Neutrophils regulate tissue neutrophilia in inflammation via the oxidant modified lipid lysophosphatidylserine*

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Background: NADPH oxidase-generated lysoPS enhances efferocytosis by macrophages.

Results: Recruited, viable lysoPShigh neutrophils are readily cleared by macrophages in vitro and in vivo via G2A signaling. LysoPShigh neutrophils also program macrophages to a highly efferocytic, resolving state.

Conclusion: LysoPS in/on exudate neutrophils orchestrates early removal of neutrophils and subsequent macrophage programming.

Significance: Neutrophil-generated lysoPS regulates tissue neutrophilia of acute inflammation.

SUMMARY

Resolution of neutrophilia characteristic of acute inflammation requires i) cessation of neutrophil recruitment, and ii) removal of tissue neutrophils. Based on in vitro studies, a role in these events was hypothesized for oxidant-generated lysophosphatidylserine (lysoPS) on recruited neutrophils signaling via the G2A receptor on macrophages. Peritoneal exudate neutrophils harvested from wild type (WT) mice had 5-fold more lysoPS (lysoPS high) than those of gp91phox-/- (lysoPS low) mice. Ex vivo engulfment of lysoPS high neutrophils (95% viable) by WT peritoneal macrophages was quantitatively similar to UV-irradiated apoptotic blood neutrophils, though the signaling pathway for the former was uniquely dependent on macrophage G2A. In contrast, lysoPS low neutrophils were poorly engulfed unless presented with exogenous lysoPS. Enhanced clearance of lysoPS high neutrophils was also seen in vivo following their adoptive transfer into inflamed peritonea of WT, but not G2A-/- mice, further supporting a requirement for signaling via G2A. To investigate downstream effects of lysoPS/G2A signaling, antibody blockade of G2A in WT mice reduced macrophage CD206 expression and efferocytosis during peritonitis. Conversely, adoptive transfer of lysoPS high neutrophils early in inflammation in gp91phox-/- mice led to accelerated development of efferocytic high, CD206 high macrophages. This macrophage reprogramming was associated with suppressed production of pro-inflammatory mediators and reduced neutrophilia. These effects were not seen if G2A was blocked or lysoPS low neutrophils were transferred. Taken together, the results demonstrate that oxidant-generated
lysoPS made by viable tissue neutrophils is an endogenous anti-inflammatory mediator working in vivo to orchestrate the “early” and rapid clearance of recruited neutrophils as well as the reprogramming of “resolving” macrophages.

Acute inflammation results in brisk neutrophil recruitment with a time course and robustness dependent on the nature of the insult and production of danger signals and neutrophil chemoattractants. When the danger is passed, e.g. microbes neutralized, signals for neutrophil recruitment subside abruptly curtailing further recruitment. Clearance of recruited neutrophils is then essential to return tissues to their normal function, and clearance must be timely before these short-lived cells disintegrate and spill their phlogistic contents (1,2). The “turn off” of neutrophil recruitment and the “turn on” of neutrophil removal are often simplistically represented as sequential processes, but, in fact, occur simultaneously. Indeed, the critical accumulation of neutrophils in tissues at any point in time is the net sum of both the processes of recruitment and removal (3).

Signals for suppression of recruitment and for neutrophil removal, are poorly understood, but are likely highly orchestrated. The removal of dying cells by macrophages in a process known as efferocytosis (4) is touted as key to both. The paradigm holds that apoptotic neutrophils are phagocytosed, and the process is actively anti-inflammatory with the production of factors such as PGE2, IL-10 and TGFβ by macrophages that in turn, suppress the production of pro-inflammatory mediators driving neutrophil recruitment (5,6). Efferocytosis is generally highly efficient such that apoptotic neutrophils and other cells rarely accumulate in the absence of defects in the process (7,8). Conversely, dysregulation of efferocytosis is often associated with chronic inflammation and autoimmunity (9,10).

Neutrophil-generated oxidants, in addition to microbial killing and inciting inflammation, may also play somewhat paradoxical anti-inflammatory roles (11,12). Indeed, deficient functioning of the NADPH oxidase is associated with exaggerated inflammation in human and murine models of Chronic Granulomatous Disease (CGD) (13-15). We have recently shown that lysophosphatidylserine (lysoPS) made in a NADPH oxidase-dependent manner in activated, live as well as aged (apoptotic) neutrophils signals to macrophages via the G-protein coupled receptor G2A for enhanced clearance in vitro (16,17). As such, we hypothesized that lysoPS-driven clearance would be a key mechanism for control of neutrophil numbers in vivo during acute inflammation. Using neutrophils recruited to the peritoneum in a well-characterized model of sterile peritonitis, it was shown that lysoPS, a downstream product of the NADPH oxidative burst: i) drives the “early” recognition and clearance of viable, nonapoptotic neutrophils by macrophages via the process of efferocytosis, ii) reprograms macrophages to a “resolving” state, and iii) suppresses production of pro-inflammatory cytokines including those implicated in neutrophil recruitment. These findings support the hypothesis that lysoPS plays a pivotal role in calibration of tissue neutrophilia.

EXPERIMENTAL PROCEDURES

Materials - All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Amiloride was from Sigma Aldrich (St. Louis, MO). Zymosan, Annexin V Alexa 488 and anti-F4/80 were from Invitrogen Life Sciences (Grand Island, NY). Anti-G2A M-20 and normal goat IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Ly6G (1A8), anti-CD115, anti-CD45.1, anti-CD45.2, isotype control antibodies, and cell proliferation dye eFluor® 450 (PBSE) were from eBioscience (San Diego, CA). Anti-CD16/32 (Fc-block) was from BD Biosciences (San Jose, CA). Anti-CD206 was from Biolegend (San Diego, CA). DeadEnd™ Colorimetric TUNEL system was from Promega (Madison, WI). IL-6 and KC ELISAs were from ElisaTech (Aurora, CO).

