THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 9, Issue of March 4, pp. 7519–7529, 2005
Printed in U.S.A.

Cytosolic Phospholipase A_2-α Is Necessary for Platelet-activating Factor Biosynthesis, Efficient Neutrophil-mediated Bacterial Killing, and the Innate Immune Response to Pulmonary Infection

cPLA$_2$-α DOES NOT REGULATE NEUTROPHIL NADPH OXIDASE ACTIVITY*

Received for publication, July 2, 2004, and in revised form, October 4, 2004
Published, JBC Papers in Press, October 8, 2004, DOI 10.1074/jbc.M407438200

Barry B. Rubin,[a,b,c] Gregory P. Downey,[b,d] Adeline Koh,[d] Norbert Degousee,[a] Farideh Ghomashchi,[e] Laxman Nallan,[e] Eva Stefanski,[a] Denis W. Harkin,[a] ChunXiang Sun,[d] Brian P. Smart,[e] Thomas F. Lindsay,[a] Vera Cherepanov,[d] Eric Vachon,[d] David Kelvin,[f] Martin Sadilek,[e] Glenn E. Brown,[g] Michael B. Yaffe,[g] Jonathan Plumb,[h] Sergio Grinstein,[h] Michael Glogauer,[i] and Michael H. Gelb[e,j]

From the [a]Division of Vascular Surgery and Toronto General Hospital Research Institute of the University Health Network, University of Toronto, Toronto, Ontario M5G 2C4, Canada, [d]Division of Respirology, Department of Medicine and Toronto General Hospital Research Institute of the University Health Network, University of Toronto, Toronto, Ontario M5S 1A8, Canada, [e]Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195, [f]Division of Experimental Therapeutics and Department of Immunology, University of Toronto, University Health Network, Toronto General Research Institute, Toronto General Hospital, Toronto, Ontario M5G 2C4, Canada, [g]Department of Surgery, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, [h]Program in Cell Biology, The Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada, and [i]Faculty of Dentistry, University of Toronto, Toronto, Ontario M5S 3E2, Canada

The role of a cytosolic phospholipase A_2-α (cPLA$_2$-α) in neutrophil arachidonic acid release, platelet-activating factor (PAF) biosynthesis, NADPH oxidase activation, and bacterial killing *in vitro*, and the innate immune response to bacterial infection *in vivo* was examined. cPLA$_2$-α activity was blocked with the specific cPLA$_2$-α inhibitor, Pyrrolidine-1 (human cells), or by cPLA$_2$-α gene disruption (mice). cPLA$_2$-α inhibition or gene disruption led to complete suppression of neutrophil arachidonate release and PAF biosynthesis but had no effect on neutrophil NADPH oxidase activation, FcγII/III or CD11b surface expression, primary or secondary granule secretion, or phagocytosis of *Escherichia coli in vitro*. In contrast, cPLA$_2$-α inhibition or gene disruption diminished neutrophil-mediated *E. coli* killing *in vitro*, which was partially rescued by exogenous arachidonic acid or PAF but not leukotriene B_4. Following intratracheal inoculation with live *E. coli in vivo*, pulmonary PAF biosynthesis, inflammatory cell infiltration, and clearance of *E. coli* were attenuated in cPLA$_2$-α(−/−) mice compared with wild type littermates. These studies identify a novel role for cPLA$_2$-α in the regulation of neutrophil-mediated bacterial killing and the innate immune response to bacterial infection.

