THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 14, pp. 12108–12122, April 8, 2011

Signaling via Macrophage G2A Enhances Efferocytosis of Dying Neutrophils by Augmentation of Rac Activity*[S]

Received for publication, September 2, 2010, and in revised form, January 14, 2011 Published, JBC Papers in Press, February 4, 2011, DOI 10.1074/jbc.M110.181800

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Phosphatidylserine (PS) and oxidized PS species have been identified as key ligands on apoptotic cells important for their recognition and removal (efferocytosis) by phagocytes, a requisite step for resolution of inflammation. We have recently demonstrated that lysophosphatidylserine (lyso-PS) generated and retained on neutrophils following short term activation of the NADPH oxidase *in vitro* and *in vivo* enhanced their clearance via signaling through the macrophage G-protein-coupled receptor G2A. Here, we investigated the signaling pathway downstream of G2A. Lyso-PS, either made endogenously in apoptosing neutrophils or supplied exogenously in liposomes along with lyso-PS^{neg} apoptotic cells, signaled to macrophages in a G2A-dependent manner for their enhanced production of prostaglandin E_2 (PGE_2) via a calcium-dependent cytosolic phospholipase A_2/cyclooxygenase-mediated mechanism. Subsequent signaling by PGE_2 via EP2 receptors activated macrophage adenylyl cyclase and protein kinase A. These events, in turn, culminated in enhanced activity of Rac1, resulting in an increase in both the numbers of macrophages efferocytosing apoptotic cells and the numbers of cells ingested per macrophage. These data were surprising in light of previous reports demonstrating that signaling by PGE_2 and adenylyl cyclase activation are associated with macrophage deactivation and inhibition of apoptotic cell uptake. Further investigation revealed that the impact of this pathway, either the enhancement or inhibition of efferocytosis, was exquisitely sensitive to concentration effects of these intermediaries. Together, these data support the hypothesis that lyso-PS presented on the surface of activated and dying neutrophils provides a tightly controlled, proresolution signal for high capacity clearance of neutrophils in acute inflammation.

A hallmark of acute inflammation is the recruitment of large numbers of neutrophils from the vasculature into tissues in response to microbial infection and/or injury. Although robust neutrophil recruitment is critical for host defense, removal of these short lived cells is imperative for resolution of inflammation and restoration of tissue function. Removal of activated and dying neutrophils is most often orchestrated by professional phagocytes, such as macrophages, in a process that has been called efferocytosis ("to carry to the grave") (1). Where defects in efferocytosis have been identified, dying neutrophils ultimately disintegrate, release phlogistic cargo (*e.g.* serine proteases and cationic proteins), and contribute to ongoing inflammation, tissue destruction, and, in some cases, autoimmunity (2–4).

Relatively little is known of the ligands presented by apoptosing neutrophils, or any apoptosing cell for that matter, that signal for their recognition and engulfment. The exofacially exposed phosphatidylserine (PS)[2] head group is the best described ligand and is recognized by an increasing number of bridge molecules and receptors on macrophages, including MFG-E8, Gas6, BAI1, Tim4, and Stabilin 2 (5–8). The consequences of PS-dependent interactions are actively anti-inflammatory, resulting in the production of mediators, such as TGFβ and prostaglandin E_2 (PGE_2) (9, 10). More recently, oxidized PS has also been shown to facilitate recognition of apoptotic cells through scavenger receptors (*e.g.* CD36) (11–14), adding to the array of possible receptors utilized by macrophages to recognize different PS species and structures. Given this, we had previously hypothesized that activation of the NADPH oxidase would enhance PS oxidation and contribute significantly to the removal of neutrophils. Surprisingly, substantial amounts of lyso-phosphatidylserine (lyso-PS) species, rather than oxidized PS species, were generated in an NADPH oxidase-dependent manner during neutrophil activation both *in vitro* and *in vivo* (15). We demonstrated further that cell-associated lyso-PS signaled to macrophages via the G-protein-coupled receptor G2A for enhanced PS-dependent removal of activated neutrophils.

In this investigation, we sought to define the signaling pathway downstream of G2A resulting in enhanced engulfment. Here, we show that the modified phosphatidylserine lyso-PS

* This work was supported, in whole or in part, by National Institutes of Health Grants AI058228 (to D. L. B.), HL34303 (to D. L. B., C. C. L., and R. C. M.), and GM61031 and HL81151 (to P. M. H.). This work was also supported by the Chronic Granulomatous Disorder Research Trust (to D. L. B.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: PS, phosphatidylserine; lyso-PS, lysophosphatidylserine (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoserine); PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine); DPI, diphenyleneiodonium; RPMΦ, resident peritoneal macrophages; MΦ, macrophages; PLA_2, phospholipase A_2; PGE_2, prostaglandin E_2; db-cAMP, dibutyryl-cAMP; COX, cyclooxygenase; PKA, cAMP-dependent protein kinase; Epac, exchange protein directly activated by cAMP; PGI_2, prostacyclin I_2; PKA, protein kinase A; $cPLA_2\alpha$, calcium-dependent cytosolic PLA_2; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecanoic acid.

was generated during neutrophil apoptosis only under conditions where the NADPH oxidase was activated. Similar to the findings of our earlier studies of activated neutrophils, lyso-PS-positive apoptotic neutrophils signaled via macrophage G2A for enhanced engulfment. Using exogenous lyso-PS supplied in liposomes to activate G2A, key signaling events and intermediaries downstream of G2A were identified and included macrophage calcium-dependent cytosolic PLA_2 ($cPLA_2\alpha$) activation and PGE_2 production leading to cyclic AMP (cAMP)-dependent protein kinase A (PKA) activation. Lyso-PS alone did not signal for these events but rather signaled in the context of other ligands, including that mimicked by carboxylate-modified beads presenting a PS head grouplike surface. Ultimately, this combined signaling led to the enhanced activation of Rac1, a Rho-GTPase required for efferocytosis (16–18). The data presented here demonstrate that lyso-PS is an enhancer of efferocytosis via macrophage G2A and define the downstream signaling pathway. These data place lyso-PS, signaling from activated and apoptotic neutrophils to macrophages via G2A, squarely in a pathway for resolution of neutrophilic inflammation.

EXPERIMENTAL PROCEDURES

Materials—All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) unless otherwise noted. Solvents (HPLC grade) were from Fisher Scientific. Diphenyleneiodonium (DPI), $cPLA_2\alpha$ inhibitor, and bromoenol lactone were from EMD Biosciences (Gibbstown, NJ). Flash Red carboxylate-modified beads (5 μm) were from Bangs Laboratories, Inc. (Fishers, IN). Anti-G2A M-20 and normal goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Dibutyryl cAMP (db-cAMP); PGE_2; iloprost; SQ22563; GW627368X; AH6809; KT5720; NS-398; indomethacin; and anti-cyclooxygenase 1 (COX1), anti-COX2, anti-EP2, and anti-EP4 antibodies were from Cayman Chemicals (Ann Arbor, MI). PKA agonist and Epac agonist were from Biolog (distributed by Axxora, San Diego, CA). Annexin V-Alexa Fluor 488 was from Molecular Probes (Eugene, OR). Lyso-PS internal standard (17:1/OH-PS) was a generous gift from Dr. Walter Shaw at Avanti Polar Lipids. EO3 monoclonal antibody, an autoantibody directed against oxidation-specific epitopes of oxidized LDL, was a generous gift of Dr. Joseph Witztum (University of California, San Diego).

Animals—Male and female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and also used from a breeding colony at National Jewish Health (Denver, CO). $G2A^{-/-}$ breeder pairs on a C57BL/6 background were a generous gift from Dr. Katherine Hedrick (University of Virginia). Breeding of homozygous negative mice was maintained at National Jewish Health. Male and female BALB/c (to serve as controls for $cPLA_2\alpha^{-/-}$) mice were obtained from Harlan Laboratories (Indianapolis, IN). $cPLA_2\alpha^{-/-}$ mice were generated using 129 embryonic stem cells in a C57BL/6 strain as described previously (19). The mixed strain was back-crossed onto a BALB/c background and used after 10 generations. Breeding of homozygous negative mice was maintained at National Jewish Health. All animals received care in accordance with the guidelines of the Institutional Animal Care and Use Committee and were maintained on food and water *ad libitum*. Mice between the ages of 8 and 16 weeks were age- and gender-matched for all experiments and used for *in vivo* studies and provision of peritoneal macrophages as outlined below.

Isolation and Culture of Murine Peritoneal Macrophages—Resident peritoneal macrophages (RPMΦ) were isolated from mice using 5 ml of sterile Hanks' balanced salt solution supplemented with 10 mM HEPES (pH 7.4) and 1 mM EDTA to lavage the peritoneum following euthanization with CO_2. Resident peritoneal cells were collected, centrifuged at 1,000 rpm for 10 min at 4 °C, resuspended in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (supplemented DMEM). For phagocytosis, cells were plated at 5×10^5 cells/well in a 24-well tissue culture plate; for PGE_2 production, cells were plated at 1×10^6 cells/well in a 24-well tissue culture plate; for Western immunoblot analysis and cAMP production, cells were plated at 2×10^6 cells/well in a 12-well tissue culture plate; and for Rac G-LISA™, cells were plated at $5–8 \times 10^6$ cells/well in a 6-well tissue culture plate. After plating, macrophages were allowed to adhere for 2 h at 37 °C in a 10% CO_2 humidified incubator after which non-adherent cells were removed by washing, and macrophages (~50% remained adherent following washes) were cultured for an additional 48 h before use. Medium was replaced 24 h after plating and 1–2 h prior to use. Thioglycollate-elicited macrophages were obtained 3 days following intraperitoneal injection of 1.5 ml of thioglycollate as described previously (15). Cells were plated at 3×10^5 cells/well and cultured as described for resident peritoneal macrophages.

Induction of Sterile Peritonitis—Mice were injected intraperitoneally with 1 mg of zymosan (in 1 ml of PBS), and peritoneal cells were harvested by lavage with sterile Hanks' balanced salt solution supplemented with 1 mM EDTA and 10 mM HEPES (pH 7.2) at the times indicated. Cell counts and cytospins were done to determine apoptosis by nuclear morphology, cell differentials, and absolute numbers of cells.

Lipid Preparation—1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine small unilamellar vesicles with or without 10, 20, or 30 mol % 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoserine (lyso-PS) or 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (lyso-PC) at a final concentration of 2,000 nmol/ml were prepared by evaporating the lipid to dryness under nitrogen in a glass tube. Dried lipids were suspended in DMEM without serum or protein supplementation by vigorous vortexing, and small unilamellar vesicles were created by sonication in a waterbath sonicator (15). Small unilamellar vesicles were stored on ice and used within 1 h of preparation. Small unilamellar vesicles were added at 100 nmol of total lipid/5×10^5 plated macrophages. In some experiments, 30 nmol of lyso-PS or lyso-PC were delivered on 0.05% fatty acid-free BSA as described previously (20). Oxidized fatty acids were also delivered at the indicated concentrations on 0.05% fatty acid-free BSA. In all cases, lipids were added to macrophage cultures at the same time that apoptotic neutrophils were added.

Lipid Extraction—Lipids were extracted by the method of Folch *et al.* (21) and as described in Frasch *et al.* (15) with the following modifications. Briefly, human neutrophils (1×10^7)

were suspended in 0.6 ml of PBS and extracted in a screw cap glass culture tube with $CHCl_3$/MeOH/distilled H_2O (2:1:0.6, v/v/v). Fifty ng of 17:1/OH-PS was added to each sample as an internal standard. The extractions were allowed to sit overnight at room temperature. The organic layer was collected and brought to dryness under a stream of nitrogen, and lipids were suspended in 115 μl of reverse phase HPLC buffer A (described below), and 50 μl were injected per HPLC run.