Animals - Male and female C57BL/6 (WT CD45.2), B6.SJL-PtprcaPep3b/BoyJ (WT CD45.1) and gp91phox-/- mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were generated from a breeding colony at National Jewish Health (Denver, Colorado). G2A-/- mice were a generous gift from Dr. Katherine Hedrick (University of Virginia) and were bred in house. 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 12 weeks were used for in vitro and in vivo experiments and were age and gender matched for all experiments.

Induction and assessment of sterile peritonitis - Mice were injected intraperitoneally (i.p.) with 1 mg zymosan (in 1 ml PBS) and peritoneal cells were harvested by lavage with ice cold, sterile Hank’s 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 cell differentials and absolute numbers. Efferocytosis was determined as described below, and apoptotic cells were determined by visual inspection of morphology or by TUNEL staining. Cells were also stained and analyzed by flow cytometry as described below. Cell free lavage supernatants were analyzed where indicated for pro-inflammatory cytokines. In some cases, exudate neutrophils were first labeled with 10 µM PBSE according to the manufacturers instructions. In experiments to test effects of adoptively transferred neutrophils on downstream macrophage programming, exudate neutrophils were resuspended at 2x10⁷ cells/ml in PBS and 0.5 ml was injected i.p. into recipient mice at the indicated times.

Lysosphosphatidylserine vesicle preparation and loading of neutrophils - 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) small unilamellar vesicles (SUVs) containing 1-oleoyl-2-hydroxy-sn-glycero-3-phosphoserine (lysoPS) at 30 mole percent were prepared as described previously for in vitro phagocytosis assays (16,17). For in vivo phagocytosis assays, POPC and lysoPS (10 mg/kg at 30 mole percent) were evaporated to dryness under nitrogen and resuspended in 0.5 ml PBS by vigorous vortexing. SUVs were then created by sonication in a water bath sonicator and mixed with exudate neutrophils for 30 minutes prior to adoptive transfer. Under these conditions, approximately 20% of the lysoPS was incorporated into the neutrophils as measured by LC/MS/MS.

In vitro phagocytosis assays - Resident peritoneal (RP) MΦ were used for in vitro phagocytosis assays. RPMΦ were isolated from mice using 5 mL of sterile Hank’s balanced salt solution (Cellgro; Kansas City, MO) supplemented with 10 mM HEPES (pH 7.2) and 1 mM EDTA to lavage the peritoneum following euthanization with CO₂. Resident peritoneal cells were collected, centrifuged at 1,000 rpm for 10 minutes at 4°C, and plated at 4x10⁵ cells/well in a 24-well tissue culture plate in DMEM supplemented with 10% heat-inactivated FBS (Atlanta Biologicals; Lawrenceville, GA), 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin. Macrophages were allowed to adhere for 2 hours at 37°C in a 10% CO₂ humidified incubator at which time non-adherent cells were removed and macrophages were cultured for an additional 48 h before use in phagocytosis assays.

Apoptotic human neutrophils were also prepared as targets for in vitro phagocytosis. 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 (18). The neutrophils were suspended at 5×10^6/ml in a HEPES buffer (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl_2, 5 mM glucose, 1 mM CaCl_2, 10 mM HEPES (pH 7.4)) supplemented with 0.05% fatty acid-free bovine serum albumin and UV-irradiated for 5 minutes on a trans-illuminator followed by incubation at 37°C for 2 h. Under these conditions UV-irradiated neutrophils were greater than 50% apoptotic as determined by nuclear morphology and Annexin V staining, and >95% propidium iodide negative as determined by flow cytometry. Though isolated human neutrophils have variable amounts of lysoPS at baseline, their lysoPS content does not change during UV irradiation-induced apoptosis, and signaling via G2A has not been demonstrated (16,17).

For these in vitro studies, target cells (2×10^6 per well), either murine exudate neutrophils or UV-irradiated apoptotic human neutrophils, with or without liposomes (100 nmoles total lipid), in 100 µl DMEM supplemented with 10% FBS were added simultaneously to macrophages. Prior studies have shown that lysoPS liposomes added either prior to or simultaneously with UV-irradiated apoptotic Jurkat cells or carboxylate-modified beads (apoptotic cell mimics) were equally effective at enhancing engulfment by macrophages (16,17). For antibody blocking experiments, 10 µg/ml anti-G2A or isotype control antibody was added for 30 minutes before target cells and liposomes were added. The macrophages and target cells were co-cultured for 60 min at 37°C in 10% CO2, washed three times with PBS, and stained with a modified Wright’s Giemsa stain (Fisher Scientific, Pittsburgh, PA). The phagocytic index (PI) was calculated by multiplying the percentage of MΦ that have phagocytosed one or more cells by the average number of engulfed cells per MΦ (17,19). A minimum of 200 MΦ were counted blindly. Each condition was tested in duplicate using at least four mice per experiment and repeated 3-10 times as indicated.

Assay of in vivo phagocytosis – Peritoneal cells were collected by lavage at indicated time points and phagocytosis was determined in a blinded fashion as above by visual inspection of cytopsin slides stained with either a modified Wright’s Giemsa stain or TUNEL staining (below). Where in vivo engulfment of adoptively transferred exudate neutrophils was assessed, exudate neutrophils were prepared as above and uptake assessed by visual inspection and blinded scoring of cytopsin slides. In some experiments, uptake of PSBE-labeled exudate neutrophils by recipient macrophages, defined as CD45.1+/Ly6G-/F4/80+, was determined by flow cytometry. Time course experiments demonstrated that ingestions of adoptively transferred neutrophils were detectible by 1 hour of dwell time in recipient mice, but that engulfment was highly reproducible following a 4 h dwell time in vivo (data not shown). Four hours was chosen for further experiments.