Bacterial pneumonia is a significant cause of morbidity and a leading cause of mortality, especially in very young, elderly, and immunocompromised individuals (1, 2). Bacterial infection of the lung stimulates an innate immune response that is characterized by the local generation of pro-inflammatory mediators, increased microvascular permeability, and the influx of leukocytes, which generate oxidants, release cytotoxic enzymes, and kill invading bacteria (3). A variety of host and bacterial mediators are responsible for attracting leukocytes to sites of bacterial infection, including cytokines, chemokines, and lipid mediators. The first step in the biosynthesis of pro-inflammatory lipid mediators such as leukotrienes and thromboxanes, which are pivotal to the development of an effective innate immune response, can be catalyzed by cytosolic phospholipase PLA$_2$-α (cPLA$_2$-α),[1] which selectively liberates arachidonic acid from the *sn*-2 position of phospholipids (4). cPLA$_2$-α translocates from the cytosol to nascent phagosomes during phagocytosis by mouse macrophages (5) and regulates neutrophil LTB_4 biosynthesis (6) and eicosanoid generation by mouse bone marrow-derived mast cells (7). In addition, arachidonic acid generated by cPLA$_2$-α is proposed to play a role in the assembly and activation of the NAPDH oxidase in profes-

* This work was supported by grants from The Physicians of Ontario through The P.S.I. Foundation (Grant 01-12 (to B. B. R.) and 98-049 (to T. F. L.)), The Canadian Institutes of Health Research Grants 53297 (to B. B. R.), MT-12255 (to G. P. D.), MT-53136 (to M. Glogauer), and MT-7075 (to S. P. G.), and The National Institutes of Health Grants HL36236 and HL50040 (to M. H. G.) and GM59281 (to M. B. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[b] Both authors contributed equally to this work.

[c] A Wylie Scholar in Academic Vascular Surgery, Pacific Vascular Research Foundation (San Francisco, CA). To whom correspondence may be addressed: Division of Vascular Surgery, 200 Elizabeth St., EC5-302a, Toronto General Hospital, Toronto, Ontario M5G 2C4, Canada. Tel.: 416-340-3645; Fax: 416-340-5029, E-mail: barry.rubin@uhn.on.ca.

[j] To whom correspondence may be addressed: Depts. of Chemistry and Biochemistry, Campus Box 351700, University of Washington, Seattle, WA 98195. Tel.: 206-543-7142; Fax: 206-685-8665; E-mail: gelb@chem.washington.edu.

[1] The abbreviations used are: cPLA$_2$-α, cytosolic phospholipase A_2-α; PMA, phorbol 12-myristate 13-acetate; d_8-arachidonic acid, [5,6,8,9,11,12,14,15-^{2}H]arachidonic acid; PAF, platelet-activating factor; BAL, bronchoalveolar lavage; d_3-PAF, PAF with a 2,2,2-trideuteroacetyl group and a *sn*-1-hexadecyl group; C_{18}-lyso-PC, 1-octadecyl-*sn*-phosphatidylcholine; OZ, opsonized zymosan; fMLP, formylmethionylleucylphenylalanine; ANOVA, analysis of variance; HBSS, Hanks' balanced salt solution; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; TNFα, tumor necrosis factor; IL, interleukin; 5-LO, 5-lipoxygenase; LTB_4, leukotriene B_4; Me, methyl; GM-CSF, granulocyte macrophage colony-stimulating factor.

sional phagocytic cells *in vitro* (8–10). These observations support a potential role for cPLA$_2$-α in the innate immune response to bacterial infection.

cPLA$_2$-α can also hydrolyze 1-*O*-alkyl phospholipids, leading to the formation of lyso-PAF, which can be acetylated to form PAF. The administration of PAF induces pulmonary edema, pulmonary neutrophil sequestration, and hypoxemia (11). In addition, PAF appears to play a central role in the pathophysiology of endotoxin, immunocomplex, intestinal ischemia-reperfusion, and acid aspiration-induced lung injury (12–15). PAF biosynthesis in response to the calcium ionophore A23187 or to phorbol 12-myristate 13-acetate was markedly attenuated in peritoneal macrophages from cPLA$_2$-α(−/−) mice compared with macrophages from wild type mice (16). The role of cPLA$_2$-α in the regulation of neutrophil PAF biosynthesis in response to a physiologically relevant agonist, such as opsonized zymosan, and the role of cPLA$_2$-α in the regulation of the innate immune response to bacterial infection *in vivo* have not been evaluated.