Reverse Phase Chromatography and Electrospray Ionization Tandem Mass Spectrometry—For PS head group species, reverse phase HPLC was performed using a Gemini 3-μm C_{18} (2 × 50-mm) column (Phenomenex, Torrance, CA) with mass spectrometric detection using a Sciex API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada). The HPLC system was operated at a flow rate of 0.2 ml/min with a mobile phase of methanol/water (65:35, v/v) with 0.05% ammonium hydroxide (solvent A) and methanol/hexane (94:6, v/v) with 0.05% ammonium hydroxide (solvent B). The HPLC method for PS analysis was an isocratic hold of 0% solvent B for 3 min followed by a gradient of 0% solvent B to 100% solvent B in 17 min and then an isocratic hold at 100% B for 5 min. The PS species were detected in the negative ion mode by monitoring for the neutral loss of serine (neutral loss of 87 atomic mass units) with a collision energy of −30 V (22). The mass range scanned for the scan of the neutral loss of 87 atomic mass units was m/z 400–900 at a rate of 3 s/scan. For PS analysis in the negative ion mode, the electrospray voltage was −4,000 V, the focusing potential was −200 V, and the declustering potential was −45 V. For PC head group species, LC/MS/MS and multiple reaction monitoring was performed as described previously (15).

Quantitation of Lyso-PS—Quantitation of Lyso-PS in the samples was performed using a standard isotope dilution curve as described previously (23). The internal standard used for this quantitative analysis was 17:1/OH-PS. Under these conditions, 18:1/OH-PS was the major species generated with minor contributions of 18:0/-OH and 16:0/OH-PS. Because all species were expected to signal equivalently (20), total lyso-PS is a sum of all species generated. Similarly, 18:0/OH-PC and 18:1/OH-PC were the major species detected with minor contributions from 16:0/OH-PC. Lyso-PC was estimated by calculating a ratio of lyso-PC/internal standard by integrating the area under the curve for each species and the internal standard.

Mass Spectrometry Eicosanoid Measurement—Macrophages were plated as described above, and supernatants were collected at the indicated times following co-culture with the indicated target in the absence or presence of lyso-PS liposomes. The supernatants were centrifuged, transferred to 2 volumes of ice-cold methanol, and stored at −20 °C until analysis. Eicosanoids, HETEs, and HODEs were extracted and quantitated by LC/MS/MS in negative ion mode using multiple reaction monitoring for specific analytes as described previously (23–25).

Preparation of Neutrophils for Lipid Extraction and Engulfment Assays—Human neutrophils were obtained from normal, healthy donors in accordance with a protocol reviewed and approved by the Institutional Review Board. Using endotoxin-free reagents and plasticware, human neutrophils were isolated by the plasma Percoll method as described previously (26). For *in vitro* studies, human neutrophils were suspended at 5 × 10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin (supplemented RPMI). Cells were apoptosed as described below and subjected to lipid extraction and LC/MS/MS analysis as described above.

For induction of apoptosis, human neutrophils were either cultured overnight at 5 × 10^6cells/ml in supplemented RPMI at 37 °C in 5% CO_2 or UV-irradiated for 5 min on a trans-illuminator followed by incubation at 37 °C for 2 h. Surface PS exposure was detected by Annexin V binding and propidium iodide staining (as a test for permeability) and flow cytometry according to the manufacturer's instructions. Apoptotic cells were pelleted, washed once, and resuspended in supplemented DMEM for addition to macrophages in phagocytosis assays described below.

Phagocytosis Assays—Phagocytosis assays were performed as described previously (15, 27). For inhibitor studies or antibody blocking experiments, 5 μg/ml anti-G2A or isotype control or inhibitors at the indicated doses were added for 30 min prior to addition of liposomes or target cells. Liposomes (100 nmol of total lipid.5 × 10^5 plated macrophages) or lipids on albumin (10–30 nmol/5 × 10^5 plated macrophages) were added as indicted simultaneously with target cells. Target cells at a ratio of 4:1 targets/plated macrophage or carboxylate-modified beads at a ratio of 2:1 beads/plated macrophage were added per well. The macrophages with target cells were co-cultured for 60 min at 37 °C in 10% CO_2, washed three times with PBS, and stained with a modified Wright's Giemsa stain (Fisher Scientific). The phagocytic index was calculated by multiplying the percentage of macrophages that have been phagocytosed by the average number of engulfed cells per macrophage (28). A minimum of 200 MΦ were counted blindly. Each condition was tested in duplicate using at least four mice per experiment and repeated three or more times.

For *in vivo* phagocytosis assays, 5 × 10^6 fluorescent (Flash Red) 5-μm carboxylate-modified beads in 1 ml of PBS or 1 ml of PBS containing 30 mol % lyso-PS liposomes (10 mg/kg of total lipid/mouse; Ref. 29) were injected intraperitoneally into WT or $G2A^{-/-}$ mice. After 1 h, mice were euthanized, and cells were harvested by lavage and stained for F4/80 to identify the macrophage population as described previously (30). Phagocytosis of beads was determined by flow cytometry and indicated as the geometric mean of beads associated with F4/80-positive cells.

Rac G-LISA™ Activity Assay—Rac activity was determined using the Rac activity G-LISA™ (Cytoskeleton, Denver, CO). Briefly, RPMΦ were harvested and plated as described above. Cells were co-cultured with the indicated target cells at a 4:1 targets/plated macrophage ratio in the absence or presence of 100 nmol of total lipid/5 × 10^5 cells for the indicated times, and cell lysates were collected according to the manufacturer's instructions. Ten micrograms of total protein was used in the G-LISA™, and Rac activity was performed according to the manufacturer's instructions. Results are described as -fold of control.

cAMP Production—RPMΦ were plated as described above and co-cultured with 2:1 carboxylate-modified beads/plated macrophage for the indicated times. Intracellular cAMP was determined using the cAMP Direct Biotrak enzyme immunoassay (GE Healthcare) according to the manufacturer's instructions. Results are described as -fold of control.

Western Immunoblot Analysis—RPMΦ were plated as stated above and treated as indicated. Cells were washed twice with ice-cold PBS and scraped in lysis buffer (50 mM HEPES (pH 7.4), 150 mM sodium chloride, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 200 μM sodium vanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 300 nM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubation on ice for 30 min, lysates were centrifuged at 15,000 rpm for 15 min, and the protein concentration in the supernatant was determined by the bicinchoninic acid method. Lysates were boiled for 5 min after addition of Laemmli electrophoresis sample buffer, and then proteins (15 μg) were separated on 10% SDS-polyacrylamide gels. After transfer onto nitrocellulose, membranes were blocked for 1 h in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20 supplemented with 5% nonfat milk and incubated with the indicated antibody (1:1,000 dilution) overnight at 4 °C. The membranes were washed and incubated with anti-rabbit horseradish peroxidase-linked secondary antibody (1:10,000 dilution) for 1 h. Detection was performed using ECL substrate (Pierce Biotechnology) following the manufacturer's instructions.

Statistical Analyses—Statistical analyses and *p* value calculations were conducted using non-paired or paired Student's *t* test. Analysis of variance was used for multiple pairwise comparisons. When significant analysis of variance was indicated, post hoc analysis using the Tukey-Kramer or Dunnett's method for multiple comparisons or comparison with an internal control, respectively, was performed (JMP statistical program (SAS Institute, Cary, NC)).

RESULTS

Enhanced Efferocytosis of "Aged" Apoptotic Neutrophils by Macrophages Is Mediated by G2A Signaling—We had previously shown that neutrophils, activated either *in vitro* or *in vivo*, were ingested in an enhanced manner by a PS-dependent efferocytic mechanism. Enhanced efferocytosis required NADPH oxidase activation and lyso-PS production in the neutrophils and signaling via G2A on macrophages. Here, we sought to determine whether "aging" neutrophils undergoing apoptosis in culture, a process in which NADPH oxidase activation has been implicated (31, 32), also stimulated enhanced clearance by macrophages. To this end, human neutrophils were aged overnight to induce apoptosis and were compared with neutrophils induced to undergo apoptosis by UV irradiation, a process that is independent of the NADPH oxidase (15). Aged neutrophils were 61.3 ± 7.6% positive for PS exposure as detected by Annexin V staining compared with 79.5 ± 1.5% positive for UV-irradiated apoptotic neutrophils (Table 1). In both cases, the mean fluorescence index was similar (Table 1), and fewer than 5% were positive for propidium iodide, a measure of membrane permeability (data not shown). When "fed" to RPMΦ, ingestion of the aged neutrophils was enhanced over that seen for macrophages fed UV-irradiated apoptotic neutrophils (Fig. 1*A*). Examination of the macrophages demonstrated frequent multiple ingestions of aged neutrophils, whereas almost all macrophages fed UV-irradiated neutrophils had only single ingestions (Fig. 1*B*) despite the fact that there was a higher percentage positive for Annexin V in the UV-irradiated neutrophils (Table 1). Quantification of single and multiple ingestions demonstrated that the enhanced ingestion of the aged neutrophils was almost entirely attributable to macrophages taking up multiple targets; nearly 50% of macrophages positive for engulfment had ingested two or more targets (Fig. 1*C*). To determine whether signals downstream of the NADPH oxidase were responsible for these differences, aged neutrophils were treated with DPI, an inhibitor of the NADPH oxidase. Although DPI did not alter PS exposure on either aged or UV-irradiated neutrophil populations (Table 1), inhibition of the oxidase during aging of neutrophils almost completely eliminated enhanced efferocytosis by macrophages and the presence of multiple ingestions (Fig. 1*C*).

TABLE 1
PS head group exposure detected by Annexin V binding
Ctr, control; UV, UV-irradiated.

	Percent positive			Geometric mean		
	Ctr	Aged	UV	Ctr	Aged	UV
Ctr	8.8 ± 3.8	61.3 ± 7.6	79.5 ± 1.5	13 ± 2.4	339 ± 101.7	400 ± 108.3
DPI	9.5 ± 4.8	64.7 ± 4.1	83.5 ± 4.0	9.8 ± 0.32	361 ± 81.8	399 ± 123.6

Because we had previously found that signaling via the macrophage G-protein-coupled receptor G2A was responsible for enhanced ingestion of neutrophils stimulated to activate their NADPH oxidase (15), we asked whether macrophage G2A played a role in the clearance of aged neutrophils. As shown in Fig. 1*D*, blockade of macrophage G2A with antibody (but not isotype control) or utilization of RPMΦ from G2A knock-out mice (Fig. 1*E*) eliminated enhanced efferocytosis of the aged neutrophils. Additionally, using the knock-out mice, we sought to test whether G2A signaling *in vivo* was of functional consequence in the resolution of acute neutrophilic inflammation. Utilizing a well characterized model of zymosan peritonitis, resolution of neutrophilia was delayed, and apoptotic neutrophils accumulated in $G2A^{-/-}$ mice. Although there were no differences between the genotypes in numbers of neutrophils or macrophages lavaged from the peritoneum at 18 h (Fig. 1*F*), the number of apoptotic neutrophils as determined by nuclear morphology, expressed either as absolute numbers or as apoptotic neutrophils per macrophage (Fig. 1*F*), was elevated in the $G2A^{-/-}$ mice. Delayed resolution of neutrophilia and further evidence of uningested apoptotic neutrophils were also evident at 48 h post-zymosan, although at this point in time, fewer macrophages were lavaged from the peritonea of $G2A^{-/-}$ mice (Fig. 1*F*; see "Discussion"). Of note, these data were almost identical to those utilizing antibody blockade of G2A in wild type mice as published previously (15). Taken together, these *in vitro* and *in vivo* data support the hypothesis that both activated (15) and apoptosing neutrophils generate signals via an NADPH oxidase-dependent mecha-

