staining with Annexin V. HEK-293T cells were transfected with bicistronic expression plasmids that contained EGFP expression genes and allowed to recover for at least 48 h before co-culturing with apoptotic cells. The transfected HEK-293T cells were co-cultured with apoptotic cells for 2 h at a ratio of 1:10, after which the cells were washed with phosphate-buffered saline containing 5 mM EDTA to remove surface-bound apoptotic cells. Two-color FACScan analysis was used to determine the percentages and geo-mean fluorescent intensity of green fluorescent phagocytes that had phagocytosed red fluorescent target cells. In some experiments, myristoylated PKC inhibitory peptide was added 30 min before the addition of apoptotic cells. The myristoylation moieties allow cell membrane permeability, permitting its use in inhibition of intracellular PKC activation. This inhibitor was nontoxic at the times and concentrations utilized as evidenced by Annexin V staining.

Luciferase Assay—Mertk-mediated suppression of NF-κB-dependent transcription was assessed using a dual luciferase strategy (31). RAW 264.7 cells were transfected with 10 μg of pNF-κB-Luc, a plasmid containing the firefly (Photinus pyralis) luciferase gene, the expression of which is driven by a basal transcriptional promoter linked to four copies of the κB motif (Clontech Laboratories, Palo Alto, CA), together with 1 μg of pRL-SV40, a Renilla (sea pansy, Renilla reniformis) luciferase control vector, the constant expression of which is dependent on the SV40 early enhancer/promoter region (Promega). The cells were allowed to attach for 4 h and then treated with or without Gas6 (150 nm), LPS (100 ng/ml), or calphostin C for the indicated times. For CD8-Mertk experiments, 4 μg of this plasmid was used per transfection, and after overnight culture in 100-mm diameter dishes, cells were re-plated the following day into 12-well plates at 1 × 105 cells/2 ml/well. Cell extracts were prepared, and luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega) in an FB12 Luminometer (Zylux, Oak Ridge, TN). Each condition was repeated in triplicate wells, and the luciferase activities in cells from each well were determined independently. The firefly luciferase activity in each sample was normalized with respect to the internal Renilla luciferase activity, and the relative level of normalized firefly luciferase activity compared with the activity in an untreated population was taken as a measure of NF-κB-dependent transcriptional activity.

Image-based Subcellular Localization of NF-κB p65 in RA W264.7 Cells—1 × 106 RAW 264.7 cells were used per sample. The cells were maintained in RPMI supplemented with 10% FBS and 2 mM L-glutamine containing penicillin/streptomycin. The cells were treated with either LPS (1 μg/ml) alone, Gas6
(150 nM) alone, or pretreated with Gas6 at different time points followed by LPS treatment. The cells were then scraped and collected in tubes, washed twice in wash buffer (2% FBS in phosphate-buffered saline), and then fixed in fixation buffer (4% paraformaldehyde in phosphate-buffered saline) for 10 min at room temperature. After washing, the cells were re-suspended in Perm Wash buffer (0.1% Triton X-100, 3% FBS, 0.1% sodium azide in phosphate-buffered saline) containing 10 μg/ml anti NF-κB p65 antibody (Santa Cruz Biotechnology) for 20 min at room temperature. The cells were then washed with Perm Wash buffer and re-suspended in Perm Wash buffer containing 7.5 μg/ml fluorescein isothiocyanate F(ab')2, donkey anti-rabbit IgG for 15 min at room temperature. Cells were washed twice in Perm Wash buffer and re-suspended in 1% paraformaldehyde containing 5 μM DRAQ5 nuclear stain (BioStatus) for 5 min at room temperature. 10,000 event image files for each sample were collected and analyzed for NF-κB nuclear translocation using an ImageStream imaging cytometer (Amnis Corp., Seattle, WA). Briefly, single cells were identified and gated at intermediate DRAQ5 intensity with high nuclear aspect ratio events. Single cells with high nuclear contrast (in-focus) and NF-κB staining were gated and analyzed for NF-κB translocation using the Similarity feature, which measures the correlation between the NF-κB and DRAQ5 image pair for each cell (32). If NF-κB is nuclear localized, the two images will be similar and have large positive values. If NF-κB is cytoplasmic, the two images will be anti-similar and have small or negative values.