Deletion of *PLA$_2$g4a*, the gene that encodes cPLA$_2$-α, attenuates the inflammatory response and lung injury associated with acid aspiration, intravenous infusion of lipopolysaccharide-zymosan, and treatment with bleomycin (17, 18). Based on these studies, which used the same *PLA$_2$g4a*(−/−) mouse as the one employed in the current series of experiments, the inhibition of cPLA$_2$-α was proposed as a potential therapeutic strategy for patients with acid aspiration or bleomycin-induced lung injury or pulmonary sepsis. Because cPLA$_2$-α plays a role in the biosynthesis of pro-inflammatory mediators and may participate in the regulation of neutrophil oxidant production and phagocytosis, we hypothesized that the inhibition of cPLA$_2$-α activity would attenuate the innate immune response to live bacterial infection. In this study, we demonstrated that cPLA$_2$-α catalyzes arachidonic acid release, initiates PAF biosynthesis, and partially regulates *Escherichia coli* killing by neutrophils *in vitro* and that exogenous arachidonic acid reverses the defect in neutrophil *E. coli* killing induced by the inhibition or genetic deletion of cPLA$_2$-α. In addition, we showed that deletion of a functional cPLA$_2$-α gene impairs PAF biosynthesis and eradication of Gram-negative bacteria from the lung *in vivo*. Our observations identify a new role for cPLA$_2$-α in the regulation of neutrophil-mediated bacterial killing and the innate immune response to Gram-negative bacterial infection. Surprisingly, we show that cPLA$_2$-α does not participate in the regulation of neutrophil oxidant production.

EXPERIMENTAL PROCEDURES

Materials—d_8-Arachidonic acid is from BioMol. d_3-PAF was synthesized using 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (Avanti Polar Lipids Inc., Alabaster, AL) and d_6-acetic anhydride (Aldrich) using standard methods. Pyrrolidine-1 and Me-Indoxam were prepared as described previously (19, 20). Dihydrorhodamine and goat anti-mouse Alexa 488 were from Molecular Probes (Eugene, OR). PMA, fMLP, cytochalasin B, and cytochalasin D were from Sigma. Aggregated human IgG was from Wisent (Montreal, Quebec, Canada). Ionomycin was from Calbiochem. Paraformaldehyde was from Canemco (St. Laurent, Quebec, Canada). The rat monoclonal antibody against mouse CD11b (clone M1/70) was from BIOSOURCE International (Nivelles, Belgium). Antisera to αCD63 and αCD66b were from Serotec (Raleigh, NC). Antiserum to αN-RAS was from Santa Cruz Biotechnology (Santa Cruz, CA). *PLA$_2$g4a*(−/−) mice were kindly provided by Dr. T. Shimizu (Department of Geriatric Medicine, University of Tokyo, Tokyo, Japan).

Preparation of Human and Mouse Neutrophils—All of the experimental protocols involving the use of mice were approved by the Animal Care Committee of the University Health Network. *PLA$_2$g4a*(−/−) mice, or their wild type littermates were anesthetized with isofluorane and euthanized by cervical dislocation. The femur and tibia were dissected, cut proximally and distally, and flushed with ice-cold 1× HBSS (supplemented with 10 mM HEPES, pH 7.5), and the eluant was centrifuged at 2,600 rpm for 30 min at 4 °C over a three-layer Percoll gradient (52, 65, and 75%) (21). The neutrophil-rich fraction was collected at the interface of the 65 and 75% layers, resuspended in 1× HBSS, and centrifuged at 1,500 rpm for 5 min at 4 °C. Pelleted cells were resuspended in 1× HBSS containing 2.5% heat-inactivated fetal bovine serum and used immediately. Human peripheral neutrophils were isolated from EDTA-anticoagulated blood obtained from healthy donors not taking any medications by dextran sedimentation (T500, Amersham Biosciences), hypotonic lysis of residual red blood cells, and centrifugation through Ficoll-Paque (Amersham Biosciences) (23). The human and murine neutrophil isolation protocols routinely yielded cell suspensions that were >90% neutrophils with >98% viability as judged by Wright stain and trypan blue exclusion (22), respectively. All of the neutrophil studies were carried out at 37 °C.