Peritoneal cell surface staining and flow cytometry - Peritoneal cells were suspended in PBS supplemented with 3% FBS at 1×10^7 cells/ml. 0.5×10^6 cells (50 µl) were incubated with Fc-block for 1 h on ice. Fifty µl of 2X Antibody solution (anti-F4/80, anti-CD115, anti-CD206 (or isotype) and anti-Ly6G) in PBS plus 3% FBS was added to cells for a final volume of 100 µl and incubated in the dark on ice for 1 h. Cells were washed twice with PBS plus 3% FBS and analyzed by flow cytometry. Neutrophils were defined as Ly6G+/F4/80+/CD115+ cells. CD206 expression was determined on monocytes/macrophages defined as Ly6G+/F4/80-/CD115+ cells. CD206 expression was determined on monocytes/macrophages defined as Ly6G+/F4/80+/CD115+. CD206 mean fluorescence intensity (MFI) was determined by dividing the geometric mean fluorescence of CD206 staining by the geometric mean fluorescence of its own isotype control staining and was expressed as fold over isotype control.

Cytokine measurement - IL-6 and KC were measured by ELISA from cell free lavage supernatants according to the manufacturer’s instructions.

TUNEL staining - Colorimetric TUNEL staining was performed on cytopsin slides according to the manufacturer’s instructions.

Statistical analyses and p-value calculations were conducted using ANOVA [JMP statistical program (SAS Institute, Cary, NC)]. The Dunnett’s and Tukey-Kramer tests were used for single and multiple comparisons respectively.
RESULTS

LysoPS generated by viable exudate neutrophils drives clearance by macrophages in a G2A dependent process - Previous investigation has shown that blood neutrophils activated in vitro can be recognized and removed by macrophages and that lysoPS can play a role in this process. Accordingly studies were initiated to explore the concept that lysoPS generated in recruited neutrophils as a consequence of NADPH oxidase activation leads to an increase in “palatability” for uptake of these viable cells by macrophages. Neutrophils isolated from an acute inflammatory reaction were first characterized for their lysoPS content as well as their viability in preparation for assessing their ability to be cleared in vitro or when re-instilled into an inflammatory reaction in vivo. Since neutrophils lacking the NADPH oxidase are deficient in lysoPS generation, these were compared to wild-type cells in this analysis (16). C57BL/6 and gp91phox-/- mice were injected with zymosan, and cells lavaged from inflamed peritonea 6-10 h later. Cell differentials showed that 89% ± 2% (mean ± S.E.) of the inflammatory cells were intact neutrophils, and that recovery was equivalent during this time frame for both genotypes (Fig. 1A). Only 6% of exudate neutrophils from either genotype contained visible zymosan. Evidence of apoptosis was rare: 2.9% for wild type and 3.1% for gp91phox-/- as determined by morphology and 6% for wild type and 8% for gp91phox-/- as determined by annexin V staining (Fig. 1A). Analysis for the presence of lysoPS by LC/MS/MS showed (as previously published; (16)) that wild type exudate neutrophils had 5 times more lysoPS (lysoPS high) than gp91phox-/- (lysoPS low) exudate neutrophils with approximately 90% the 18:0 and 10% the 18:1 species. As both are thought to signal via G2A (20,21), their summated concentration in the wild type neutrophils was 3.1 µM based on an average cellular volume of 330 fl per neutrophil (22) (Fig 1B). Lysophosphatidylcholine levels did not differ between wild type and gp91phox-/- neutrophils (data not shown).

To address the hypothesis that lysoPS comprises a significant clearance signal on emigrated neutrophils, the harvested exudate neutrophils were first co-cultured with resident peritoneal macrophages. After 1 h, cultures were washed and macrophages assessed for phagocytosis of neutrophils. While ingestions of exudate lysoPS<sub>high</sub> wild type neutrophils were readily visible, engulfment of exudate lysoPS<sub>low</sub> gp91phox-/- neutrophils were significantly fewer (Fig. 2A). As a reference comparison, uptake of apoptotic neutrophils was determined. Human neutrophils isolated from peripheral blood and induced to undergo apoptosis by UV irradiation (approximately 60-80% positive by annexin V staining and less than 5% propidium iodide positive), were also co-cultured with the macrophages. Quantitatively, lysoPS<sub>high</sub> (and non-apoptotic) wild type exudate neutrophils were only slightly less “palatable” than apoptotic human neutrophils.

Next, signaling for the apparent palatability of exudate neutrophils was investigated. Previous studies had demonstrated a role for lysoPS signaling via the G2A receptor on macrophages (16,17), and thus, blocking antibodies directed against G2A and macrophages from mice genetically deficient in G2A were employed. As shown in Fig. 2B, treatment of peritoneal macrophages with anti-G2A, but not isotype antibodies, significantly blocked the ingestion of lysoPS<sub>high</sub> exudate neutrophils to levels seen for gp91phox-/- neutrophils and had no effect on the minimal uptake seen with the gp91 phox-/- cells. UV-irradiated apoptotic human neutrophils do not make lysoPS (Methods), and have been shown not to signal via G2A (16,17). As expected, anti-G2A antibody did not reduce their removal. Likewise, when presented to peritoneal macrophages from G2A<sup>−/−</sup> mice, engulfment of lysoPS<sub>high</sub> exudate neutrophils, but not the apoptotic cells, was markedly reduced (Fig. 2C).

We have previously shown that lysoPS is not released from the activated neutrophil and have suggested that it is presented on its surface (16). Accordingly, gp91phox-/- exudate neutrophils were presented with lysoPS containing liposomes (30 mole %) before testing their palatability to macrophages (16,17). As shown in Fig. 2D, lysoPS liposomes fully restored the palatability of gp91phox-/- exudate neutrophils to levels of wild type exudate neutrophils. Similar addition of exogenous lysoPS to wild type neutrophils did not further enhance their clearance suggesting that signaling by endogenous lysoPS in/on these exudate neutrophils was already maximal.

Engulfment of apoptotic cells (efferocytosis)
has been shown to be a process akin to macropinocytosis and dependent on the Na/H+ antiporter (23,24). To determine whether lysoPS-mediated engulfment occurred by a similar process, engulfment assays in the presence of amiloride to block the antiporter were conducted. Amiloride suppressed the uptake of both viable exudate neutrophils as well as apoptotic neutrophils but had no effect on macrophage phagocytosis of killed opsonized Candida albicans (data not shown). Thus, engulfment driven by lysoPS appears to converge with efferocytosis driven by other signals on apoptotic cells (25). Together, these data demonstrate that i) viable exudate neutrophils are rendered “palatable” to macrophages likely via a macropinocytotic mechanism, and ii) this palatability is driven by NADPH oxidase-derived lysoPS on the neutrophil signaling to G2A on the macrophage.