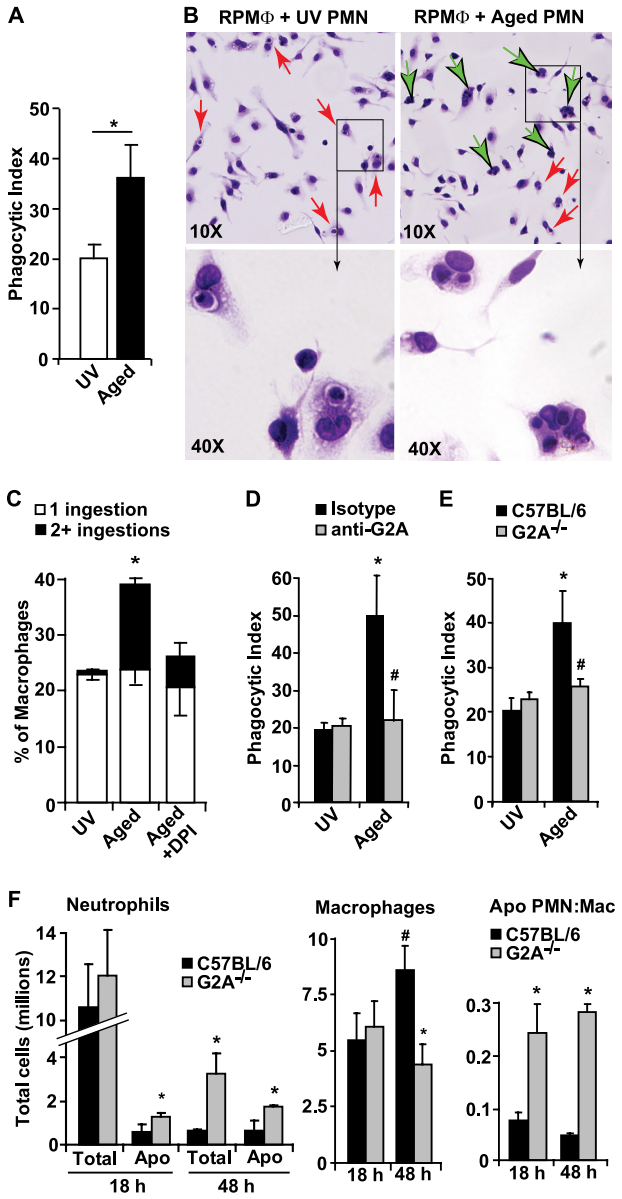


FIGURE 1. **Enhanced efferocytosis of aged apoptotic neutrophils is dependent on signaling through macrophage G2A and results in multiple ingestions.** *A*, UV-irradiated apoptotic or aged apoptotic neutrophils were co-cultured with RPMΦ from C57BL/6 mice, and the phagocytic index was determined after 1 h. $n = 4$; *, $p < 0.05$ compared with UV-irradiated cells. *B*, shown are representative images of cytospins from *A*. Single ingestions are identified by *red arrows*, and multiple ingestions are identified by *green arrows*. *C*, single and multiple (two or more targets/MΦ) ingestions were quantitated for RPMΦ co-cultured with the indicated apoptotic neutrophils generated in the absence or presence of 6.6 μM DPI. $n = 3$; *, $p < 0.05$ for multiple ingestions compared with UV-irradiated cells; #, $p < 0.05$ compared with aged cells. *D*, WT RPMΦ were pretreated for 30 min with 5 μg/ml normal goat IgG (isotype control) or anti-G2A blocking antibody and then co-cultured with apoptotic neutrophil targets as indicated, and phagocytic indices were determined. $n = 5$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with isotype control in the same group. *E*, RPMΦ from WT or G2A$^{-/-}$ mice were co-cultured with the indicated apoptotic neutrophil targets, and phagocytic indices were determined. $n = 5$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with WT RPMΦ fed aged apoptotic neutrophils. *F*, WT or G2A$^{-/-}$ mice were injected intraperitoneally with 1 mg of zymosan. Differentials and cell counts were determined from cytospins at the indicated times. Apoptotic neutrophils were determined microscopically by nuclear morphology. $n = 4$; *, $p < 0.05$ compared with C57BL/6 in the same treatment group; #, $p < 0.05$ compared with the 18-h C57BL/6 control. *PMN*, polymorphonuclear neutrophils; *Apo*, apoptotic; *Mac*, macrophages. Data represent mean ± S.E.

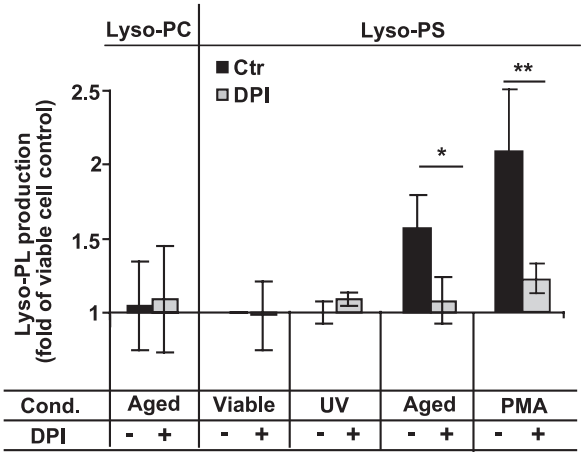


FIGURE 2. **Lyso-PS, but not other activators of G2A, is generated in NADPH oxidase-dependent manner in neutrophils induced to undergo apoptosis by overnight culture.** Lyso-PC was measured by LC/MS/MS in neutrophils undergoing apoptosis by aging in the absence or presence of 6.6 μM DPI and expressed as -fold change from freshly isolated neutrophils (viable control) of the same donor. Lyso-PS was measured by LC/MS/MS and quantitated in neutrophils either freshly isolated, undergoing apoptosis by UV irradiation or aging, or activated by phorbol 12-myristate 13-acetate (*PMA*) (20 ng/ml; 30 min) in the absence or presence of 6.6 μM DPI to inhibit the NADPH oxidase. Results are presented as -fold increase over viable control from the same donor ($n = 5$–10; **, $p < 0.001$; *, $p = 0.013$ by paired *t* test). *PL*, phospholipid; *Ctr*, control; *Cond.*, condition. Data represent mean ± S.E.

nism that activate G2A on macrophages for high capacity clearance.

Activation of NADPH Oxidase during Neutrophil Aging Generates Lyso-PS but Not Other Activators of G2A, and Only Lyso-PS Enhances Efferocytosis—To further investigate G2A-dependent signaling, the generation of known activators of G2A was analyzed by LC/MS/MS and multiple reaction monitoring in aged neutrophils. Murine G2A is activated by lyso-PS and in some cases by lyso-PC as well (20, 29). Lyso-PC was identified (see "Experimental Procedures") but did not increase with neutrophil aging nor were levels altered by DPI treatment of the cells (Fig. 2). Lyso-PS was quantitated in lipid extracts by LC/MS/MS monitoring the neutral loss of 87 atomic mass units in negative ion mode (specific for the PS head group). Although absolute amounts produced showed variability from donor to donor as has been demonstrated previously (15), neutrophils undergoing apoptosis by aging showed a 1.5–2-fold increase in lyso-PS (Fig. 2). This production was similar to that observed in neutrophils stimulated with either short term phorbol 12-myristate 13-acetate stimulation (shown) or opsonized zymosan and, as in earlier studies, was always cell-associated and undetectable in the culture supernatants (15). In all instances, lyso-PS production was dependent on a functioning NADPH oxidase as demonstrated in cells treated with DPI. In contrast and as shown previously (15), neutrophils induced to undergo apoptosis by UV irradiation did not produce lyso-PS above background levels (and are referred to as lyso-PS^{neg} apoptotic cells in all subsequent experiments) (Fig. 2). Although murine G2A, unlike the human counterpart, reportedly does not respond to 9- and 13-HODE and 12- and 15-HETE (33, 34), these too were measured in aged neutrophils, and none were detected (data not shown). Finally, to assess the potential contribu-

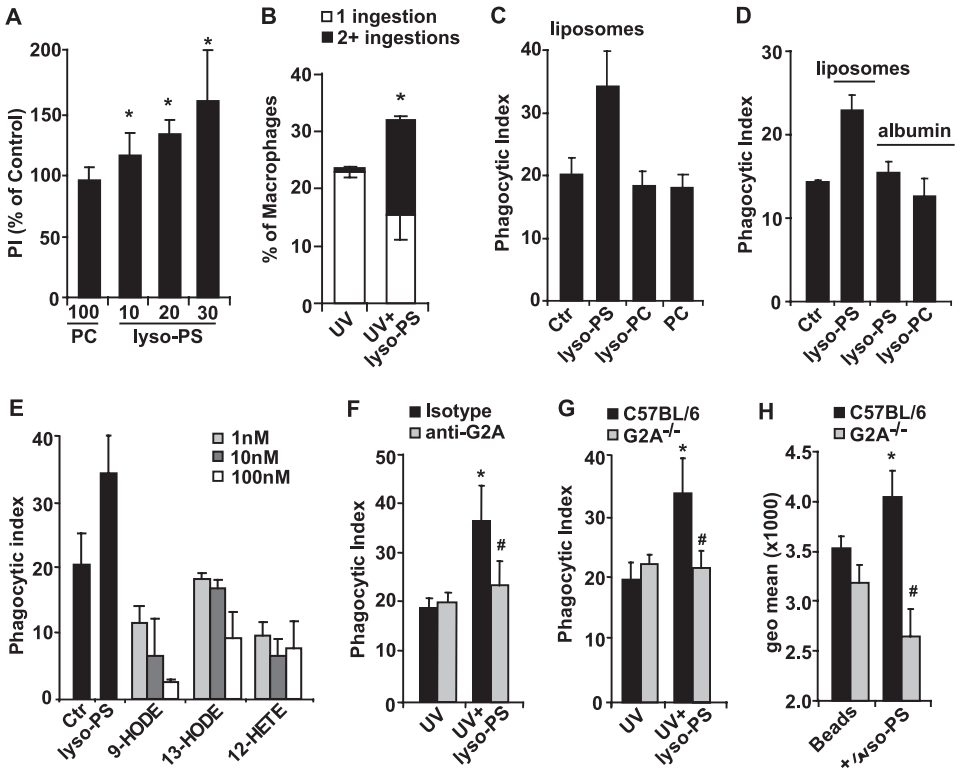


FIGURE 3. **Exogenous addition of lyso-PS, but not other activators of G2A, enhances efferocytosis in G2A-dependent manner.** WT RPMΦ (*A–F*) were co-cultured with UV-irradiated apoptotic neutrophils in the absence or presence of added lipids as follows, and phagocytic indices were determined after 1 h. *A*, liposomes containing carrier lipid (PC) or the indicated dose (mol %) of lyso-PS. $n = 3$; *, $p < 0.05$ compared with PC control. *B*, liposomes containing 30 mol % lyso-PS where indicated. Multiple ingestions were quantitated as in Fig. 1*C*. $n = 3–4$; *, $p < 0.05$ compared with UV-irradiated cells. *C*, PBS (*Ctr*) or liposomes of 30 mol % lyso-PS, lyso-PC, or PC carrier lipid alone. *D*, 30 nmol of lyso-PS or lyso-PC delivered in liposomes or on 0.05% albumin as indicated. *E*, lyso-PS liposomes (30 mol %) or the indicated concentration of 9-HODE, 13-HODE, or 12-HETE delivered on 0.05% albumin. *F*, lyso-PS liposomes (30 mol %) following pretreatment with isotype control or anti-G2A as in Fig. 1*C*. *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with isotype control in the same treatment group. *G*, RPMΦ from WT and G2A$^{-/-}$ mice were co-cultured with UV-irradiated apoptotic neutrophils in the presence of lyso-PS liposomes (30 mol %) as indicated. $n = 3–5$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with C57BL/6 in the same treatment group. *H*, *in vivo* phagocytosis by F4/80-positive macrophages was determined by flow cytometry 1 h following intraperitoneal injection of fluorescent carboxylate-modified beads (see "Experimental Procedures"). Shown is the average geometric mean of F4/80-positive macrophages associated with beads. $n = 4$; *, $p < 0.05$ compared with WT beads alone; #, $p < 0.05$ compared with WT following the same treatment. *Ctr*, control; *PI*, phagocytic index. Data represent mean ± S.E.

tion by oxidized phospholipid species to enhanced efferocytosis, oxidized PC and oxidized PS species were measured by multiple reaction monitoring. Few oxidized PC species were detected, and the amounts of these species did not change with aging in culture or DPI treatment (supplemental Table 1). Additionally, 10 transitions for oxidized PS species that were identified during short term phorbol 12-myristate 13-acetate stimulation were examined (15), and none were detected under these culture conditions (data not shown). To further rule out contributions to efferocytic signaling via oxidized phospholipids, an antibody shown to recognize various oxidized PC species (see "Experimental Procedures") and inhibit uptake of apoptotic thymocytes (35, 36) had no effect on efferocytosis of aged apoptotic neutrophils nor did it inhibit lyso-PS enhancement of efferocytosis (data not shown).