Analysis of Arachidonic Acid and PAF Mass—Human neutrophils (10^7/sample) or mouse neutrophils (10^6/sample) were stimulated with various agonists (see figure and table legends). After stimulation, 38 pmol of d_3-PAF and 22 pmol of d_8-arachidonic acid were added as internal standards and the entire sample (cells + medium) was extracted by the method of Bligh and Dyer (24). One-tenth of the Bligh and Dyer (24) organic phase was processed for arachidonic acid analysis, and the remainder was processed for PAF analysis. For arachidonic acid analysis, the solvent was removed (SpeedVac, Savant Instruments), 0.5 ml of water was added, the sample was extracted with Dole solvent, and arachidonic acid was quantified as its pentafluorobenzyl ester by gas chromatography/mass spectrometry (25). For PAF analysis, the sample was concentrated to 100–200 μl (SpeedVac) and transferred to a conical insert of a Hewlett Packard HPLC auto-injector vial. After complete removal of solvent, the residue was dissolved in 50 μl of 88% methanol/water and 40 μl was injected onto the HPLC column (Zorbax Extend C-18, 2.1 × 100 mm, 3.5 m, 80 Å, Agilent Technologies), which was developed at 200 ml/min with 88% methanol, 12% water, and 0.5% concentrated NH_4OH for 4 min and then with 88% methanol, 12% hexane, and 0.5% concentrated NH_4OH for more than 11 min. The column eluant was directly infused into a Sciex API III+ mass spectrometer operating in a negative ion mode. Multiple reaction-monitoring mode was used with the monitoring of 508/59 (d_0-PAF), 508/283 (C_{18}-lyso-PC), and 511/62 (d_3-PAF). 508 is the d_0-PAF minus methyl negative ion, and 59 is the acetate ion. 508 is also the minus methyl anion for C_{18}-lyso-PC, and 283 is the stearate anion (26). The collision energy was 25 volts, the collision gas thickness was 270 × 1013 molecules/cm^2, and the orifice voltage was −95 volts. The resolution setting on the first quadrupole (RE1) was 103 volts, and the delta mass on the same quadrupole (DM1) was −0.4 volts. The resolution setting on the second mass filter (third quadrupole) was RE3 = 110 volts, DM3 = 0.5 volts.

Measurement of Neutrophil NADPH Oxidase Activity—Opsonized zymosan (OZ) was prepared from human serum depleted of IgG by ammonium sulfate (28.5%) precipitation (27). Extracellular superoxide release by human neutrophils was measured with the cytochrome *c* assay (23). For some studies, luminol-dependent chemiluminescence (27) was used to monitor reactive oxygen species production by neutrophils. For chemiluminescence studies, reactions were initiated by adding 4 × 10^3 neutrophils in 25 μl of PBS containing 100 mg/ml bovine serum albumin and 150 mM luminol to 75 μl of the same buffer (prewarmed to 37 °C) ± inhibitors and/or agonists as indicated in the legend for Fig. 4, *panels E–H*. Chemiluminescence events were measured in a Berthold Autolumat LB953 luminometer. Data are the mean luminescence events ×10^{-5} integrated over 15 min. Murine neutrophil H_2O_2 production was assessed with dihydrorhodamine-123 (1 μM), a membrane-permeable probe that is oxidized by H_2O_2 to yield fluorescent rhodamine-123. Dihydrorhodamine-123 was incubated at 37 °C for 10 min with murine neutrophils (1 × 10^6) before treatment with agonists (23). The mean fluorescence index of >10^4 cells was determined by flow cytometric analysis (fluorescence-activated cell sorter, BD Biosciences) from gated regions on dot plots for each sample (28).