Presentation of Data—Unless stated otherwise, the results shown are from a single experiment representative of three-five independent experiments.

RESULTS

Mertk Post-receptor Signaling for Phagocytosis of Apoptotic Cells and Down-modulation of NF-κB Both Depend on Tyrosine Phosphorylation—Mertk functions in at least two aspects of apoptotic cell clearance, both of which require the intact tyrosine kinase domain and downstream tyrosine phosphorylation signaling. As shown in Fig. 1A, when Mertk was expressed in β5-integrin-expressing HEK 293T cells and co-cultured with red-labeled PKH-26 CEM apoptotic T cells, WT Mertk stimulated phagocytosis by ~54%, but this effect was suppressed to 27% by the similar expression of a K614M Mertk kinase-dead mutant (CD8-MertkKD) containing a wild-type ectodomain. In similarly conceived experiments to test the ability of Mertk to suppress LPS-inducible NF-κB reporter activity, we used a RAW264.7 macrophage cell line. As shown in Fig. 1 (B and C), activation of Mertk, either by addition of ligand Gas6 (Fig. 1B), or expression of CD8 Mertk (constitutively activated Mertk) (Fig. 1C) readily suppress LPS-inducible activation of the NF-κB reporter. In all experiments in this report, a positive response was defined as >50% decrease in reporter gene transcription in triplicate samples, based on a repeated pattern of inhibition observed with WT Mertk in multiple experiments. In the case of Gas6 pretreatment (Fig. 1B), suppression of LPS-inducible activation required at least a 30-min pretreatment. Notably, when we co-stimulated cells with Gas6 and LPS at the same time point (t = 0), the Gas6 had no suppressing effect on LPS, suggesting that at least 30 min pretreatment was required.
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In addition to PI3K, signaling via Mertk also results in the concomitant activation of PLCγ2 and PKC (39, 40). Although PLCγ isozymes are unique in that they contain two tandem Src homology (SH) 2 domains, which allow interaction with proteins that contain specific phosphorylated tyrosine residues, it is also well known that full activation of PLCγ requires phosphatidylinositol 3,4,5-trisphosphate and PH domain-dependent recruitment to the plasma membrane (41). Activated PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate resulting in the activation of PKC. Previous studies have shown that the challenge of Mertk-expressing cells with apoptotic cells stimulates tyrosine phosphorylation of PLCγ2, but not PLCγ1, and PLCγ2 association with Mertk appears to play a critical role in Mertk-mediated phagocytosis in murine peritoneal macrophages and in J774 cells (40). To investigate the effect of Mertk Y867F on the phosphorylation and activation of PLCγ2, we expressed CD8-Mertk and CD8-MertkY867F, as well as other major autophosphorylation site mutants, Mertk Y544F, Mertk Y825F, and Mertk Y924F in HEK 293T cells (Fig. 2C). As illustrated in the figure, HEK 293 cells expressing CD8-Mertk displayed constitutively increased tyrosine phosphorylation of PLCγ2, which was completely abrogated by the CD8-Mertk Y867F mutant or CD8-MertkKD, but not by other Tyr-to-Phe mutants (upper panel). All CD8-Mertk mutants were expressed at comparable levels with the exception of the kinase-dead

to inhibit a critical NF-κB activating step later in the pathway. Moreover, this effect of Mertk trans-inhibition on LPS-TLR4 signaling was sustainable when we transiently co-expressed a constitutively active CD8-Mertk with the NF-κB reporter, but not CD8-MertkKD (Fig. 1C). These data illustrate that the dual role of Mertk as a phagocytic receptor and its ability to suppress LPS-inducible NF-κB reporter activation upon LPS stimulation are both dependent on Mertk kinase activity and tyrosine phosphorylation.