To investigate the signaling pathway in macrophages downstream of G2A, we developed a model system in which to mimic signaling by aged neutrophils. Known activators of G2A were supplied exogenously and tested for effects on RPMΦ efferocytosing lyso-PS^{neg} UV-irradiated neutrophils. As published previously and shown in Fig. 3*A*, lyso-PS supplied in liposomes dose-dependently enhanced engulfment of these targets. Notably, liposomes containing 30 mol % lyso-PS closely mimicked the enhanced efferocytosis observed with aged neutrophils: similar to aged apoptotic neutrophils (compare with Fig. 1*C*), lyso-PS enhanced efferocytosis of UV-irradiated neutrophils, and enhancement was attributable to multiple ingestions by efferocytosing macrophages (Fig. 3*B*). In the presence of lyso-PS liposomes, the percentage of RPMΦ eating two or more targets increased to ~50% of the macrophages positive for engulfment and was similar to the distribution observed in macrophages co-cultured with aged apoptotic neutrophils (see above). As published previously, lyso-PS had no effect on the uptake of viable cells or opsonized cells (data not shown; Ref. 15), demonstrating that the effect was specific for efferocytosis.

By comparison and as noted in earlier studies (15), lyso-PC offered in liposomes had very little effect on efferocytosis even at 30 mol % (Fig. 3*C*), and neither lysophospholipid at the same molar concentration offered on small amounts of albumin (0.05%) was able to enhance efferocytosis (Fig. 3*D*) (see "Discussion"). Similarly, HODEs and HETEs, although demonstrated to be activators of human G2A but not as yet for murine G2A (33, 34), were added to co-cultures up to doses of 1 μM. Under these conditions, either no effect or inhibition of efferocytosis was observed (Fig. 3*E*). Thus, although several lipids have been shown to lead to G2A activation using various end points, *e.g.*

calcium mobilization or migration (20, 34, 37, 38), in our studies shown here, only lyso-PS presented in the context of liposomes or membranes (exogenously added or endogenously produced, respectively) resulted in enhanced efferocytosis.

As expected, enhanced efferocytosis by exogenously supplied lyso-PS was abrogated by antibody blockade of G2A but not isotype control antibodies (Fig. 3*F*). Furthermore and as shown in our previous studies (20), G2A signaling was likely via its engagement of $G\alpha_i$ in that pertussis toxin inhibited lyso-PS-enhanced efferocytosis (data not shown). Likewise, RPMΦ from $G2A^{-/-}$ mice showed no enhancement of efferocytosis in response to lyso-PS provided in liposomes (Fig. 3*G*). This was further demonstrated in $G2A^{-/-}$ and wild type mice *in vivo*. For these experiments, carboxylate-modified beads, which serve as apoptotic cell mimics supplying a PS-like surface (15, 39) (but no lipid), were injected into the naïve peritonea. RPMΦ were harvested by lavage 1 h later, and engulfment was analyzed by flow cytometry. Although the percentage of macrophages positive for bead uptake was no different between the strains in the absence of lyso-PS (35 ± 1% for wild type *versus* 35 ± 6.2% for $G2A^{-/-}$), differences in the mean fluorescence index as a measure of multiple bead ingestion were apparent and significantly increased in wild type mice but not in $G2A^{-/-}$ mice (Fig. 3*H*). Together, these data support that lyso-PS signaling via G2A was both necessary and sufficient to enhance efferocytosis. Furthermore, these experiments defined conditions for lyso-PS-enhanced efferocytosis mediated via macrophage G2A that closely mimicked conditions where the G2A activating signal, lyso-PS, was endogenously produced and supported high capacity neutrophil clearance. Exogenously supplied lyso-PS liposomes (30 mol %) were then used in subsequent experiments to investigate signaling downstream of macrophage G2A.

Signaling via G2A Enhances Rac1 Activity—It is known that activation of the Rho-GTPase Rac1 is required for engulfment of apoptotic cells (16, 18, 40). Because engulfment capacity appeared to be increased with activation of G2A, we hypothesized that signaling through G2A would augment Rac1 activity. Rac1 activity over a time course was measured using the Rac G-LISA™. RPMΦ were co-cultured with UV-irradiated lyso-PS^{neg} apoptotic cells alone or delivered with lyso-PS liposomes as a G2A activator. As shown in Fig. 4*A*, Rac1 activity increased in macrophages following addition of UV-irradiated apoptotic cells within 20 min and slowly declined over time, returning to base-line levels by 60 min. In the presence of lyso-PS liposomes, however, Rac1 activity was significantly enhanced by 2-fold over UV-irradiated apoptotic cells alone with maximal levels evident at 20 min of co-culture. Lyso-PS liposomes alone had little effect on macrophage Rac1 activation. This observation supports our earlier report that stimulation of G2A alone is insufficient to promote phagocytosis of viable or IgG opsonized targets and requires additional signals from the apoptotic or activated neutrophil (15) (see above and "Discussion").

To confirm that augmentation of Rac1 activity was dependent on signaling through G2A, Rac1 activity was determined in RPMΦ from $G2A^{-/-}$ mice. As shown in Fig. 4*B*, Rac1 activation at 20 min induced by UV-irradiated lyso-PS^{neg} apoptotic cells was similar to that of WT macrophages. In the presence of the G2A activator lyso-PS, however, there was no augmentation of Rac1 activity in $G2A^{-/-}$ RPMΦ compared with those of WT. These results strongly suggest that concomitant G2A signaling can augment macrophage Rac1 activity and, in turn, result in enhanced efferocytosis and multiple ingestions.

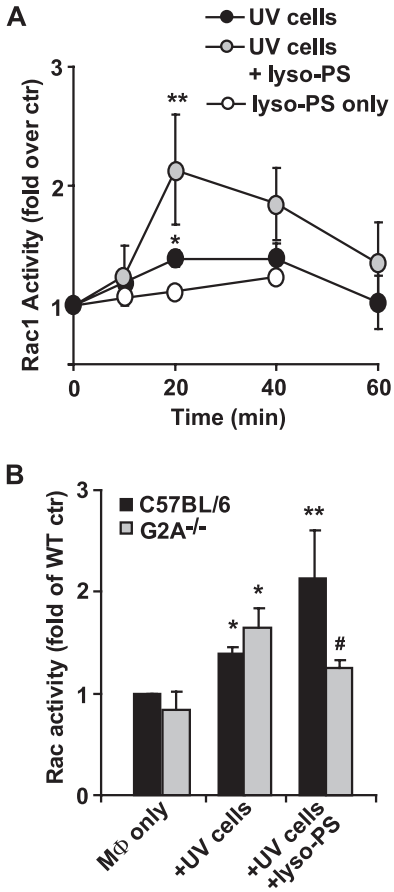


FIGURE 4. **Signaling through G2A augments Rac1 activity.** *A*, Rac1 activity by G-LISA™ was determined in C57BL/6 RPMΦ treated for the indicated times with UV-irradiated apoptotic neutrophils (*UV cells*) alone, UV-irradiated cells plus lyso-PS liposomes (30 mol %), or lyso-PS liposomes alone. Data are represented as -fold over control. $n = 4$; *, $p = 0.002$ compared with UV-irradiated cells at time 0; **, $p = 0.03$ compared with UV-irradiated cells alone at 20 min. *B*, Rac1 activity was determined in C57BL/6 or $G2A^{-/-}$ RPMΦ after 20 min of co-culture in the absence or presence of UV-irradiated apoptotic neutrophils with or without lyso-PS liposomes. $n = 3$; *, $p < 0.05$ compared with MΦ only for each genotype, respectively; **, $p < 0.05$ compared with its own genotype treated with UV-irradiated cells; #, $p < 0.05$ compared with C57BL/6 in the same treatment group. *ctr*, control. Data represent mean ± S.E.

Signaling via G2A Enhances Efferocytosis and Rac1 Activity via cAMP/PKA Pathway—Next we sought to elucidate the signaling pathway downstream of G2A resulting in enhanced Rac1 activity and efferocytosis. Recently, a pivotal role for cAMP elevation in macrophages in resolving acute peritoneal inflammation has been demonstrated, and this elevation was absent in mice lacking a functional NADPH oxidase (41, 42). Given these data, we hypothesized that under normal circumstances lyso-PS produced in neutrophils following activation of the NADPH might signal to the macrophage for cAMP production, resulting in enhanced engulfment.

First, a phosphodiesterase-resistant analog of cAMP, db-cAMP, was used over a concentration curve to mimic lyso-PS effects. As shown in Fig. 5*A*, very low doses of db-cAMP enhanced uptake of UV-irradiated lyso-PS^{neg} apoptotic cells. In