LysoPS drives “early” neutrophil clearance in vivo - To examine palatability of emigrated neutrophils in vivo, lysoPS$^{\text{high}}$ or lysoPS$^{\text{low}}$ exudate neutrophils from wild type or gp91phox$^{-/-}$ mice, respectively, were harvested, washed, pre-mixed with lysoPS liposomes (Methods) or not, and instilled into inflamed peritonea of C57BL/6 or G2A$^{-/-}$ mice 5 days after peritonitis induction with zymosan. Four days was chosen because endogenous neutrophils are largely cleared from inflamed peritonea in both recipient genotypes at this time. Four hours after instillation, peritoneal cells were lavaged and analyzed. The numbers of recovered macrophages from mice receiving exudate neutrophils did not differ in comparison to mice injected with only PBS. Ingestions of neutrophils were investigated on cytospins. As shown in Fig. 3A, lysoPS$^{\text{high}}$ exudate neutrophils from wild type mice were ingested at significantly greater frequency than exudate neutrophils from gp91phox$^{-/-}$ mice. As in the in vitro experiments, pre-mixing of gp91phox$^{-/-}$ neutrophils with lysoPS restored their in vivo palatability to that of wild type lysoPS$^{\text{high}}$ neutrophils. Conversely, few exudate neutrophils of either genotype were engulfed following instillation into peritonea of G2A$^{-/-}$ mice (Fig. 3B). Further, as in the in vitro experiments, pre-mixing of neutrophils with exogenous lysoPS liposomes did not enhance the clearance of either wild type or gp91phox$^{-/-}$ exudate neutrophils in the inflamed peritonea of G2A$^{-/-}$ mice.

Because of the 4-hour dwell time of the instilled neutrophils in the peritonea for these experiments, cytopsins of the harvested cells from recipient mice were stained to identify whether the cells had become apoptotic. TUNEL positive free neutrophils were rarely seen regardless of donor genotype, and with or without pre-mixing with lysoPS liposomes (Fig. 3C). In contrast, quantification of TUNEL positive neutrophils inside the macrophages replicated the ingestion data generated by visual inspection. Thus, TUNEL positive phagosomes were seen with significantly greater frequency following the transfer of lysoPS$^{\text{high}}$ vs. lysoPS$^{\text{low}}$ neutrophils (Fig. 3D and E). Importantly, when gp91phox$^{-/-}$ neutrophils were pre-mixed with lysoPS liposomes, TUNEL positive ingestions equaled that for lysoPS$^{\text{high}}$ wild type neutrophils. These data support the hypothesis that lysoPS is a major “eat me” signal for clearance of viable emigrated neutrophils in vivo, and suggest that neutrophil apoptosis as detectable by TUNEL staining occurs within the phagosome after uptake (Discussion) (26,27).

The clearance of instilled exudate neutrophils during earlier stages of the inflammatory process was also investigated. For these experiments, wild type CD45.1 mice were used as recipients at 18 h post zymosan, near the peak of endogenous neutrophil accumulation in the peritoneum (Fig. 4A). Wild type or gp91phox$^{-/-}$ CD45.2 neutrophils were labeled with PBSE, adoptively transferred, and their ingestion by CD45.1$^{+/+}$/F480$^{+/+}$/Ly6G$^{-/-}$ recipient macrophages assessed following 4 h of dwell time in the peritonea. As shown in Fig. 4B, PBSE$^{+}$ recipient macrophages were twice as frequent when lysoPS$^{\text{high}}$ wild type exudate neutrophils were instilled in comparison to lysoPS$^{\text{low}}$, gp91phox$^{-/-}$ exudate neutrophils; 7% vs. 3.5% of recipient macrophages, respectively. Pre-mixing the neutrophils with lysoPS prior to transfer normalized the engulfment of gp91phox$^{-/-}$ neutrophils to that of wild type, and slightly enhanced that of wild type neutrophils. These same differences in palatability detected by flow cytometry, both at baseline and following pre-mixing with lysoPS, were also confirmed on blinded scoring of cytopsins by visual inspection (Fig. 4C).

Given that G2A is expressed on both resident and recruited macrophages (16), we asked whether
blockade of endogenous lysoPS signaling would diminish neutrophil removal and delay resolution of neutrophilia in the inflammatory reaction. Blocking antibody to G2A (or isotype control antibody) was administered to wild type mice 18 h after induction of peritonitis with zymosan and neutrophil clearance investigated 30 h later. As predicted, neutrophil ingestions by macrophages were significantly decreased following anti-G2A treatment (Fig. 5A). This reduction in clearance was associated with increased recovery of neutrophils in lavage, both viable and morphologically apoptotic, following the G2A blockade (Fig. 5B).

Effects of lysoPS/G2A signaling on macrophage programming for enhanced efferocytosis - Efferocytic capability has been generally associated with “alternative activation” or programming of macrophages to a “resolving” state. Acquisition of CD206 expression on macrophages appears to track with efferocytic capability during the resolution of zymosan-induced peritonitis (28,29). Accordingly, we asked whether antibody blockade of lysoPS signaling resulted in diminished expression of CD206 on macrophages. As shown in Fig. 5C and D, treatment of wild type mice with anti-G2A (but not isotype antibodies) reduced the subsequent expression of CD206 on macrophages. As further proof of concept, inflammatory macrophages from G2A−/− mice were also investigated for efferocytosis and CD206 expression at the same time point during zymosan-induced peritonitis (28,29). Diminished efferocytosis (Fig. 5A) and CD206 expression (Fig. 5D) were demonstrated for G2A−/− macrophages relative to wild type, though inflammation in this genotype is somewhat delayed (17) making head-to-head comparisons inexact (Discussion). Of note, CD36 and 15-lipoxygenase, additional markers associated with “alternative activation” and implicated in apoptotic cell engulfment (30,31), were also evaluated but neither protein was altered in this system (data not shown).