Phagocytosis of Apoptotic Cells and Mertk Connection to Cytoskeletal Assemblages Is Controlled by the Tyr-867 Autophosphorylation Docking Site—Previous studies demonstrated that the suppression of LPS-inducible NF-κB signaling and TNF-α production by apoptotic cells requires contact dependent licensing but does not necessarily require phagocytosis (33–35). We were therefore interested whether we could provide a molecular rationale for this distinction by uncovering Mertk-specific post-receptor signaling. Because both phagocytosis and the suppression of LPS-inducible pro-inflammation required the kinase activity of Mertk, we posited that specific proteins may interact with tyrosine phosphorylated motifs in the intracellular domain of Mertk to initiate signal transduction. This notion was also prompted by studies by Georgescu et al. showing the existence of multiple autophosphorylation site initiated signals in transfected BaF3 cells, most notably that the phosphorylated motif (pYAXNX) beginning at pTyr-867 directly bound Grb2, and that Grb2 subsequently recruited the p85 subunit of PI3K (30). PI3Ks have been shown to be involved in multiple Mertk-related biological events, including cell survival (36), as well as phagocytosis of rod outer segments (37) and apoptotic cells (38). To investigate the importance of Mertk autophosphorylation Tyr-867 docking site for phagocytosis and cytoskeletal signaling, we generated wild-type Mertk and an Y867F Mertk mutant with a functional ectodomain for binding Gas6. As shown in Fig. 2A, Gas6-inducible activation of Mertk resulted in Akt Ser-473 phosphorylation, whereas expression of MertkY867F abrogated this effect to baseline levels. Similar results were obtained when we expressed constitutively activated CD8-Mertk (data not shown). Moreover, when these cells were challenged with apoptotic T cells, β5 integrin-expressing HEK-293T cells expressing MertkY867F showed decreased phagocytosis of apoptotic cells relative to β5-HEK-293T cells expressing wild-type Mertk. Phagocytosis was reduced to baseline levels (Fig. 2B, 38.37% compared with 24.13%).

FIGURE 2. The kinase activity and Tyr-867 residue of Mertk is required for Akt activation and tyrosine phosphorylation of PLCγ2. A, HEK 293T cells were transfected with 40 ng of DNA encoding Mertk or Mertk Y867F, and transiently transfected cells were stimulated with 150 nm of Gas6 for 5 min. Detergent lysates were prepared (20 μg) and analyzed by Western blot for phospho-Akt (anti-pS473Akt) or total Akt levels (lower panel). B, HEK-293T cells were transfected with vector, bicistronic pIRES2-EGFP-Mertk, or pIRES2-EGFP-MertkY867F mutant plasmids, and phagocytosis was performed as described in Fig. 1C. HEK-293T cells were transfected with plasmid encoding empty vector (pLXSN), CD8-Mertk, CD8 MertkKD, or one of the autophosphorylation mutants as indicated. After 48 h, detergent lysates were immunoprecipitated with anti-PLCγ2 mAb, and immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine Ab (α-pY99) (upper panels) or anti-PLCγ2 mAb to verify equivalent IP in each lane. In the lower panels, replicate samples were immunoprecipitated with anti-FAK antisera, and samples were immunoblotted with antibody specific for phosphoFAK(Tyr-861). The blot was subsequently re-probed with antisera to FAK to verify loading. Gels are typical of several independent experiments.
Mertk, a mutant that is known to alter the expression of Mertk (Fig. 2C, top lane). These data demonstrate that Mertk\(^{\text{Y867F}}\) is a common docking site for the co-activation of both PI3K and PLC\(\gamma\)2, which likely cooperate to influence downstream signals.