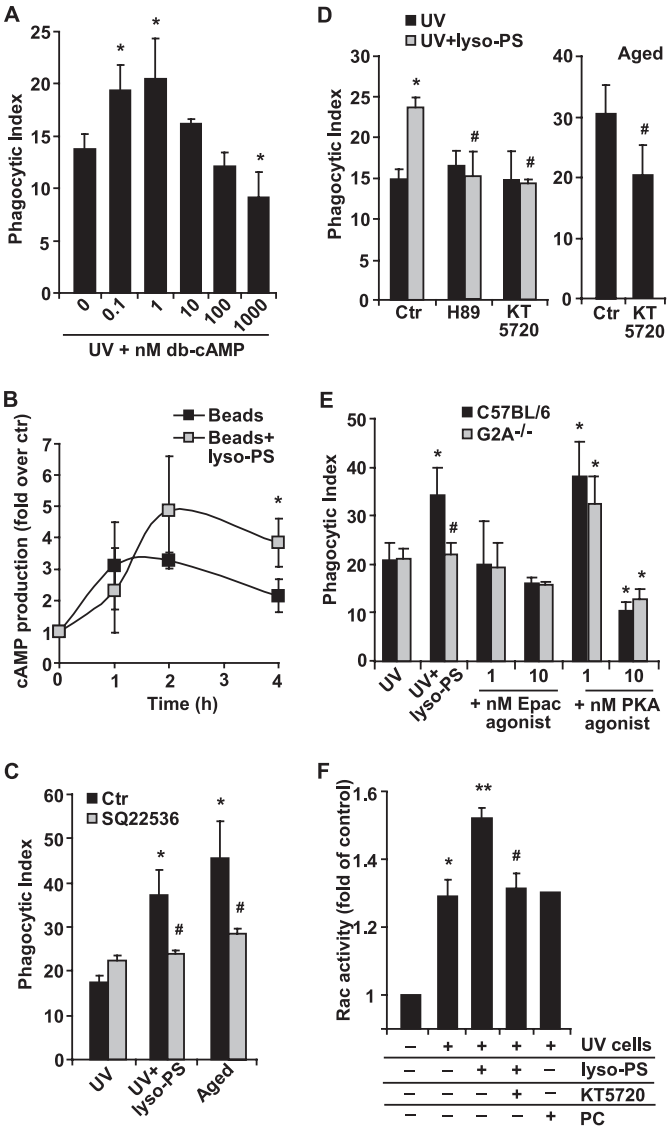


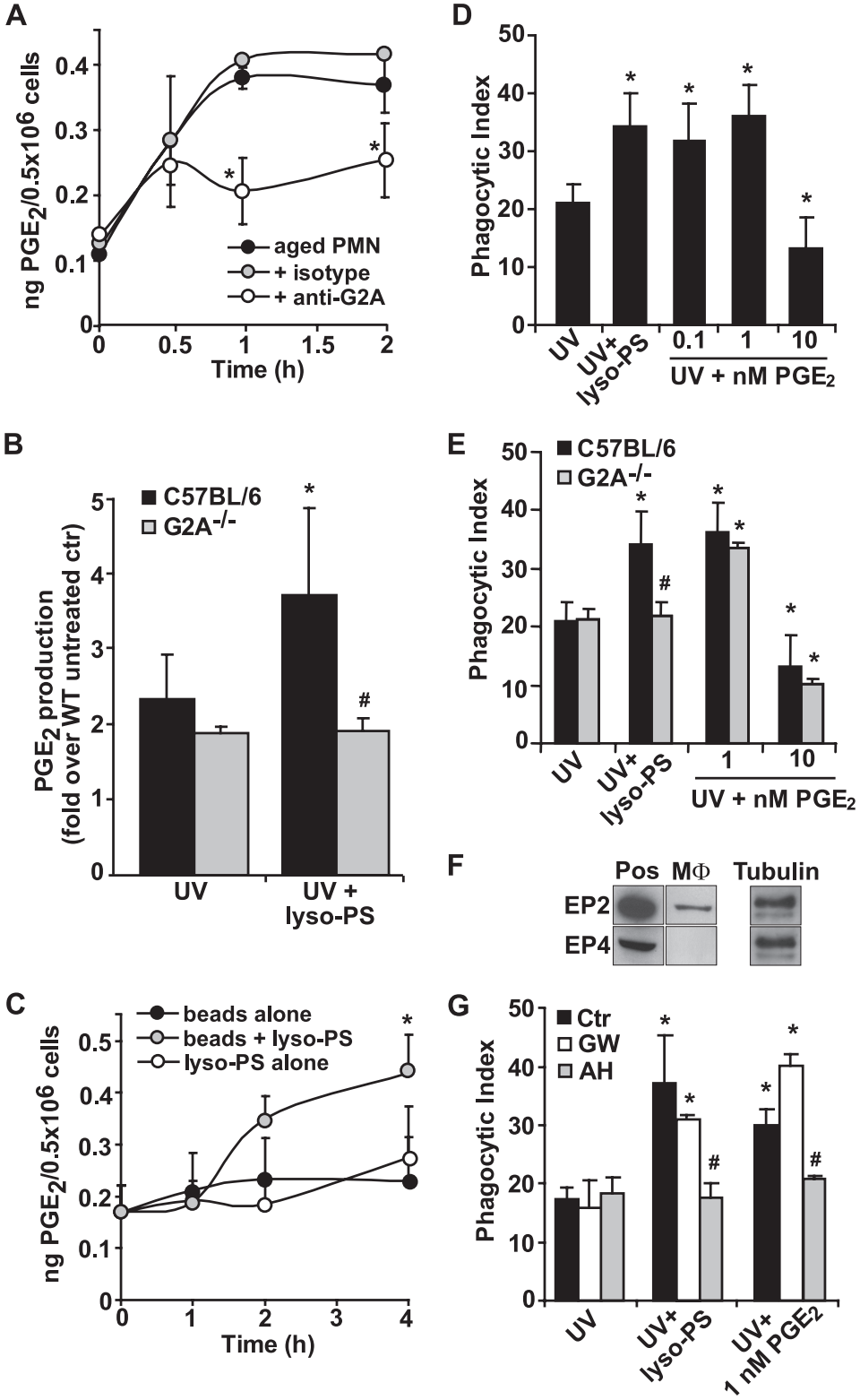
FIGURE 5. **Signaling through G2A enhances efferocytosis via cAMP production and PKA-dependent enhancement of Rac1 activity.** *A*, phagocytic indices were determined as in Fig. 1 using RPMΦ co-cultured with UV-irradiated apoptotic neutrophils in the absence or presence of the indicated concentrations of db-cAMP. $n = 4$; *, $p < 0.05$ compared with 0 nM db-cAMP. *B*, intracellular cAMP was measured at the indicated times in RPMΦ following co-culture with carboxylate-modified beads in the absence or presence of lyso-PS liposomes (30 mol %). Data are displayed as -fold over control. $n = 4$; *, $p = 0.035$ compared with beads alone at 4 h. *C* and *D*, phagocytic indices were determined for RPMΦ pretreated for 30 min in the absence or presence of 10 μM SQ22563 to inhibit adenylyl cyclase or 10 μM H89 or 2 μM KT5720 to inhibit PKA and then co-cultured with apoptotic cells and lyso-PS liposomes as indicated. $n = 3$–5; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with untreated control for each group, respectively. *E*, C57BL/6 or G2A$^{-/-}$ RPMΦ were pretreated with the indicated concentrations of either the Epac agonist or PKA agonist for 15 min and then co-cultured with UV-irradiated cell targets, and phagocytic indices were determined as above. $n = 4$; *, $p < 0.05$ compared with respective UV-irradiated cells; #, $p < 0.05$ compared with WT ingesting UV-irradiated cells + lyso-PS. *F*, Rac1 activity was determined as in Fig. 4 in RPMΦ that had been pretreated for 30 min in the absence or presence of 2 μM KT5720 prior to addition of UV-irradiated apoptotic cells and lyso-PS or PC (carrier) liposomes. $n = 3$; *, $p < 0.05$ compared with macrophages alone; **, $p < 0.05$ compared with UV-irradiated cells alone; #, $p < 0.05$ compared with UV-irradiated cells + lyso-PS. *Ctr*, control. Data represent mean ± S.E.

contrast, treatment with higher doses of db-cAMP, known to deactivate macrophages, suppressed engulfment in keeping with a previous report (43). Intracellular cAMP production was also measured over a time course of co-culture of apoptotic cells or carboxylate-modified beads (15, 39) in the absence or presence of G2A activation by lyso-PS liposomes. Although the levels measured were variable between experiments, there was a consistent 1.5–2-fold increase in cAMP production in the presence of lyso-PS, *e.g.* as shown at 2 and 4 h for macrophages co-cultured with beads alone *versus* beads with lyso-PS liposomes (Fig. 5*B*). As additional confirmation of the role of adenylyl cyclase in lyso-PS signaling, we used the inhibitor SQ22536 to block activation of adenylyl cyclase in RPMΦ. In the presence of the inhibitor, G2A-mediated enhanced efferocytosis was clearly abrogated (Fig. 5*C*). This was demonstrated both for the enhanced uptake of lyso-PS^{neg} apoptotic cells delivered with lyso-PS liposomes and for aged apoptotic neutrophils that present their own endogenously produced G2A activator. Adenylyl cyclase inhibition had no effect on the uptake of UV-irradiated lyso-PS^{neg} apoptotic cells. These results support the hypothesis that lyso-PS signals through adenylyl cyclase activation via a cAMP-dependent pathway and that this is distinct from signaling for engulfment by apoptotic cells in the absence of lyso-PS.

Downstream effectors of cAMP, PKA and Epac proteins, were investigated for their role in mediating lyso-PS-enhanced engulfment. First, the compounds H-89 and KT5720, both reported to be inhibitors of PKA, were tested. As shown in Fig. 5*D*, in the presence of the G2A activator, lyso-PS, enhanced uptake was blocked by both inhibitors, suggesting that PKA might be a downstream target of cAMP induced by the G2A signaling pathway. In contrast, pretreatment of macrophages with either inhibitor had no effect on the uptake of UV-irradiated lyso-PS^{neg} apoptotic cells. Similarly, pretreatment of macrophages with KT5720 reduced the uptake of aged neutrophils to levels comparable with the those observed for UV-irradiated cells, again suggesting that endogenously produced lyso-PS in aged neutrophils likely activated this pathway (Fig. 5*D*). To confirm PKA involvement rather than activation of an Epac protein and to rule out a nonspecific effect of the inhibitors, we took advantage of recently available agonists specific for either PKA or Epac (44). Given the dose-dependent effects of the cAMP analog above, concentration effects of each were investigated. At a higher dose, the PKA agonist was inhibitory for engulfment of lyso-PS^{neg} UV-irradiated cells just as was seen for higher doses of db-cAMP (Fig. 5, *A* and *E*). In contrast, at a lower dose, the PKA agonist enhanced uptake of lyso-PS^{neg} apoptotic cells in a manner mimicking enhancement observed using lyso-PS liposomes and low dose db-cAMP. Neither dose of the Epac agonist had any effect on uptake of UV-irradiated lyso-PS^{neg} apoptotic cells. Similar effects of PKA/Epac agonism downstream of G2A were also demonstrated using RPMΦ from G2A$^{-/-}$ mice: treatment with a low dose of the PKA agonist enhanced engulfment, whereas a higher dose was inhibitory, and Epac agonism was unable to enhance uptake (Fig. 5*E*). These data support a role for PKA rather than Epac in the G2A signaling pathway and show that this pathway can be salvaged downstream of G2A in the G2A$^{-/-}$ macrophages. Finally, to determine whether G2A-dependent enhancement of Rac1 activity required PKA activation, Rac1 activity was determined in RPMΦ pretreated with the PKA inhibitor KT5720. As expected, augmented Rac1 activity mediated by G2A activation

was blocked by the PKA inhibitor, supporting dependence on PKA for enhanced Rac1 activation by G2A signaling (Fig. 5*F*).

G2A Signaling Upstream of Adenylyl Cyclase—Apoptotic cell recognition by macrophages can result in their production of prostanoids, such as PGE_2 (9, 10, 45), that might be expected to signal for activation of adenylyl cyclase (*e.g.* signaling via the prostaglandin receptors EP2 and/or EP4, which are known to couple to $G\alpha_s$ heterotrimeric G-proteins). RPMΦ were co-cultured with aged apoptotic neutrophils over a time course, and supernatants were analyzed for a panel of eicosanoids by LC/MS/MS (see "Experimental Procedures"). Only PGE_2 was detected with levels peaking at a concentration of ~0.4 ng/5 ×



10^5 cells (1.1 nM under these conditions) by 1 h and sustained to 2 h following co-culture (Fig. 6*A*). Culture of aged apoptotic neutrophils alone, in the absence of macrophages, showed no increase in PGE_2 production (data not shown). In the presence of the anti-G2A blocking antibody, PGE_2 production in response to aged neutrophils was reduced by ~50% (0.2 ng/5 × 10^5 cells or 0.5 nM), suggesting that signaling via G2A on these cells augmented PGE_2 production. This was confirmed using $G2A^{-/-}$ RPMΦ where no additional enhancement of PGE_2 production was observed following co-culture with lyso-PS^{neg} apoptotic cells in the presence of the G2A activator, lyso-PS (Fig. 6*B*). Similar enhancement of PGE_2 production was elicited from wild type macrophages treated with lyso-PS liposomes along with lyso-PS^{neg} apoptotic neutrophils as was seen for aged apoptotic neutrophils (Fig. 6*B*). Finally, to rule out any contribution from the apoptotic cells themselves, carboxylate-modified beads serving as apoptotic cell mimics were investigated. Similar levels of PGE_2, although somewhat delayed in production, were detected in supernatants of macrophages co-cultured with lyso-PS liposomes plus carboxylate-modified beads (Fig. 6*C*), and production was significantly higher than for either beads or lyso-PS liposomes alone; the levels were largely unchanged from levels associated with macrophages incubated alone. Increases in the levels of other eicosanoids were not detected in response to apoptotic cells or carboxylate-modified bead mimics in the presence or absence of lyso-PS. Together, these data suggested that macrophages that had ingested apoptotic cells in the presence of the G2A activator, lyso-PS (either generated endogenously or provided exogenously), generate more PGE_2 than macrophages that had ingested lyso-PS^{neg} apoptotic cells and that activation of G2A enhanced production of PGE_2, leading to augmented Rac1 activity via cAMP production and subsequent PKA activation. These findings were somewhat surprising given that PGE_2, like db-cAMP, has been associated with macrophage deactivation and inhibition of apoptotic cell uptake (43, 45). Thus, the effect of exogenously added PGE_2 on apoptotic cell uptake was investigated. Similar to the stimulatory effects of db-cAMP, exogenous addition of PGE_2 at very low doses (0.1–1.0 nM) enhanced the uptake of UV-irradiated lyso-PS^{neg} apoptotic cells (Fig. 6*D*). As reported in the literature, higher doses of PGE_2 were suppressive (45). $G2A^{-/-}$ macrophages showed identical responses: enhanced uptake of lyso-PS^{neg} apoptotic cells was observed with a low dose, whereas a higher dose of PGE_2 resulted in suppressed uptake (Fig. 6*E*). The latter data further demonstrate that the signaling pathway in the $G2A^{-/-}$ mice is intact downstream of the receptor and can be bypassed with effectors of G2A signaling. Although no change in 6-keto-PGF1α (a stable byproduct of PGI_2) was detected in culture supernatants, stimulation of the prostaglandin I2 receptor in macrophages with the PGI_2 analog iloprost also enhanced uptake of UV-irradiated apoptotic cells at low doses although not as robustly, suggesting that other prostanoids coupled to adenylyl cyclase-activating G-proteins are also capable of enhancing uptake (data not shown).

PGE_2-specific receptors have been identified and include EP1, EP2, EP3, and EP4 (46), and it has been reported that macrophages can express both EP2 and EP4 (47, 48). Western blot analysis of the RPMΦ from C57BL/6 mice demonstrated expression of EP2 but not EP4 (Fig. 6*F*). To determine whether EP2 was utilized in the lyso-PS signaling pathway, macrophages were pretreated with an EP2 receptor antagonist (AH6809). An EP4 receptor antagonist (GW627368X) was also used as a control. As shown in Fig. 6*G*, the EP2 receptor antagonist blocked the enhancing effects of both G2A activation (by lyso-PS) and low dose PGE_2, whereas the EP4 receptor antagonist had no effect in accordance with the receptor expression data.