Given these findings associating blockade of lysoPS/G2A signaling with diminished macrophage programming for efferocytic capability, we next sought to determine whether adoptively transferred lysoPS<sup>high</sup> exudate neutrophils could drive the acquisition of the resolving macrophage marker, CD206, on the macrophages. Adoptive transfer of lysoPS<sup>high</sup> neutrophils into wild type mice 12 h after initiation of the peritonitis was found to variably enhance expression of CD206 assessed 24 h later (Fig. 6A). We suspect that in these wild-type mice, the presence of signaling by the endogenous lysoPS<sup>high</sup> neutrophils and relatively high levels of CD206 expression on the macrophages may have reduced detection of an enhancement. Therefore, to further test for effects of lysoPS signaling on macrophage programming, we turned to lysoPS deficient gp91<sub>phox</sub>−/− mice as recipients of lysoPS<sup>high</sup> exudate neutrophils. As the murine model of CGD, these mice demonstrate delayed expression of CD206 on inflammatory macrophages, deficient efferocytosis and exaggerated inflammation during zymosan-induced peritonitis (Fig. 6B; compare timecourse to Fig. 4A) (28). Adoptive transfer of lysoPS<sup>high</sup> neutrophils into these mice at 12 h significantly increased the subsequent expression of CD206 on macrophages 24 h later, resulting in levels comparable to that of wild type macrophages (Fig. 6C). This enhancement was not seen following transfer of lysoPS<sub>low</sub> gp91<sub>phox</sub>−/− neutrophils.

LysoPS-driven macrophage reprogramming restores “normal” resolution of inflammation in the murine model of CGD. Neutrophilia (Fig. 6B), and the levels of pro-inflammatory cytokines, are both prolonged in the CGD gp91<sub>phox</sub>−/− mice (28,32,33). We therefore hypothesized that transfer of lysoPS expressing viable neutrophils would not only lead to their efficient intrinsic removal (Fig. 3-4) and the reprogramming of macrophages (Fig. 6), but would also increase efferocytosis and decrease production of proinflammatory chemokines and cytokines. Adoptive transfer of CD45.1 lysoPS<sup>high</sup> neutrophils into CD45.2 gp91<sub>phox</sub>−/− recipients allowed us to track donor versus recipient neutrophil accumulation and disappearance over time. At 36 h after transfer, only 3% of harvested peritoneal neutrophils were CD45.1 donor neutrophils while 97% were CD45.2 endogenous neutrophils. Macrophage numbers were unaltered regardless of the adoptively transferred neutrophil genotype (Fig. 7A), though again,
macrophage CD206 expression was enhanced following transfer of lysoPS\textsuperscript{high} neutrophils (Fig. 7B). Accompanying the enhanced CD206 expression, efferocytic ingestions were also significantly increased in macrophages from gp91\textsuperscript{phox}\textsuperscript{-/-} mice given lysoPS\textsuperscript{high} neutrophils, and were comparable to levels seen in wild type mice. PBS or adoptively transferred lysoPS\textsuperscript{low} neutrophils had no effect. Given the reduction in accumulated neutrophils, we also assessed levels of mediators associated with neutrophil recruitment. As shown (Fig. 7C), decreased KC and IL-6 levels were found in peritoneal lavage of gp91\textsuperscript{phox}\textsuperscript{-/-} recipients receiving lysoPS\textsuperscript{high} exudate neutrophils relative to levels seen in mice receiving either PBS or lysoPS\textsuperscript{low} neutrophils (Discussion).

The role of lysoPS signaling via G2A for these events was verified by antibody blockade. Treatment of the mice with antibody to G2A (but not isotype) 2 h prior to the adoptive transfer resulted in reversal of the heightened CD206 expression (Fig. 8A) and efferocytosis (Fig. 8B) induced by the lysoPS\textsuperscript{high} neutrophils. The resulting levels following G2A blockade were comparable to those of mice receiving lysoPS\textsuperscript{low} neutrophils. Additionally, the reductions in mediator levels evident in gp91\textsuperscript{phox}\textsuperscript{-/-} mice adoptively transferred with lysoPS\textsuperscript{high} neutrophils were also reversed by G2A blockade (Fig. 8C). Taken together, the results demonstrate that provision of lysoPS\textsuperscript{high} neutrophils enhanced gp91\textsuperscript{phox}\textsuperscript{-/-} macrophage programming to a resolving state associated with improved efferocytosis and reduced neutrophilia.

**DISCUSSION**

Our results support the hypothesis that neutrophils themselves govern their own “early” clearance in acute inflammation, and that lysoPS is the major signal determining their “palatability” for removal by macrophages. Using antibodies to block G2A signaling and macrophages from G2A\textsuperscript{-/-} mice, a requirement for macrophage G2A was clearly demonstrated (Figs. 2, 5 and 8). Of note, it is thought that lysoPS (as well as its other related lipid activators, e.g. lysoPC) signals indirectly through this receptor rather than as a direct ligand (20,34,35). We have previously shown that lysoPS signaling downstream of G2A activates cPLA\textsubscript{2} and COX for the production of PGE\textsubscript{2}. PGE\textsubscript{2}, in turn, signals to adenylyl cyclase for production of cAMP and the activation of PKA and ultimately Rac (17). The activation of Rac is required for macropinocytosis, the clearance of apoptotic cells and of activated, viable neutrophils, and there appears to be considerable convergence between these pathways (Fig. 2) (4,17,25). Precedence for the ingestion of viable cells by macrophages is found in other inflammatory contexts (36,37). As such, lysoPS-driven clearance likely explains the earlier findings of Lagasse et al. in which neutrophils rendered resistant to apoptosis by the overexpression of Bcl-2 were cleared from inflamed peritonea with the same kinetics as unaltered neutrophils (38). Furthermore, and as shown here, ingested viable cells have been well documented to undergo subsequent apoptosis within the macrophage phagosome (26,27).