Mertk-dependent Tyr-867 Signal Transduction Converges on PKC: Requirement of PKC for Mertk Signaling to FAK and the Actin Cytoskeleton—Because PKC is a substrate of diacylglycerol, a product of phosphatidylinositol-specific phospholipase C, and PKC is required for phagocytosis of apoptotic cells in macrophages (39, 42), we next investigated whether PKC activation is required for Mertk signaling to the cytoskeleton and phagocytosis of apoptotic cells. Previous studies have also shown that PKC can impinge on \(\alpha\)v\(\beta\)5 integrin and cytoskeletal linkages (39, 43), possibly to regulate integrin binding to MFG-E8. To investigate the function of PKC in Mertk downstream signaling, we first used a myristoylated cell-permeable PKC-inhibitory peptide, myr-RFARKGALRQKKNV. As shown in Fig. 3A, Mertk-mediated uptake of apoptotic cells was significantly impaired by pretreatment with this peptide (decreased phagocytosis in Mertk-expressing HEK cells 35.03\% \pm 1.61\% versus 22.57\% \pm 0.29\%), whereas the decrease by the PKC-inhibitory peptide was <10\% in HEK cells without Mertk expression (right side of panel). Moreover, pretreatment of cells with the pharmacological inhibitor, calphostin C, also strongly diminished Gas6-inducible tyrosine phosphorylation of both cytoskeletal proteins p130\(\text{cas}\) and FAK\(^{\text{Y861}}\), which have been previously implicated in Mertk-mediated phagocytosis (26) (Fig. 3B, panels i and ii). Finally, to assess whether these signals were initiated from the Tyr-867 autophosphorylation site, we examined the phosphorylation status of FAK on Tyr-861, because this was shown previously to be required for binding \(\beta\)5 integrin. As indicated in Fig. 2C, both KD Mertk and Mertk\(^{\text{Y867F}}\) significantly reduced FAK\(^{\text{Y861}}\) phosphorylation, to background levels, although the effects of Y867F substitution were not complete. The findings that either calphostin C pretreatment, or mutation at Tyr-867, abrogated FAK\(^{\text{Y861}}\) phosphorylation link common upstream elements and integrate PI3K and PLC\(\gamma\)2 to PKC, and subsequently PKC to cytoskeletal reorganization and phagocytosis (Figs. 2C and 3A).

Inhibition of NF-\(\kappa\)B by Gas6/ Mertk Is Independent of Tyr-867 and PKC—Having provided evidence that the autophosphorylation site mutant Tyr-867 disrupted a

\[ \text{Mertk} \rightarrow \text{PI3K} \rightarrow \text{PLC}\(\gamma\)2 \rightarrow \text{PKC} \rightarrow \text{FAK} \]  

\[ \text{Mertk} \rightarrow \text{PI3K} \rightarrow \text{PLC}\(\gamma\)2 \rightarrow \text{PKC} \rightarrow \text{FAK} \]  

The next obvious question was to assess whether autophosphorylation site Tyr-867, or the above pathway, is required for the anti-inflammatory activity of Mertk. Previous studies by Matsushima and colleagues showed that Mertk suppresses LPS-mediated NF-\(\kappa\)B activation in macrophages, and in mice, Mertk mutant mice are more susceptible to LPS and have increased production of TNF-\(\alpha\) when challenged with endotoxin (18). However, recent studies have shown that apoptotic cell contact, without uptake, is sufficient to decrease NF-\(\kappa\)B reporter activation and TNF-\(\alpha\) production (34, 35). Therefore, to investigate the relationship between LPS/TLR4 signaling and Mertk autophosphorylation site docking mutants, we assessed the ability of CD8-Mertk and mutants to suppress LPS-mediated NF-\(\kappa\)B activation as described in Fig. 1. As shown in Fig. 4, expression of CD8-Mertk in RAW264.7 cells showed a persistent and reproducible 50–75\% decrease in NF-\(\kappa\)B reporter activation when these cells were stimulated with LPS. Most notably, however, Mertk\(^{\text{Y867F}}\) did not reverse the ability of Mertk to suppress LPS-inducible NF-\(\kappa\)B activation (Fig. 4), although interestingly, none of the major auto-phosphorylation site mutants appeared to impinge on the ability of Mertk to trans-inhibit LPS signaling (Fig. 4).
Because the major auto-phosphorylation sites were dispensable for the Merk function in NF-κB suppression, we next examined the potential role of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in Merk. ITIMs have the minimal consensus sequence (V/I)/XXX(L/V), or may be more broadly defined by the sequence (V/I)/L(S)XXX(L/V/I/S) (44). Lemke and colleagues first noted the presence of an ITIM in Axl, but we identified three additional candidate ITIMs at positions Tyr-671, Tyr-680, and Tyr-689 in Merk using the ProSORT method. ITIMs are present in signaling proteins that down-modulate immune responses by binding cellular phosphatases, Shp-1, and Shp-2 (45), and can inhibit 1b phosphorylation and degradation (46). Because Shp-1 (−/−) mice develop a similar autoimmunity phenotype as Merk knockouts (47), it is therefore reasonable to assess the role of Shp proteins in Merk signaling. To address their potential role in Merk/TLR4 cross-talk, we mutated each Tyr independently to Phe to generate Y671F, Y680F, and Y689F mutants. As shown in Fig. 4, expression of each of these single mutants did not reverse the Merk trans-inhibition effect on LPS-inducible NF-κB activation. Moreover, when WT Merk or each of the ITIM mutants was co-expressed with HA-Shp-1, we failed to detect a stable interaction between Merk and Shp-1 (data not shown).