PGE_2 synthesis by prostaglandin-E synthase first requires liberation of arachidonic acid by the action of a phospholipase A_2 followed by COX modification (49). Several phospholipase A_2s are known to be active in macrophages, including calcium-independent PLA_2 as well as a $cPLA_2\alpha$, which has specific substrate specificity for arachidonic acid esterified in the *sn*-2 position. Therefore, to test whether these PLA_2s were activated following G2A activation, RPMΦ were pretreated with the $cPLA_2\alpha$ inhibitor (a pyrrolidine derivative) or the calcium-independent PLA_2 inhibitor bromoenol lactone, and phagocytosis of UV-irradiated lyso-PS^{neg} apoptotic cells in the absence or presence of lyso-PS liposomes was determined. As shown in Fig. 7*A*, inhibition of $cPLA_2\alpha$ had no effect on uptake of UV-irradiated apoptotic cells in the absence of lyso-PS liposomes but suppressed lyso-PS-dependent enhancement. In contrast, bromoenol lactone had no effect, suggesting that signaling through G2A activates $cPLA_2\alpha$, liberating arachidonic acid for subsequent PGE_2 synthesis. These results were confirmed using RPMΦ from mice deficient for $cPLA_2\alpha$. Macrophages from WT (BALB/c in this case) or $cPLA_2\alpha^{-/-}$ mice were fed UV-irradiated lyso-PS^{neg} apoptotic cells in the absence or pres-

FIGURE 6. **Signaling through G2A augments PGE_2 production.** *A*, RPMΦ were pretreated in the absence or presence of isotype control or anti-G2A blocking antibody for 30 min and then co-cultured with aged apoptotic neutrophils. PGE_2 production was measured in supernatants at the indicated times by LC/MS/MS (see "Experimental Procedures"). $n = 4$; *, $p < 0.05$ compared with aged polymorphonuclear neutrophils (*PMN*). *B*, C57BL/6 or $G2A^{-/-}$ RPMΦ were co-cultured with UV-irradiated apoptotic cells in the absence or presence of lyso-PS liposomes, and PGE_2 production was measured in supernatants after 1 h. Data are presented as -fold over WT MΦ control. $n = 3$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with WT MΦ treated with UV-irradiated cells + lyso-PS. *C*, RPMΦ were co-cultured with carboxylate-modified beads in the absence or presence of lyso-PS liposomes, and PGE_2 production was measured in the supernatants at the indicated times. $n = 3$; *, $p < 0.05$ compared with beads alone. *D* and *E*, phagocytic indices of RPMΦ were determined as in Fig. 1with additional conditions. *D*, C57BL/6 RPMΦ were treated with lyso-PS or the indicated doses of PGE_2 15 min before addition of UV-irradiated apoptotic cells. $n = 3$–4; *, $p < 0.05$ compared with UV-irradiated cells. *E*, RPMΦ from C57BL/6 or $G2A^{-/-}$ mice were treated with the indicated concentrations of PGE_2 or lyso-PS liposomes as described in *D*, and phagocytic indices were determined as above. $n = 3$; *, $p < 0.05$ compared UV-irradiated cells; #, $p < 0.05$ compared with WT MΦ ingesting UV-irradiated cells + lyso-PS. *F*, expression of EP2 and EP4 was investigated by Western immunoblot analysis in RPMΦ. *Pos*, positive control; *M*Φ, RPMΦ whole cell lysates; *Tubulin*, loading control for MΦ. Data are representative of three independent experiments. *G*, RPMΦ were pretreated for 30 min in the absence or presence of either GW627368X (*GW*; 10 μM), an EP4 antagonist, or AH6809 (*AH*; 10 μM), an EP2 antagonist, and then co-cultured with UV-irradiated apoptotic cells with and without lyso-PS liposomes and PGE_2 as indicated. $n = 3$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with untreated UV-irradiated cells + lyso-PS or UV-irradiated cells + PGE_2 for each group, respectively. *Ctr*, control. Data represent mean ± S.E.

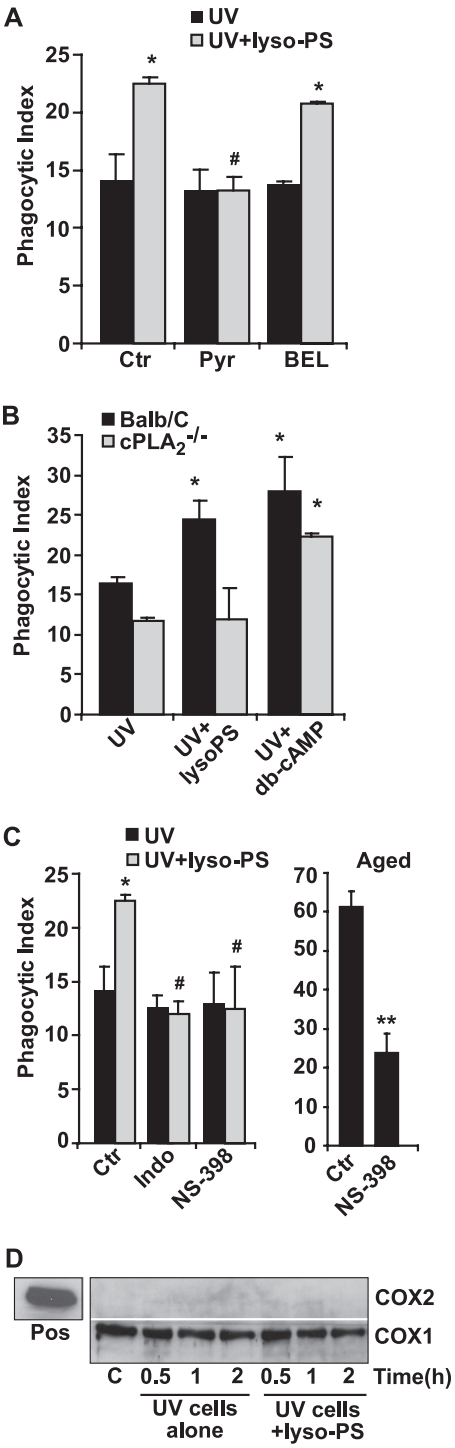


FIGURE 7. **Enhanced efferocytosis by G2A requires activation of cPLA$_2\alpha$.** *A*, RPMΦ were pretreated for 30 min in the absence or presence of pyrrolidine (*Pyr*; 2 μM), a cPLA$_2\alpha$ inhibitor, or bromoenol lactone (*BEL*; 0.5 μM), a calcium-independent PLA$_2$ inhibitor, and then co-cultured with lyso-PS liposomes and UV-irradiated apoptotic cells, and phagocytic indices were determined as before. $n = 3$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with UV-irradiated cells + lyso-PS. *B*, phagocytic indices were determined in RPMΦ from BALB/c (WT) or cPLA$_2\alpha^{-/-}$ mice following the addition of UV-irradiated apoptotic cells in the absence or presence of lyso-PS liposomes or 1 nM db-cAMP. $n = 3$; *, $p < 0.05$ compared with its own genotype treated with UV-irradiated cells. *C*, RPMΦ were treated for 30 min in the absence or presence of COX inhibitor indomethacin (*Indo*; 20 μM) or NS-398 (10 μM) and then co-cultured with either UV-irradiated apoptotic cells with and without lyso-PS liposomes or aged apoptotic neutrophils, and phagocytic indices were determined. $n = 3$; **, $p < 0.05$ compared UV-irradiated cells; #, $p < 0.05$ compared with UV-irradiated cells + lyso-PS; *, $p < 0.001$ compared with aged cells. *D*, COX1 and COX2 expression was determined by Western immunoblot analysis in whole cell lysates of RPMΦ left untreated or treated with UV-irradiated apoptotic cells in the absence or presence of 30 mol % lyso-PS liposomes for the indicated times. *Pos*, positive control for COX2 expression; *C*, control. Data are representative of three independent experiments. *Ctr*, control. Data represent mean ± S.E.

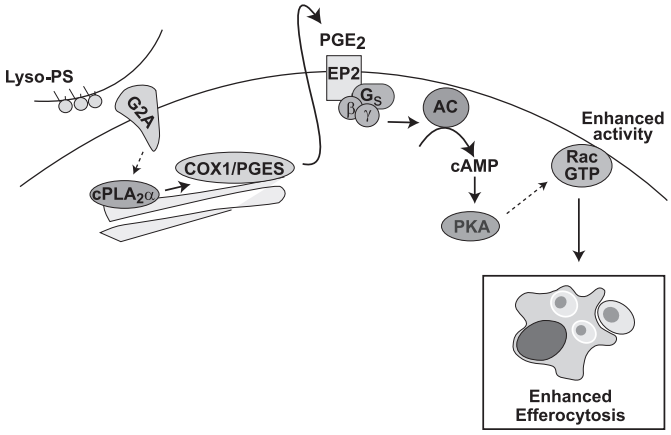


FIGURE 8. **Proposed signaling pathway for G2A-mediated enhancement of apoptotic cell engulfment.** *PGES*, prostaglandin-E synthase; *AC*, adenylyl cyclase.

ence of G2A activation by lyso-PS, and the phagocytic indices were determined. Macrophages from cPLA$_2\alpha^{-/-}$ mice showed no enhancement of apoptotic cell uptake with lyso-PS treatment (Fig. 7*B*) in accordance with the inhibitor studies and again supported the hypothesis that lyso-PS stimulation activates cPLA$_2\alpha$. Enhanced engulfment by macrophages from the cPLA$_2\alpha^{-/-}$ mice was observed following treatment with 1 nM db-cAMP, a downstream effector in the proposed pathway, demonstrating that the signaling pathway in MΦ from these mice is intact downstream of cPLA$_2\alpha$ (Fig. 7*B*).

Finally, COX activity is required for further processing of released arachidonic acid for PGE$_2$ production. Two COX enzymes exist: COX1, which is constitutively expressed, and COX2, which has inducible expression (50). The inhibitors indomethacin and NS-398 were used to test for involvement of the COX enzymes. Pretreatment of macrophages with either inhibitor abrogated G2A-dependent enhanced uptake of UV-irradiated lyso-PS^{neg} apoptotic targets (Fig. 7*C*). Enhanced uptake of aged apoptotic neutrophils was similarly abrogated, suggesting activation of a COX enzyme (Fig. 7*C*). Only COX1 was evident in these resident peritoneal macrophages at base line even following G2A activation with lyso-PS (Fig. 7*D*). As there was no detectible COX2 expression under any of the culture conditions, the data support that PGE$_2$ production is likely due to the activity of COX1.

Inflammatory Macrophages Also Respond to G2A Activation via This Pathway—Taken together, these results support the signaling pathway G2A → cPLA$_2\alpha$ → COX1 → PGE$_2$ → cAMP → PKA, which augments Rac1 activity and enhanced uptake in resident peritoneal macrophages (Fig. 8). Because we had previously shown that other macrophage populations, including inflammatory macrophages (thioglycollate-elicited) respond to lyso-PS liposomes via G2A signaling for enhanced efferocytosis (15), we sought to determine whether the key

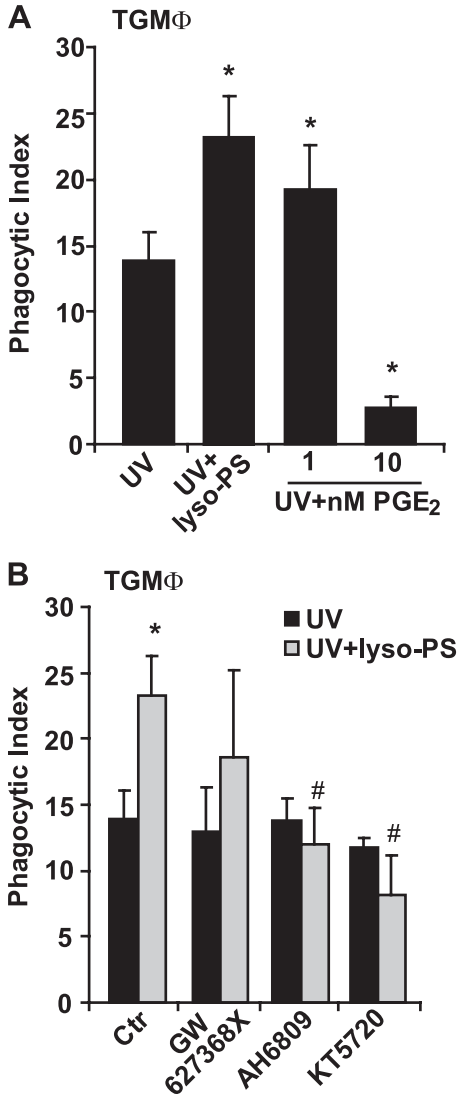


FIGURE 9. **G2A activation in inflammatory macrophages enhances phagocytosis via the same proposed pathway.** *A*, thioglycollate-elicited MΦ (*TGM*Φ) were pretreated with either lyso-PS liposomes or the indicated dose of PGE_2 for 15 min and then co-cultured with UV-irradiated apoptotic cells, and phagocytic indices were determined. $n = 3$; *, $p < 0.05$ compared with UV-irradiated cells. *B*, thioglycollate-elicited macrophages were pretreated with a 10 μM concentration of either the EP4 (GW627368X) or EP2 (AH6809) receptor antagonist or 2 μM KT5720 for 30 min and then co-cultured with UV-irradiated apoptotic cells in the absence or presence of lyso-PS liposomes, and phagocytic indices were determined as above. $n = 3$; *, $p < 0.05$ compared with UV-irradiated cells; #, $p < 0.05$ compared with UV-irradiated cells + lyso-PS. *Ctr*, control. Data represent mean ± S.E.