Bacterial Killing Assay—A single colony of *E. coli DH5α* was cultured in Luria-Bertani broth overnight at 2,500 rpm and 37 °C in an orbital-incubated shaker (Lab-Line MaxQ 5000 floor shaker; Barnstead/Lab-Line), spun at 2,500 rpm for 10 min, and resuspended in HBSS^{++} at 10^9 cells/ml. Bacteria were then opsonized for 30 min at 37 °C in heat-inactivated bovine serum, washed, and resuspended in HBSS^{++} at 3 × 10^7 cells/ml.

Human neutrophils were resuspended in HBSS^{++} at 10^7 cells/ml and incubated with vehicle (Me_2SO, 0.1%) or Pyrrolidine-1 (10 μM) for 5 min followed by incubation with vehicle (Me_2SO, 0.1%), arachidonic acid (5 μM), or PAF (500 nM) for 5 min at 37 °C as indicated in the figure legends. Human neutrophils (100 μl) were then co-incubated with *E. coli* (100 μl) in a Thermomixer (FinePCR, Finemould Precision Ind Co.) for 1 h at 37 °C.

Murine neutrophils from *PLA$_2$g4a*(+/+) or *PLA$_2$g4a*(−/−) mice were resuspended in HBSS^{++} at 10^7 cells/ml and incubated with vehicle (Me_2SO, 0.1%), MK886 (1 μM), or U75302 (10 μM) for 5 min at 37 °C and then incubated with Me_2SO (0.1%), arachidonic acid (5 μM), PAF (500 nM), or LTB_4 (10 nM) for 5 min at 37 °C as indicated in the figure legends. Murine neutrophils (100 μl) were then co-incubated with *E. coli* (100 μl) in a Thermomixer for 1 h at 37 °C. At the end of the incubation period, samples were pelleted by centrifugation and lysed by incubation in 0.2% Triton X-100. Bacterial viability was assessed by 10^1-10^5-fold serial dilution culture of samples done in triplicate.

E. coli Pneumonia Model and Collection of Bronchoalveolar Lavage (BAL) Fluid—Mice were anesthetized with intraperitoneal ketamine (8 mg/kg) and Rompun (1 mg/kg), placed in supine position, and swabbed with 70% alcohol along the anterior neck, and the trachea was exposed. 50 μl of E+9 *E. coli* DH5α in PBS was then injected into the trachea with a 30-gauge needle, and the wound was closed with 5-0 Dermalon suture. After 3 h, mice were re-anesthetized with intraperitoneal ketamine and Rompun. The anterior surface of the abdomen was soaked with alcohol, the skin was incised, and the mice were euthanized by transection of the abdominal aorta. The neck wound was then re-sterilized with alcohol, and the trachea was exposed and cannulated with a 20-gauge needle followed by infusion of 1.0 ml of PBS at room temperature. After 10 s, the trachea was aspirated and the BAL was placed on ice. The collection of BAL fluid was then repeated a total of five times, and the BAL fluid was pooled. Blood was obtained in a sterile fashion from cPLA$_2$-α(+/+) and cPLA$_2$-α(−/−) mice immediately before and 3 h after intratracheal administration of live *E. coli*. Bacterial viability in BAL fluid, lung tissue, and blood was assessed by serial dilution culture (29). All of the samples were analyzed in triplicate.

Measurement of E. coli Phagocytosis by Murine Neutrophils—E. coli X1-blue bacteria were grown overnight, subcultured, and incubated with fluorescein isothiocyanate (1 mg/ml) for 10 min with constant rotation at 25 °C. After two washes with PBS, bacteria were fixed with glutaraldehyde (1%) for 20 min, washed twice with PBS, and then opsonized with mouse serum for 30 min. Opsonized bacteria were washed twice in PBS and resuspended in HBSS at 10^7/ml.