**A Post-nuclear Merk Signaling Pathway Is Responsible for NF-κB Cross-inhibition**—Finally, to rule out the possibility that another cytoskeletal pathway may directly impinge on NF-κB, we next investigated the effect of Merk on 1b phosphorylation and NF-κB translocation. Under normal conditions NF-κB is bound by the inhibitory protein 1b in the cytoplasm. Upon LPS stimulation, 1b becomes phosphorylated and degraded allowing p65 and p50 heterodimers to translocate into the nucleus. When we examined the effect of Gas6 pretreatment on LPS-inducible 1b phosphorylation and degradation (Fig. 5A), we found no differences in the 1b phosphorylation status or expression with or without Gas6 pretreatment. Further, we also analyzed the subcellular localization of the p65 subunit of NF-κB upon Gas6 pretreatment and LPS stimulation. Using multispectral imaging flow cytometry on thousands of cells per sample, we observed that p65 translocates to the nucleus as a result of LPS stimulation with or without Gas6 pretreatment (Fig. 5, B and C). Moreover, pretreatment of RAW264.7 cells with Cal-C and Gas6, a combination that effectively inhibited phagocytosis, did not impinge on the ability of Merk activation to suppress NF-κB activation (Fig. 5D) or translocation (not shown). These studies suggest that, in contrast to the tyrosine phosphorylation-mediated events associated with Merk and the cytoskeleton (Tyr-867), the tyrosine phosphorylation-mediated events initiated by Merk that impinge on NF-κB transcription are regulated in a post-nuclear fashion.

**DISCUSSION**

Efficient phagocytosis is the ultimate fate of apoptotic cells in complex tissues, and defective clearance of apoptotic cells has been linked to autoimmunity and persistent inflammatory diseases. Because Merk is perhaps the best characterized receptor implicated in clearance and in promoting anti-inflammatory and immunosuppressive effects, we undertook this study to characterize the post-receptor events downstream of Merk that are responsible for cytoskeletal signaling, and in turn, to identify whether the NF-κB-mediated immune modulatory functions occur by overlapping signaling events. Our present studies demonstrate these events can be molecularly uncoupled and dissociated. We identified a phagocytic cytoskeletal-linked signaling pathway, initiated by docking at the autophosphorylation Tyr-867 site that involved phosphorylation of Akt and PLCγ2, activation of PKC, and PKC-mediated tyrosine phosphorylation of FAK-Tyr-861 (Fig. 6). Previous studies have shown that FAK-Tyr-861 phosphorylation is coupled to β5 integrin and involved in a pathway to engulf apoptotic cells (26). In contrast, the ability of Merk to suppress LPS signaling at the level of NF-κB trans-activation required a Tyr-867-independent pathway, also independent of PKC, and apparently exclusively targeted to the nucleus. Our present results are consistent with a model in which phagocytosis is not necessary to mediate anti-inflammation and offers a molecular example of how phagocytosis can be dissociated from immune licensing and anti-inflammation.