intermediaries of the G2A signaling pathway identified here were utilized in thioglycollate-elicited macrophages as well. As shown in Fig. 9*A*, lyso-PS provided in liposomes with lyso-PS^{neg} apoptotic cells enhanced engulfment by the inflammatory macrophages. Similarly, low dose PGE_2 also enhanced engulfment of lyso-PS^{neg} apoptotic cells, whereas a higher dose was inhibitory. Finally, G2A-mediated enhanced uptake was abrogated in the presence of the EP2 receptor antagonist AH6809 (but not the EP4 antagonist GW627368X) and the PKA inhibitor KT5720 (Fig. 9*B*). Together, these data support that the G2A signaling pathway defined in resident macrophages is also operative in inflammatory macrophages.

DISCUSSION

Not all apoptotic cells signal equivalently to macrophages for efferocytosis. Differential display of signals for tethering and recognition, "enhancers," and so-called "don't eat me" signals alters responses of phagocytic cells (51). The data presented here support the hypothesis that the presence of lyso-PS, either generated endogenously or provided exogenously on liposomes, significantly increased both the number of macrophages ingesting apoptotic cells and the number of cells ingested per macrophage (Figs. 1, *A* and *B*, and 3*B*). Conversely, apoptosis of neutrophils (and Jurkat cells; data not shown) induced by UV irradiation, an admittedly non-physiologic (but widely used) stimulus, did not increase lyso-PS production, and uptake by macrophages was consequently reduced. As such, we hypothesize that generation of lyso-PS and concomitant signaling through macrophage G2A may contribute to high capacity clearance of neutrophils that may promote timely resolution of acute inflammation where neutrophils often vastly outnumber macrophages (52). Given both their numbers and the potential for tissue injury by their toxic intracellular cargo if not recognized and removed, it is critical that these cells are cleared in a facile manner.

Lipid activators of G2A are not thought to be direct ligands but rather likely alter the oligomerization of the receptor (20, 53, 54), and there are significant and as yet unresolved discrepancies between investigations regarding the ability of various lipids to activate this receptor depending on species (human *versus* murine), end point used, and means of delivering lipid (20, 34, 55). Although this investigation cannot entirely rule out signaling from other described activators of G2A, their levels were either undetectable in apoptotic neutrophils (*i.e.* HETEs and HODEs) or did not change with neutrophil aging or inactivation of the neutrophil oxidase (*i.e.* lyso-PC), nor did their exogenous addition to macrophage cultures enhance efferocytosis (Fig. 3, *C–E*). By contrast, lyso-PS produced in both activated (15) and aged neutrophils was readily detectible and generated in a manner dependent on activation of the NADPH oxidase as would accompany their recruitment to tissue *in vivo*. As such, the data support that lyso-PS was the most likely endogenously produced activator of G2A signaling for high capacity neutrophil clearance (Fig. 2). We hypothesize that of the lyso-PS produced in aging or activated neutrophils only a fraction is likely presented on the cell surface where it and other so-called "tethering or ticking signals" (51) are highly efficient in enhancing efferocytosis. Much less efficient on a mole per mole basis was lyso-PS provided in liposomes added simultaneously with lyso-PS^{neg} UV-irradiated neutrophils to macrophage cultures. Furthermore, lyso-PS added on albumin was ineffective altogether (Fig. 3*D*). It is important to point out that the data support that lyso-PS signaling via G2A required other ligands presented on the apoptotic cell surface: in the most simplified system investigated here, carboxylate-modified beads were used to mimic apoptotic cells by presenting a PS head grouplike surface as a recognition signal (Figs. 3*H*, 5*B*, and 6*C*) (15, 39). In contrast, stimulating macrophages with lyso-PS alone did not activate the pathway intermediaries defined in this investigation of enhanced efferocytosis. For instance,

lyso-PS liposomes did not stimulate PGE_2 production or Rac1 activation (Figs. 6*C* and 4*A*) (and discussed below). We hypothesize that G2A activation and additional apoptotic cell recognition signals must be presented to macrophages in a carefully orchestrated context. Further elucidation of this co-signaling will undoubtedly require development of highly sensitive technologies and simplified manipulable model systems.

Enhancement of efferocytosis over base-line uptake in this investigation appeared to be mediated entirely by G2A on macrophages. In addition to antibody blockade studies, macrophages from $G2A^{-/-}$ mice were unresponsive to lyso-PS either endogenously generated in aged neutrophils or exogenously added in liposomes. Accordingly, $G2A^{-/-}$ macrophages were unable to propagate lyso-PS signaling for generation of distal signaling intermediaries (*e.g.* PGE_2 production or augmentation of Rac1 activation) but were fully functional for enhanced responses when provided these intermediaries downstream of the G2A receptor. We have demonstrated that G2A is present on inflammatory macrophage populations as well as resident populations and that signaling by lyso-PS enhanced efferocytosis by all macrophage populations studied to date (15). Thus, enhancement of efferocytosis by G2A signaling may be fairly ubiquitous among macrophages and serve to differentially augment various recognition pathways that are utilized by distinct macrophage populations (27). G2A is also widely expressed on other hematopoietic cells, including monocytes, dendritic cells, and neutrophils, but also endothelial cells and keratinocytes (56, 57). These observations suggest that G2A activators, such as lyso-PS presented on activated and apoptosing neutrophils, and other activators may signal in other contexts as well (Figs. 1 and 3) (20, 34, 37, 38).

An understanding of signaling downstream of G2A for enhanced efferocytosis was entirely unknown, and elucidation of this pathway was the impetus of this investigation. The data presented here support the pathway G2A → $cPLA_2$ → COX1 → PGE_2 → EP2 → adenylyl cyclase → PKA, which enhances Rac1. Several observations deserve further discussion. First, it has long been known that apoptotic cells stimulate the production of prostanoids (9, 10, 45). Enhanced production of PGE_2 but not other eicosanoids by macrophages in response to G2A activation by lyso-PS has been demonstrated under several conditions. G2A activation by lyso-PS on aged neutrophils resulted in ~380 ± 16 pg of PGE_2/5 × 10^5 macrophages, translating to 1.1 nM. At least half of this PGE_2 production required signaling from G2A as shown by blocking antibodies (Fig. 6*A*) and in $G2A^{-/-}$ resident peritoneal macrophages (Fig. 6*B*). Minimal signaling requirements were further defined using G2A-sufficient macrophages; lyso-PS provided along with apoptotic cell mimics (carboxylate-modified beads) resulted in PGE_2 production similar to that seen for aged neutrophils (although in a delayed time course) (Fig. 6*C*), suggesting sufficiency of these signals working together.

Interestingly, the data also demonstrate that activation of the distal adenylyl cyclase-driven pathway may be exquisitely dose- and/or context-dependent. Although PGE_2 was generated by macrophages co-cultured with lyso-PS^{neg} apoptotic cells to levels about 50% (~0.5 nM) of the levels seen in the presence of G2A activation by lyso-PS (either cell-associated or exogenously provided), the distal machinery in this circumstance did not appear to be functionally activated. In the absence of G2A activation, blockade of the EP2 receptor, adenylyl cyclase, or PKA had no effect on efferocytosis (Figs. 6*G* and 5, *C* and *D*, respectively). Furthermore, the uptake of lyso-PS^{neg} UV-irradiated cells was unaffected by inhibition or absence of $cPLA_2$ in the macrophages, whereas G2A-mediated enhancement by stimulation with lyso-PS under these conditions was completely abrogated (Fig. 7, *A* and *B*). Dose dependence was also demonstrated for exogenously supplied PGE_2 and db-cAMP; low doses, mimicking those generated in response to G2A activation, likewise enhanced efferocytosis (Figs. 6*D* and 5*A*, respectively) and were able to bypass loss of G2A signaling in both the $G2A^{-/-}$ and $cPLA_2^{-/-}$ macrophages (Figs. 6*E* and 7*B*). Conversely, higher doses of PGE_2 or db-cAMP suppressed efferocytosis in keeping with previous reports (43, 45). Stimulation of G2A with lyso-PS, under the conditions investigated thus far, has not inhibited responses in macrophages even with pretreatment of up to several hours (15). Determinants temporally or spatially controlling G2A-mediated prostanoid production and signaling or adenylyl cyclase and PKA activation and/or impinging signaling from other pathways activated by G2A have yet to be elucidated.

The molecular mechanism of efferocytosis involves reorganization of the actin cytoskeleton to produce membrane ruffles, allowing for engulfment of the target in a process that differs from phagocytosis of opsonized particles and more closely resembles macropinocytosis (58). This process ultimately requires activation of the Rho family GTPase Rac1 (18, 59). In a variety of settings, RhoA activation negatively regulates efferocytosis (40, 60–62). Furthermore, it has been demonstrated that both Rac1 and RhoA activity can be mutually antagonistic (40, 60, 63). The data presented here demonstrate that G2A activation by endogenously produced or exogenously provided activators, such as lyso-PS, along with lyso-PS^{neg} apoptotic cells significantly enhanced Rac1 activation via the G2A-mediated signaling pathway. The molecular mechanism(s) by which Rac1 activity is enhanced is under current investigation.

G2A activation signals, such as lyso-PS, join other signals, such as adenosine, as activators of adenylyl cyclase with potential for generation of "proresolution" macrophages (41). Unlike these other stimuli, we have only detected lyso-PS in/on neutrophils and not in supernatants (data not shown; Ref. 15). Thus, we hypothesize that its effects are focused on macrophages that are in direct contact with the neutrophils. This requirement for cell-cell contact may confine this early signal, allowing for a carefully titrated host response, *e.g.* as pathogens are contained. Furthermore, G2A activation and other early signals elevating PGE_2 and macrophage levels of cAMP may contribute to the production of a "second wave" of enhancers of apoptotic cell clearance, such as the lipoxins, resolvins, and protectins (64). Notably, they appear later in inflammation than lyso-PS, and their generation appears to be induced by PGE_2/cAMP. These proresolution lipids are released into the milieu as disseminated anti-inflammatory signals. Finally, using various types of apoptotic cells, it has been shown that their stimulation of alveolar macrophages leads to generation of PGE_2 and cAMP and PKA activation, resulting in macrophage deac-

tivation and suppression of host defense to subsequent bacterial infection (45). We suggest that the effects are concentration- and context-dependent. Such findings underscore the critical importance of elucidating signaling by apoptotic cells and responses of macrophages that may be either helpful or harmful to the host depending entirely on context.

To date, investigation of lyso-PS generation and signaling *in vivo* has been limited to the zymosan-induced peritonitis model (15). In that system, lyso-PS production in neutrophils increased and peaked at a time immediately preceding their rapid clearance, and apoptotic neutrophils were infrequent (Fig. 1*F* and Ref. 15), supporting the hypothesis that in resolving inflammation neutrophil removal is highly efficient. Blockade of G2A signaling with antibodies administered at peak neutrophil accumulation delayed their removal and led to a significant increase in the numbers of apoptotic neutrophils (15). These findings were also evident in $G2A^{-/-}$ mice (Fig. 1*F*). We hypothesize that the loss of G2A signaling contributes to the accumulation of apoptotic cells, a feature generally indicative of defects in clearance found in many models of chronic inflammation and autoimmunity (15, 30, 42, 53, 65–67). We also speculate that dysregulation at any of these various steps of the G2A signaling pathway defined here (*e.g.* G-protein-coupled receptor desensitization or inactivation of the NADPH oxidase) may contribute to exaggerated neutrophilia and chronicity of inflammation.