In addition to its rapid signaling for neutrophil clearance, lysoPS signaling from the activated neutrophil to the macrophage has longer-term, downstream consequences. Macrophages exposed to lysoPS\textsuperscript{high} neutrophils demonstrate a G2A-mediated shift in programming to a CD206\textsuperscript{high} “resolving” functional capacity associated with enhanced efferocytosis and diminished production of pro-inflammatory cytokines, including those involved in neutrophil recruitment itself. We note that this lysoPS-driven programming shift was most readily demonstrated in gp91\textsuperscript{phox}\textsuperscript{-/-} mice where the altered macrophage programming state is delayed, efferocytosis is diminished and the production of KC, IL-6 and IL-1\beta is prolonged (28,33,39-41). By contrast, these resolution events occur quite rapidly in wild type mice, e.g. KC levels recede within 6 h (28), and neutrophil engulfment is readily evident by 18 h (Fig. 4). We hypothesize that endogenous lysoPS signaling contributes significantly to these events in “normal” resolution of acute inflammation. Accordingly, blockade of G2A signaling in wild type mice i) reduced macrophage expression of the CD206 marker, ii) diminished cell removal, and iii) increased the numbers of viable and uningested, apoptotic neutrophils (Fig. 5). Unfortunately, attempts to examine even earlier blockade of endogenous lysoPS signaling from neutrophil immigrants with anti-G2A antibody were confounded by delayed recruitment of both neutrophils and monocyte/macrophages to the peritoneum (data not shown). G2A is present on
most hematopoietic cells including neutrophils, resident and recruited macrophages as well as on endothelium, and the G2A receptor plays multiple roles in inflammation as is borne out in the knockout mice (35,42,43).

Future investigation will use the gp91$^{\text{phox}-/-}$ model to determine the signaling pathway from G2A to the macrophage reprogramming. The changes noted, including CD206 expression, are downstream targets of PPARY activation, and we have previously shown that PPARY expression and activation are delayed in these gp91$^{\text{phox}-/-}$ mice (28). Other candidates are also possible. PGE$_2$, cAMP and PKA (44-46) are all lysoPS signaling intermediaries (17), and have been shown to lead to the development of “resolving” macrophages. Thus, there are several likely intermediaries of the lysoPS/G2A signaling pathway.

Notably, in an attempt to drive both lysoPS-mediated neutrophil clearance and altered macrophage programming, lysoPS liposomes alone were instilled into inflamed peritoneum at various time points following zymosan-induced peritonitis (data not shown). These did not reproduce the findings of adoptively transferred lysoPS$^{\text{high}}$ neutrophils. Given that we do not know the pharmacokinetics of lysoPS in vivo, our liposome concentrations or timing may have been inadequate. However, we think it is likely that the presentation of lysoPS on the neutrophil surface itself is critical. We have previously shown that endogenous lysoPS produced by neutrophils is always cell-associated, raising the possibility that lysoPS is locally concentrated on the cell surface and/or co-presented to macrophages with other, as yet, unidentified signals (47,48). In support of this, earlier studies showed that lysoPS liposomes added with apoptotic cells, e.g. Jurkat cells, enhanced their G2A-mediated engulfment while lysoPS addition to viable cells did not, suggesting that a second signal was needed (16). Further, lysoPS liposomes significantly enhanced the uptake of apoptotic cell mimics, carboxylated beads presenting a PS headgroup-like surface, perhaps indicating the need for co-signaling via a macrophage receptor for the PS headgroup (16,17). An intriguing possibility is that autocrine signaling by lysoPS via G2A on the recruited neutrophil (20) may upregulate other “eat me” signals on these cells. Thus, while we have shown a requirement for lysoPS/G2A signaling in the removal of activated neutrophils, we have not proven that it acts alone in this setting.

There is growing recognition that oxidants play significant anti-inflammatory roles in inflammation (12,14,49) though the exact mechanisms have not been elucidated. We have previously shown that lysoPS was the predominant modified PS species identified in activated neutrophils, and while roles for several phospholipases in its production were ruled out, an absolute requirement of an active NADPH oxidase was demonstrated (16,17). Here we have shown that lysoPS plays a critical role in the control and resolution of neutrophilia. Conversely, suppression of the neutrophil NADPH oxidase, and certainly its absence as in CGD, likely contribute to poor neutrophil removal and exacerbation of inflammation (28,33,41). From our own and others investigations tying the loss of the NADPH oxidase to exaggerated inflammation in CGD, two hypotheses regarding defects in efferocytosis have emerged: i) oxidase deficient neutrophils undergo delayed apoptosis with deficient “eat me” signaling, i.e. do not develop their normal palatability (16,39,41,50), and ii) CGD inflammatory macrophages do not become appropriately programmed for efferocytosis or turn off inflammatory mediator production (28,40,41). Noteworthy, are the implications of the data here that link both hypotheses to deficient lysoPS signaling from the activated neutrophil. Hence, restoration of lysoPS on the CGD neutrophil may restore these anti-inflammatory pathways in this disorder. Indeed, the development of chronic inflammation in both human and murine CGD, and autoimmunity in CGD patients and both the gp91$^{\text{phox}-/-}$ and G2A$^{-/-}$ murine models (13,35,51,52) suggests that loss of lysoPS signaling on neutrophils may have additional consequences. Similarly, defects in the programming towards resolving macrophages have been identified in a number of inflammatory disorders and linked to deficient efferocytosis, chronic inflammation and autoimmunity, e.g. in COPD, severe asthma, and Systemic lupus erythematosus (7,53,54). The data here suggest that proper exploitation of lysoPS signaling may be a tractable means of therapeutic intervention in such disorders.
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The abbreviations used are: LysoPS, lysophosphatidylserine; МΦ, macrophages; neut, neutrophil, RPMΦ, resident peritoneal macrophage; WT, wild type; gp91, gp91 phox−/−; CGD, Chronic Granulomatous Disease; cPLA₂α, calcium-dependent cytosolic PLA₂; PKA protein kinase A PGE₂, prostaglandin E₂, i.p.; intraperitoneally.
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FIGURE LEGENDS

Figure 1. Characterization of exudate neutrophils harvested from WT and gp91phox−/− mice 6-10 h after intraperitoneal zymosan injection. A. Total numbers of exudate and apoptotic neutrophils harvested from the two genotypes. Apoptotic neutrophils were detected by Annexin V staining or nuclear morphology on cytospins. B. Lipids from exudate neutrophils collected from WT or gp91phox−/− mice were extracted and lysoPS was quantified by LC/MS/MS. n=8-10; *p<0.001. Data represent mean ± S.E.