A number of studies suggest that Merk can impinge on the cytoskeleton and trigger internalization of integrin-bound apoptotic cells (26). In this study, we found that Merk-inducible tyrosine phosphorylation of Akt, PLCγ, and FAK require a single autophosphorylation docking site at Tyr-867. Previous studies in BaF3 pro-B-lymphocytic cells showed that Merk Tyr-867 was required for interleukin-3-dependent cell growth and provided a direct binding site for Grb2, which indirectly recruits the p85 subunit of PI3K (30). Consistent with this view, we observed that the Y867F mutation blocked Gas6-inducible Akt phosphorylation, a direct substrate for PI3K. Presently, it is not known how Tyr-867 induces PLCγ2 activation, because the motif corresponding to Tyr-867 does not conform to a consensus phosphotyrosine motif for the SH2 domain of PLCγ2. It is known that PLCγ2 activation not only requires tyrosine phosphorylation, but also phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate lipids, so binding of PLCγ to a different site on the membrane may indeed depend on PI3K activation and phosphatidylinositol 3,4,5-trisphosphate at the inner leaf of the membrane (41). Curtis and colleagues showed that PLCγ2 can associate with tyrosine phos-
cytoskeletal proteins involved in integrin-mediated cell spreading and actin rearrangement, supported by the fact that PS liposomes stimulate translocation of PKC isoforms to cytoskeletal and membrane fractions. Finnemann’s group has also shown that PS-opsonized rod outer segment particle binding to αvβ5 integrin requires PKC activation, and its recruitment into a detergent-insoluble fraction (43). The results here show that FAKTyro861 phosphorylation, a prerequisite for integrin binding, is dependent on PKC, and an upstream signal originating from MertkTyro867 suggests that PKC activation may be a penultimate step in the link between Mertk activation and αvβ5 integrin engagement.

Concomitant with phagocytosis, apoptotic cells elicit potent anti-inflammatory responses, but recent cell biology experiments indicate that phagocytosis can be physically uncoupled from the anti-inflammatory signals from apoptotic cells. For example, Cvetanovic et al. (35) showed, using pharmacological inhibitors of engulfment (i.e. cytochalasin D), that for suppression of pro-inflammatory phagocytes need only to contact apoptotic cells. This is referred to by Lacy-Hulbert and colleagues as contact-dependent licensing (34). Using a well characterized model in which apoptotic cells down-modulate LPS-dependent NF-κB responsiveness, we propose a molecular definition for how engulfment and anti-inflammation can be dissociated in phagocytosis-dependent pathway that diverge at the Mertk Tyr-867 autophosphorylation site. It will be most interesting to investigate a phenotype in MertkY867F transgenic mice in terms of their propensity toward autoimmunity and chronic inflammation, to test the relationship between contact-dependent licensing and phagocytosis on the inflammatory autoimmune outcomes.

We also found a notable difference in the subcellular compartmentalization whereby these signaling pathways take place. The phagocytosis-dependent pathway was expectedly cytoplasmic in nature, and converged on PKC and cytoskeletal assemblages, whereas the anti-inflammatory pathway appears to be manifested by nuclear inhibition of NF-κB transcription. Whereas 30-min pretreatment of Gas6 suppressed LPS-mediated

Although it was not established whether this is a direct interaction or whether binding was inhibited by Wortmannin, a PI3K inhibitor.

Despite a lack of a clear understanding of how PI3K and PLCγ2 are initially engaged by the autophosphorylation site Tyr-867, the involvement of these pathways in Mertk-mediated phagocytosis and PKC activation is supported by the fact that Y867F mutation (and calphostin C) suppressed FAKTyro861 phosphorylation, which represents a direct linkage to the actin cytoskeleton. Previously, we have shown that FAK, when phosphorylated on Tyr-861, stably associates with β5 integrin, thereby engaging the αvβ5 integrin pathway for phagocytosis. Additional studies have shown that PKC activation is required for PS-dependent phagocytosis of apoptotic thymocytes (39). A specific role of PKC in phagocytosis may involve changes in