Acknowledgments—We thank Dr. Robert Barkley and Chris Johnson for mass spectrometry analysis of eicosanoids and oxidized fatty acids and Saritha Suram for technical assistance.

REFERENCES

1. deCathelineau, A. M., and Henson, P. M. (2003) *Essays Biochem.* **39,** 105–117
2. Mevorach, D., Mascarenhas, J. O., Gershov, D., and Elkon, K. B. (1998) *J. Exp. Med.* **188,** 2313–2320
3. Taylor, P. R., Carugati, A., Fadok, V. A., Cook, H. T., Andrews, M., Carroll, M. C., Savill, J. S., Henson, P. M., Botto, M., and Walport, M. J. (2000) *J. Exp. Med.* **192,** 359–366
4. Fadok, V. A., Bratton, D. L., Guthrie, L., and Henson, P. M. (2001) *J. Immunol.* **166,** 6847–6854
5. Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) *Nature* **417,** 182–187
6. Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., and Nagata, S. (2007) *Nature* **450,** 435–439
7. Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., Haney, L. B., Ma, Z., Klibanov, A. L., Mandell, J. W., and Ravichandran, K. S. (2007) *Nature* **450,** 430–434
8. Park, S. Y., Jung, M. Y., Kim, H. J., Lee, S. J., Kim, S. Y., Lee, B. H., Kwon, T. H., Park, R. W., and Kim, I. S. (2008) *Cell Death Differ.* **15,** 192–201
9. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998) *J. Clin. Investig.* **101,** 890–898
10. Freire-de-Lima, C. G., Xiao, Y. Q., Gardai, S. J., Bratton, D. L., Schiemann, W. P., and Henson, P. M. (2006) *J. Biol. Chem.* **281,** 38376–38384
11. Arroyo, A., Modrianský, M., Serinkan, F. B., Bello, R. I., Matsura, T., Jiang, J., Tyurin, V. A., Tyurina, Y. Y., Fadeel, B., and Kagan, V. E. (2002) *J. Biol. Chem.* **277,** 49965–49975
12. Greenberg, M. E., Sun, M., Zhang, R., Febbraio, M., Silverstein, R., and Hazen, S. L. (2006) *J. Exp. Med.* **203,** 2613–2625
13. Kagan, V. E., Gleiss, B., Tyurina, Y. Y., Tyurin, V. A., Elenström-Magnusson, C., Liu, S. X., Serinkan, F. B., Arroyo, A., Chandra, J., Orrenius, S., and Fadeel, B. (2002) *J. Immunol.* **169,** 487–499
14. Savill, J., Hogg, N., and Haslett, C. (1991) *Chest* **99,** 6S–7S
15. Frasch, S. C., Berry, K. Z., Fernandez-Boyanapalli, R., Jin, H. S., Leslie, C., Henson, P. M., Murphy, R. C., and Bratton, D. L. (2008) *J. Biol. Chem.* **283,** 33736–33749
16. Albert, M. L., Kim, J. I., and Birge, R. B. (2000) *Nat. Cell Biol.* **2,** 899–905
17. Reddien, P. W., and Horvitz, H. R. (2000) *Nat. Cell Biol.* **2,** 131–136
18. Tosello-Trampont, A. C., Brugnera, E., and Ravichandran, K. S. (2001) *J. Biol. Chem.* **276,** 13797–13802
19. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) *Nature* **390,** 622–625
20. Frasch, S. C., Zemski-Berry, K., Murphy, R. C., Borregaard, N., Henson, P. M., and Bratton, D. L. (2007) *J. Immunol.* **178,** 6540–6548
21. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497–509
22. Pulfer, M., and Murphy, R. C. (2003) *Mass Spectrom. Rev.* **22,** 332–364
23. Hall, L. M., and Murphy, R. C. (1998) *J. Am. Soc. Mass Spectrom.* **9,** 527–532
24. Gijón, M. A., Zarini, S., and Murphy, R. C. (2007) *J. Lipid Res.* **48,** 716–725
25. Suram, S., Gangelhoff, T. A., Taylor, P. R., Rosas, M., Brown, G. D., Bonventre, J. V., Akira, S., Uematsu, S., Williams, D. L., Murphy, R. C., and Leslie, C. C. (2010) *J. Biol. Chem.* **285,** 30676–30685
26. Haslett, C., Guthrie, L. A., Kopaniak, M. M., Johnston, R. B., Jr., and Henson, P. M. (1985) *Am. J. Pathol.* **119,** 101–110
27. Fadok, V. A., Savill, J. S., Haslett, C., Bratton, D. L., Doherty, D. E., Campbell, P. A., and Henson, P. M. (1992) *J. Immunol.* **149,** 4029–4035
28. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) *J. Immunol.* **148,** 2207–2216
29. Yan, J. J., Jung, J. S., Lee, J. E., Lee, J., Huh, S. O., Kim, H. S., Jung, K. C., Cho, J. Y., Nam, J. S., Suh, H. W., Kim, Y. H., and Song, D. K. (2004) *Nat. Med.* **10,** 161–167
30. Fernandez-Boyanapalli, R., Frasch, S. C., Riches, D. W., Vandivier, R. W., Henson, P. M., and Bratton, D. L. (2010) *Blood* **116,** 4512–4522
31. Coxon, A., Rieu, P., Barkalow, F. J., Askari, S., Sharpe, A. H., von Andrian, U. H., Arnaout, M. A., and Mayadas, T. N. (1996) *Immunity* **5,** 653–666
32. Kasahara, Y., Iwai, K., Yachie, A., Ohta, K., Konno, A., Seki, H., Miyawaki, T., and Taniguchi, N. (1997) *Blood* **89,** 1748–1753
33. Obinata, H., Hattori, T., Nakane, S., Tatei, K., and Izumi, T. (2005) *J. Biol. Chem.* **280,** 40676–40683
34. Obinata, H., and Izumi, T. (2009) *Prostaglandins Other Lipid Mediat.* **89,** 66–72
35. Chang, M. K., Bergmark, C., Laurila, A., Hörkkö, S., Han, K. H., Friedman, P., Dennis, E. A., and Witztum, J. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 6353–6358
36. Chang, M. K., Binder, C. J., Miller, Y. I., Subbanagounder, G., Silverman, G. J., Berliner, J. A., and Witztum, J. L. (2004) *J. Exp. Med.* **200,** 1359–1370
37. Peter, C., Waibel, M., Radu, C. G., Yang, L. V., Witte, O. N., Schulze-Osthoff, K., Wesselborg, S., and Lauber, K. (2008) *J. Biol. Chem.* **283,** 5296–5305
38. Yang, L. V., Radu, C. G., Wang, L., Riedinger, M., and Witte, O. N. (2005) *Blood* **105,** 1127–1134
39. Kiss, R. S., Elliott, M. R., Ma, Z., Marcel, Y. L., and Ravichandran, K. S. (2006) *Curr. Biol.* **16,** 2252–2258
40. Nakaya, M., Tanaka, M., Okabe, Y., Hanayama, R., and Nagata, S. (2006) *J. Biol. Chem.* **281,** 8836–8842
41. Bystrom, J., Evans, I., Newson, J., Stables, M., Toor, I., van Rooijen, N., Crawford, M., Colville-Nash, P., Farrow, S., and Gilroy, D. W. (2008) *Blood* **112,** 4117–4127
42. Rajakariar, R., Newson, J., Jackson, E. K., Sawmynaden, P., Smith, A., Rahman, F., Yaqoob, M. M., and Gilroy, D. W. (2009) *J. Immunol.* **182,** 3262–3269
43. Rossi, A. G., McCutcheon, J. C., Roy, N., Chilvers, E. R., Haslett, C., and Dransfield, I. (1998) *J. Immunol.* **160,** 3562–3568
44. Aronoff, D. M., Canetti, C., Serezani, C. H., Luo, M., and Peters-Golden, M. (2005) *J. Immunol.* **174,** 595–599
45. Medeiros, A. I., Serezani, C. H., Lee, S. P., and Peters-Golden, M. (2009) *J. Exp. Med.* **206,** 61–68
46. Sugimoto, Y., and Narumiya, S. (2007) *J. Biol. Chem.* **282,** 11613–11617
47. Aronoff, D. M., Peres, C. M., Serezani, C. H., Ballinger, M. N., Carstens,

J. K., Coleman, N., Moore, B. B., Peebles, R. S., Faccioli, L. H., and Peters-Golden, M. (2007) *J. Immunol.* **178,** 1628–1634
48. Ikegami, R., Sugimoto, Y., Segi, E., Katsuyama, M., Karahashi, H., Amano, F., Maruyama, T., Yamane, H., Tsuchiya, S., and Ichikawa, A. (2001) *J. Immunol.* **166,** 4689–4696
49. Hara, S., Kamei, D., Sasaki, Y., Tanemoto, A., Nakatani, Y., and Murakami, M. (2010) *Biochimie* **92,** 651–659
50. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) *Annu. Rev. Biochem.* **69,** 145–182
51. Henson, P. M., and Bratton, D. L. (2009) in *Phagocyte-Pathogen Interactions: Macrophages and the Host Responses to Infection* (Russel, D. G., and Gordon, S., eds) pp. 341–365, ASM Press, Washington, D. C.
52. Nathan, C. (2006) *Nat. Rev. Immunol.* **6,** 173–182
53. Kabarowski, J. H. (2009) *Prostaglandins Other Lipid Mediat.* **89,** 73–81
54. Wang, L., Radu, C. G., Yang, L. V., Bentolila, L. A., Riedinger, M., and Witte, O. N. (2005) *Mol. Biol. Cell* **16,** 2234–2247
55. Yin, H., Chu, A., Li, W., Wang, B., Shelton, F., Otero, F., Nguyen, D. G., Caldwell, J. S., and Chen, Y. A. (2009) *J. Biol. Chem.* **284,** 12328–12338
56. Bolick, D. T., Whetzel, A. M., Skaflen, M., Deem, T. L., Lee, J., and Hedrick, C. C. (2007) *Circ. Res.* **100,** 572–580
57. Hattori, T., Obinata, H., Ogawa, A., Kishi, M., Tatei, K., Ishikawa, O., and Izumi, T. (2008) *J. Invest. Dermatol.* **128,** 1123–1133
58. Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A., and Henson, P. M. (2001) *J. Exp. Med.* **194,** 781–795
59. Nakaya, M., Kitano, M., Matsuda, M., and Nagata, S. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 9198–9203
60. Tosello-Trampont, A. C., Nakada-Tsukui, K., and Ravichandran, K. S. (2003) *J. Biol. Chem.* **278,** 49911–49919
61. Richens, T. R., Linderman, D. J., Horstmann, S. A., Lambert, C., Xiao, Y. Q., Keith, R. L., Boé, D. M., Morimoto, K., Bowler, R. P., Day, B. J., Janssen, W. J., Henson, P. M., and Vandivier, R. W. (2009) *Am. J. Respir. Crit. Care Med.* **179,** 1011–1021
62. Morimoto, K., Janssen, W. J., Fessler, M. B., McPhillips, K. A., Borges, V. M., Bowler, R. P., Xiao, Y. Q., Kench, J. A., Henson, P. M., and Vandivier, R. W. (2006) *J. Immunol.* **176,** 7657–7665
63. Kinchen, J. M., and Ravichandran, K. S. (2007) *J. Cell Sci.* **120,** 2143–2149
64. Serhan, C. N., and Savill, J. (2005) *Nat. Immunol.* **6,** 1191–1197
65. Fernandez-Boyanapalli, R. F., Frasch, S. C., McPhillips, K., Vandivier, R. W., Harry, B. L., Riches, D. W., Henson, P. M., and Bratton, D. L. (2009) *Blood* **113,** 2047–2055
66. Le, L. Q., Kabarowski, J. H., Weng, Z., Satterthwaite, A. B., Harvill, E. T., Jensen, E. R., Miller, J. F., and Witte, O. N. (2001) *Immunity* **14,** 561–571
67. Sanford, A. N., Suriano, A. R., Herche, D., Dietzmann, K., and Sullivan, K. E. (2006) *Rheumatology* **45,** 178–181