Figure 2. LysoPS drives engulfment of viable exudate neutrophils by macrophages in vitro. A. Exudate neutrophils from WT or gp91phox−/− mice, or human neutrophils induced to undergo apoptosis by UV-irradiation, were adoptively co-cultured with RPM, and the phagocytic index was determined after 1 h. n=8; *p<0.05 compared to WT neutrophils. B. RPM were treated with 10 µg/ml blocking antibodies to G2A (or IgG isotype control) prior to co-culture with neutrophils. n=6; *p<0.05 compared to WT neutrophils + isotype control. C. RPM from either WT or G2A−/− mice were co-cultured with neutrophils. n=4; *p<0.05 compared to WT neutrophils co-cultured with WT RPM. D. LysoPS liposomes were added as indicated to co-cultures. n=6; *p<0.05 compared to WT neutrophil control in the absence of lysoPS liposomes. Data represent mean ± S.E.

Figure 3. LysoPS drives engulfment of viable exudate neutrophils by macrophages in vivo. Exudate neutrophils (5x10⁶) from WT and gp91phox−/− mice, with or without lysoPS lipidosome premixing (see Methods) were adoptively transferred into the peritonea of WT or G2A−/− recipient mice on Day 5 following induction of peritonitis. After 4 h, peritoneal cells were collected by lavage and the phagocytic index determined by visual inspection of cytospins. A-B. n=5; *p<0.05 compared to WT neutrophil control. C-E. Lavaged cells were TUNEL stained and analyzed for free TUNEL+ neutrophils (C) and phagocytic index of MΦ containing TUNEL+ material in phagosomes (D). n=5; *p<0.05 compared to WT neutrophil control. E. Representative photographs of cytospins from (C-D). Red arrows indicate TUNEL+ material in phagosomes. The dotted line in panels A, B and D represents the background phagocytic index from control mice that did not receive adoptive transfer of exudate neutrophils. Data represent mean ± S.E.

Figure 4. LysoPS drives engulfment of viable exudate neutrophils by macrophages early in inflammation. A. Timecourse of total neutrophils and macrophages after induction of zymosan peritonitis in WT mice. B. Six-hour exudate neutrophils (5x10⁶) from CD45.2 WT and gp91phox−/− mice were harvested, PBSE labeled, pre-mixed with lysoPS liposomes or not, and adoptively transferred into recipient CD45.1 wild type mice at 18 h into zymosan-induced peritonitis. After 4 h, peritoneal cells were collected by lavage and MΦ of recipient mice defined as F4/80+/CD45.1+/Ly6G− were analyzed for ingested PBSE+ exudate neutrophils. Shown are representative density plots from 3 independent experiments demonstrating recipient macrophages positive for PBSE following adoptive transfer of WT neutrophils pre-mixed or not with lysoPS liposomes (middle row), and gp91phox−/− neutrophils pre-mixed or not with lysoPS liposomes (bottom row). Upper right – Bar graph shows summated data of PSBE+ recipient macrophages under each condition. C. Phagocytic indices for these same harvests were determined by visual inspection of cytospins. Dotted lines in panels B and C represent the background phagocytosis in control wild type mice that received PBS alone (no neutrophils). n=3; *p<0.05 compared to WT neutrophil control. Data represent mean ± S.E.

Figure 5. Endogenous lysoPS signaling via G2A promotes the development of CD206high efferocytic macrophages and resolution of neutrophilia. WT mice were injected i.p. with 100 µg blocking antibody to G2A (or IgG isotype control or PBS) at 18 h after induction of zymosan peritonitis. Thirty hours later, peritoneal cells were collected by lavage and analyzed. For comparison, cells from G2A−/− mice at 48 h into zymosan-induced peritonitis were also analyzed. A. Phagocytic indices were determined
by visual inspection of cytospins. \(n=6-8; \ ^*p<0.05\) compared to WT control in the absence of antibody blockade; \(^{**}p<0.05\) compared to WT control. B. Total neutrophils and total apoptotic neutrophils (as determined by morphology from cytospins) were quantified. \(n=5; \ ^*p=0.03\) compared to WT PBS control; \(^{**}p<0.02\) compared to WT PBS control. C. CD206 expression on F4/80+/LY6G- MΦ was determined by staining with anti-CD206 (solid line) or CD206 isotype control antibody (shaded gray) and analyzed by flow cytometry (shown are representative histograms from 6 independent experiments). D. Geometric means of CD206 expression was determined and expressed as fold over CD206 isotype control staining and summated for WT and G2A\(^{-/-}\) peritoneal MΦ. \(n=6; \ ^*p<0.02\) compared to WT control; \(^{**}p=0.01\) compared to WT control. Data represent mean ± S.E.