Multiple Signaling Outcomes by the Mertk Receptor

Figure 5. Mertk-mediated inhibition of NF-κB activity is a post-nuclear signaling event. A, RAW264.7 cells were either pretreated with Gas6 alone or LPS alone, or pretreated with Gas6 and then treated with LPS (first three lanes). Cells were lysed with 1% HNTG buffer and analyzed by immunoblotting with anti-pIκB antibody and total IκB antibody. B, RAW 264.7 cells grown to 70% confluence were either pretreated with LPS alone or first pretreated with Gas6 for 30 min and then treated with LPS (1 μg/ml) for an additional 30 min. Cells were collected, immunofluorescently stained for NF-κB expression and with the DRAQ5 nuclear dye, and analyzed for NF-κB nuclear translocation using the ImageStream imaging cytometer as described under “Materials and Methods.” NF-κB/DRAQ5 similarity histograms of single cells from untreated, Gas6-treated, and Gas6 plus LPS-treated samples are shown in B. The percentage of cells with nuclear localized NF-κB is indicated in the upper right corner. Brightfield, NF-κB (green), DRAQ5 (red), and NF-κB/DRAQ5 composite images of representative cells are shown below each plot. C, the percentage of cells with nuclear-translocated NF-κB for each sample in the time course is plotted here. D, RAW 264.7 cells were transfected with pNF-κB luc plasmid; 36 h post transfection, cells were pretreated with 1 μM calphostin C for 1 h alone or co-pretreated with Gas6 (150 nm) for 30 min as indicated. Subsequently, cells were treated with LPS (100 ng/ml) for 16 h and lysed for assaying luciferase activity.
ated NF-κB transcription, we found no evidence that such treatment altered IκB phosphorylation/degradation or inhibited RelA/p65 translocation into the nucleus. Indeed, Gas6 itself had a slight activating effect on NF-κB, which is consistent with its role in other cell types in mediating interleukin-3-dependent survival (30). In the study by Georgescu et al., the NF-κB activation mechanism was linked to Grb2 binding to Tyr-867, but the cross-talk to the LPS-TLR4 pathway described here is clearly different from the above. In addition, our present studies showing that Mertk impinges on a post-IκB-dependent pathway are also in contrast to recent studies by Matsushima and colleagues (29) who showed that, following long term treatment with apoptotic cells, macrophages show inhibition at the level of IκB in the cytosolic fraction. The reasons for these differences are not completely clear, although additional studies show that LPS can induce negative feedback loops by inducing micro RNA that decrease TRAF6 and IRAK1 (48, 49). Therefore, there may be two levels of regulation, early and late, that target distinct inhibitory steps. Our data suggest that the most proximal receptor-mediated events that inhibit TLR signaling occur in a post-nuclear compartment.

There are several possible mechanisms for how Mertk impinges on nuclear NF-κB regulation. Interesting studies by Martin et al. (e.g. Ref. 50) suggested that the nuclear availability of CBP may affect whether p65/RelA can transactivate target genes. In this model, these investigators propose that glycoscan synthase kinase 3β plays a central role in cofactor availability by shifting the binding of CBP from p65 to CREB. According to this idea, inhibition of glycoscan synthase kinase 3β by Akt culminates in stable association of CBP with phospho-CREB and decreases the association of the CBP with p65/RelA. Although the cofactor-availability idea may be attractive, it is clearly complex and dynamic. One question that arises here is why Mertk Tyr-867, which blocked PI3K activation and Akt phosphorylation, did not decrease NF-κB reporter activation. However, a further study by Tenner et al. also showed nuclear inhibition of NF-κB during C1q-mediated anti-inflammatory signaling and blockade of LPS signaling (51). They suggested that C1q induced CREB phosphorylation and association with p65 but also that C1q favored a dimeric p50/p50 NF-κB complex that bound to κB DNA elements but did not promote transcription. Further studies will be required to assess whether Gas6 or apoptotic cells mediate events that promote inhibitory post-translational modifications to p65 or p50. For example, certain phosphorylation sites (i.e. 435 and 505) in p65 indubitably phosphorylated by expression of the ARF tumor suppressor can inhibit p65 nuclear-binding activity. NF-κB can also be regulated by phosphorylation, acetylation, and ubiquitination (52). Clearly, further studies to ascertain how Gas6 impinges on the nuclear NF-κB circuit are warranted and likely to uncover novel targets for inflammation and innate immune regulation.

In conclusion, we have provided a molecular explanation of how engulfment can be dissociated from the potent anti-inflammatory events that govern the interactions of apoptotic or effete cells with phagocytes. The former is regulated by events initiated at the autophosphorylation site Tyr-867, involving PKC, and is largely a cytosolic directed pathway. The latter is functionally dissociable, independent of Tyr-867 and PKC, and appears to be regulated within the nucleus. These studies may be helpful in the rational design of novel therapeutics for chronic inflammation.

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