Murine neutrophils (10^6 cells/ml) were resuspended in prewarmed HBSS containing LysoTracker (100 nM) and incubated for 1 h at 37 °C. Cells were then washed twice (to remove excess dye), added to microtubes, and incubated for 20 min at 37 °C on a Thermomixer. After mixing equal volumes of neutrophils and opsonized bacteria, cells were incubated at 37 °C and observed under a ×63 immersion objective with a heating plate at 37 °C. Images were acquired by a Leica fluorescence microscope and analyzed with Openlab 3.1.5 software. Phagocytosis rates (percent of neutrophils phagocytosing at least one *E. coli* bacterium) were based on counts between 300 and 2000 neutrophils/experiment.

Measurement of Primary and Secondary Neutrophil Granule Secretion—Freshly isolated human neutrophils were incubated with vehicle (Me_2SO 0.1%) or Pyrrolidine-1 (10 μM) for 10 min at 37 °C as indicated in the figure legends. After washing, cells were incubated with cytochalasin D (10 μM) and aggregated human IgG or with ionomycin (2 μM) for a further 10 min at 37 °C, fixed with paraformaldehyde, rinsed, and then incubated with either 1:20 αN-RAS (irrelevant IgG), 1:50 αCD63, or 1:100 αCD66b primary antibodies for 1 h on ice. After washing, neutrophils were incubated with 1:1000 goat anti-mouse Alexa 488 secondary antibody for 1 h on ice, washed again, and analyzed by flow cytometry (FACScan, 10,000 events for each sample). The relative fluorescence index for each sample is reported.

Cytometric Bead Array—TNFα, interferon-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and MCP-1 levels in BAL fluid were measured with cytometric bead array kits according to the manufacturer's protocols (BD Biosciences) (30). Specific capture beads for cytokines and chemokines were mixed with 50 μl of BAL fluid or standards, and multiple phycoerythrin-conjugated detection antibodies were added. Following a 3-h incubation period with recombinant protein standards or test samples, the acquisition of sample data were performed using a two-color flow cytometer. Results were generated in graphical and tabular format using BD Biosciences cytometric bead assay analysis software. The assessment of neutrophil CD11b (21) and FcγII/III (31) surface expression was carried out exactly as described previously.

Statistical Analysis—The effect of treatment with increasing concentrations of Pyrrolidine-1 on arachidonic acid release and PAF biosynthesis and the effect of Pyrrolidine-1 or genetic deletion of *PLA$_2$g4a* on neutrophil-mediated bacterial killing were assessed by analysis of variance (ANOVA). A comparison between specific subgroups was made by paired Student's *t* tests (two-tailed) and assumed unequal variance. All of the data were presented as the mean ± S.D. A p value < 0.05 was considered to be significant.

RESULTS

cPLA$_2$-α Mediates Neutrophil Arachidonate Release—To ascertain the importance of cPLA$_2$-α in arachidonic acid release, human neutrophils incubated with the specific cPLA$_2$-α inhibitor Pyrrolidine-1, which does not inhibit cPLA$_2$-γ, calcium-independent group VI PLA$_2$ or group IIA, group V- or group X-secreted PLA$_2$ activity (19), or cPLA$_2$-β *in vitro*,[2] were stimulated with various agonists and the liberation of arachidonic acid was quantified by gas chromatography/mass spectrometry. Exposure of human neutrophils to OZ led to an ~10-fold increase in arachidonic acid release that was fully blocked by Pyrrolidine-1 with an IC_{50} of ~0.1 μM (Fig. 1*A*). Pyrrolidine-1 did not block basal arachidonic acid release or cause arachidonic acid release in the absence of agonist (Fig. 1*A*). Among the set of secreted PLA$_2$ enzymes, human neutrophils express only groups V and X PLA$_2$ (6). Me-Indoxam (10 μM), a potent cell-impermeable inhibitor of groups V and X PLA$_2$ *in vitro* (20, 32), had no effect on OZ-stimulated arachidonic acid release and did not cause arachidonic acid release in the absence of agonist (Fig. 1*A*). As shown in Fig. 1*B*, Pyrrolidine-1 blocked OZ-stimulated arachidonic acid release at all of the time points (1, 5, and 10 min), whereas Me-Indoxam had no effect on OZ-stimulated arachidonic acid release. Stimulation of human neutrophils with fMLP in the presence of cytochalasin B (Fig. 1*C*) or GM-CSF (Fig. 1*D*) led to a 3–4-fold increase in arachidonic acid release, and this was fully blocked by Pyrrolidine-1 with an IC_{50} of ~0.1 μM. Me-Indoxam had no effect on cytochalasin B + fMLP or GM-CSF + fMLP-stimulated arachidonic acid release by human neutrophils (Fig. 1, *C* and *D*).