**Figure 6.** Adoptive transfer of lysoPS\(^{\text{high}}\) exudate neutrophils enhance macrophage CD206\(^{\text{high}}\) expression during peritonitis. A. LysoPS\(^{\text{high}}\) (WT) exudate neutrophils (1x10\(^7\)) (or PBS) were adoptively transferred into peritoneal WT recipient mice at 12 h into zymosan-induced peritonitis. Peritoneal cells were collected by lavage 24 h later and CD206 expression on F4/80+/LY6G- MΦ determined by flow cytometry and expressed as in Fig. 5. Left- histograms from a single experiment. Staining with anti-CD206 (solid line) or CD206 isotype control antibody (shaded gray). Right- Summated expression data. \(n=8\). B. Left – Timecourse of total neutrophils and macrophages following induction of zymosan peritonitis in gp91\(^{\text{pox/-}}\) mice. Right - Timecourse of CD206 expression on macrophages in WT or gp91\(^{\text{pox/-}}\) mice following induction of zymosan peritonitis. \(n=8-10; \ ^*p<0.001\) compared to gp91\(^{\text{pox/-}}\). C. Representative histograms from WT PBS control or gp91\(^{\text{pox/-}}\) recipients. Lower left: summated CD206 expression data are shown for gp91\(^{\text{pox/-}}\) recipient MΦ. Dotted line represents CD206 expression on MΦ from PBS treated WT mice. \(n=8; \ ^*p=0.0001\) compared to WT PBS control; \(^{**}p<0.0002\) compared to CD206 expression on PBS treated gp91\(^{\text{pox/-}}\) mice. Data represent mean ± S.E.

**Figure 7.** In addition to driving macrophage programming, lysoPS\(^{\text{high}}\) exudate neutrophils reduce subsequent neutrophilia and pro-inflammatory mediator levels in gp91\(^{\text{pox/-}}\) recipients. gp91\(^{\text{pox/-}}\) recipient mice were adoptively transferred with either lysoPS\(^{\text{high}}\) or lysoPS\(^{\text{low}}\) neutrophil (1x10\(^7\)) at 12 h into zymosan-induced peritonitis and 36 h later peritoneal lavage analyzed. A. total neutrophil and MΦ counts. \(n=3-6; \ ^*p<0.05\) compared to PBS control. B. Left-Geometric mean of CD206 expression on recipient gp91\(^{\text{pox/-}}\) MΦ expressed as fold over their respective isotype controls. \(n= 3-6; \ ^*p<0.05\) compared to WT PBS control; \(^{**}p<0.01\) compared to gp91\(^{\text{pox/-}}\) PBS control. Right-Phagocytic index determined by visual inspection of cytospins. \(n= 3-6; \ ^*p<0.001\) compared to WT PBS control; \(^{**}p<0.001\) compared to gp91\(^{\text{pox/-}}\) PBS control. C. Levels of KC (left) and IL-6 (right) were measured in cell-free lavage supernatants from gp91\(^{\text{pox/-}}\) mice. \(n=3-6; \ ^*p<0.05\) compared to PBS control. Data represent mean ± S.E.

**Figure 8.** Signaling to G2A by lysoPS\(^{\text{high}}\) exudate neutrophils is required for enhanced efferocytic macrophage programming and reductions in pro-inflammatory mediator levels in gp91\(^{\text{pox/-}}\) recipients. gp91\(^{\text{pox/-}}\) recipient mice were injected i.p. with 100 µg blocking antibody to G2A (or IgG isotype control) 18 h after induction of zymosan-induced peritonitis. WT exudate neutrophils were adoptively transferred 2 h after antibodies and peritoneal cells were collected by lavage 24 h later and analyzed. A. CD206 expression on MΦ was determined and geometric means represented as fold over isotype control is shown. \(n=5; \ ^*p<0.005\). B. Phagocytic indices were determined by visual inspection of cytospins. \(n=5; \ ^*p<0.02\). C. IL-6 was determined in cell free lavage supernatants from gp91\(^{\text{pox/-}}\) recipient mice. \(n=5; \ ^*p<0.005\). Data represent mean ± S.E.
Figure 1

Neutrophil lysoPS regulates tissue neutrophilia

A

Total neut

Apo neut (Annexin V)

Apo neut (morphology)

Total PMN (millions)

WT

gp91^phox^-/-

B

ng lysoPS/10^7 neut

WT neut

gp91^phox^-/- neut

*
Figure 2

Neutrophil lysoPS regulates tissue neutrophilia.

A

Phagocytic Index

+WT neut  +gp91 neut  +Apo hNeut

B

Phagocytic Index

+WT neut  +gp91 neut  +Apo hNeut

C

Phagocytic Index

+WT neut  +Apo hNeut

D

Phagocytic Index

+WT neut  +gp91 neut
Figure 3

Neutrophil lysoPS regulates tissue neutrophilia.

A. WT recipient

|         | Ctr | +lysoPS |
|---------|-----|---------|
| +WT neut | 16  | 14      |
| +gp91 neut | 12  | 10      |

B. G2A−/− recipient

|         | Ctr | +lysoPS |
|---------|-----|---------|
| +WT neut | 8   | 6       |
| +gp91 neut | 6   | 4       |

C. TUNEL pos neut (%)

|         | Ctr | +lysoPS |
|---------|-----|---------|
| +WT neut | 2.5 | 2       |
| +gp91 neut | 1.5 | 1       |

D. Phagocytic Index (TUNEL+ neut inside Mφ)

|         | Ctr | +lysoPS |
|---------|-----|---------|
| +WT neut | 16  | 14      |
| +gp91 neut | 12  | 10      |

E. 

| PBS | + WT neut | + gp91phox−/− neut | + gp91phox−/− neut + lysoPS |

Images showing cellular effects with various treatments.
Neutrophil lyoPS regulates tissue neutrophilia.
Neutrophil lysoPS regulates tissue neutrophilia

Figure 5

A

Phagocytic Index

|       | IgG | aG2A |
|-------|-----|------|
| WT    |     |      |
| G2A−/−|     |      |

B

|       | Total neuts | apo neuts |
|-------|-------------|-----------|
| PBS   | **          |           |
| IgG   | *           |           |
| aG2A  |             | **        |

C

% Max

PBS

IgG

aG2A

D

MΦ CD206

|       | IgG | aG2A |
|-------|-----|------|
| WT    |     |      |
| G2A−/−|     |      |

MΦ CD206 MFI (fold over isotype)

|       |     | aG2A |
|-------|-----|------|
| WT    |     |      |
| G2A−/−|     |      |
Figure 6

Neutrophil lysoPS regulates tissue neutrophilia
Neutrophils regulate tissue neutrophilia in inflammation via the oxidant modified lipid lysophosphatidylserine

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