Arachidonic acid release by quiescent neutrophils from *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice was similar (Table I). Exposure of neutrophils from *PLA$_2$g4a*(+/+) mice to OZ resulted in a 4- and 7-fold increase in arachidonic acid release above base-line levels from two different preparations of mouse neutrophils, respectively. In contrast, exposure to OZ had no effect on arachidonate release by neutrophils from *PLA$_2$g4a*(−/−) mice (Table I). Taken together, these studies provide direct evidence that cPLA$_2$-α catalyzes agonist-induced arachidonic acid release by neutrophils.

cPLA$_2$-α Activity Is Necessary for Neutrophil PAF Biosynthesis—PAF biosynthesis (cell-associated and extracellular) by human neutrophils or by neutrophils from the bone marrow of *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice was measured by HPLC-tandem mass spectrometry as shown in Fig. 2*A* and described under "Experimental Procedures." Exposure to OZ induced a 7-fold increase in human neutrophil PAF biosynthesis (Fig. 2*B*) that progressed linearly with time (Fig. 2*B*, *insert*). Preincubation with Pyrrolidine-1 inhibited PAF production by human neutrophils in a dose-dependent fashion with an IC_{50} of ~0.1 μM and near complete inhibition at 10 μM, and it had no effect on the low level of human neutrophil PAF biosynthesis detected in the absence of OZ (Fig. 2*B*). Treatment with Me-Indoxam (10 μM) had no effect on OZ-dependent neutrophil PAF biosynthesis (13.8 ± 1.8 *versus* 14.0 ± 0.7 pmol/10^6 neutrophils/10 min, OZ alone *versus* OZ + Me-Indoxam, $p = 0.90$).

Quiescent neutrophils from *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice did not generate detectable levels of PAF (Table II). Exposure to OZ increased PAF biosynthesis by neutrophils from *PLA$_2$g4a*(+/+) mice but did not increase PAF biosynthesis by neutrophils from *PLA$_2$g4a*(−/−) mice. Interestingly, PAF biosynthesis by neutrophils derived from mouse bone marrow was significantly lower than PAF biosynthesis by circulating human neutrophils after exposure to OZ. Because the detection limit of PAF in these experiments was ~100 fmol, we could not deter-

[2] M. H. Gelb and C. C. Leslie, unpublished observations.

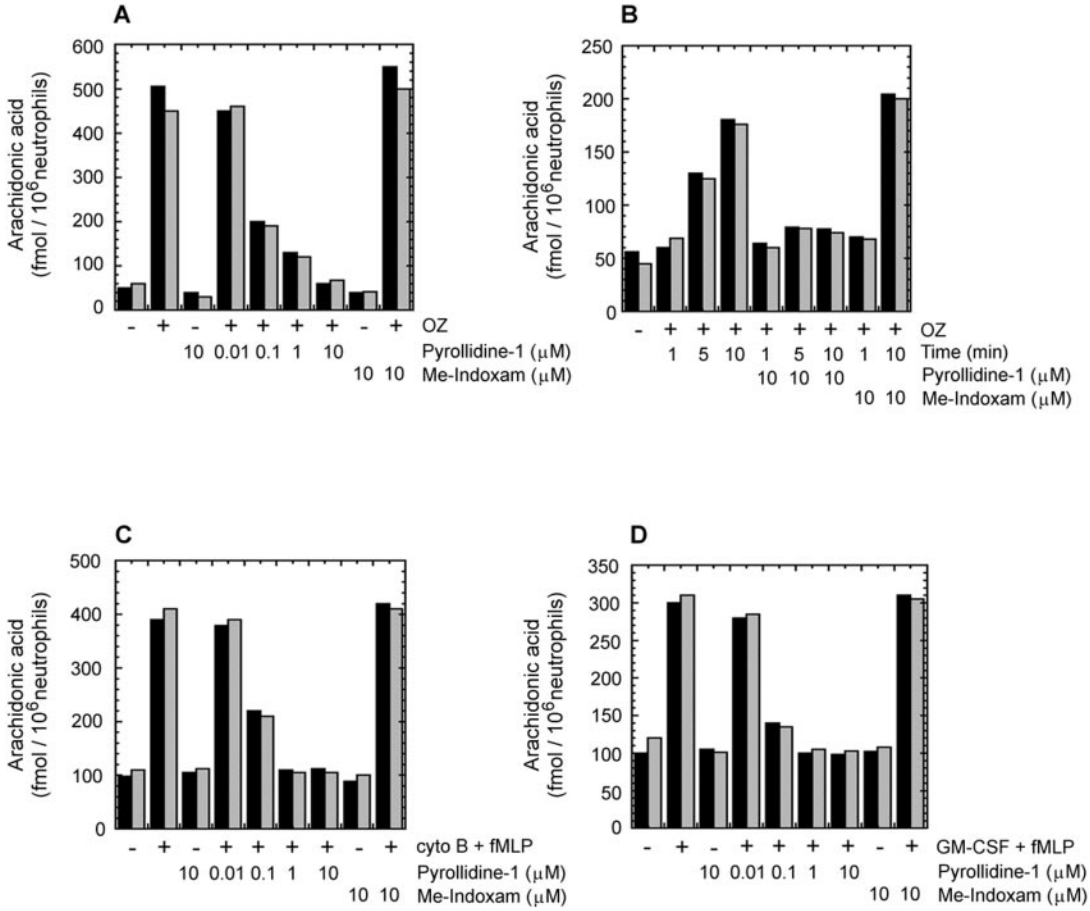

FIG. 1. **cPLA$_2$-α catalyzes arachidonic acid release from OZ- and fMLP-stimulated human neutrophils.** *A*, neutrophils were preincubated for 5 min with the indicated concentrations of Pyrrolidine-1 or Me-Indoxam and then treated with vehicle or OZ (5 mg/ml) for 10 min as indicated. *B*, neutrophils were preincubated for 5 min with Pyrrolidine-1 (10 μM) or Me-Indoxam (10 μM) and then stimulated with OZ for 1, 5, or 10 min as indicated. *C*, neutrophils were preincubated for 5 min with the indicated concentrations of Pyrrolidine-1 or Me-Indoxam and then treated with vehicle or cytochalasin B (5 μM) for 2 min followed by fMLP (1 μM) for 10 min. *D*, neutrophils were preincubated for 5 min with the indicated concentrations of Pyrrolidine-1 or Me-Indoxam and then treated with vehicle or GM-CSF (1 nM) for 60 min followed by fMLP (1 μM) for 10 min. In each panel, *black* and *gray bars* show results from duplicate experiments that are representative of ≥5 independent studies. Arachidonic acid mass release was measured by gas chromatography/electron capture mass spectrometry. *cyto b*, cytochrome *b*.

TABLE I
Arachidonate release by OZ-stimulated mouse neutrophils

Genotype	PLA$_2$g4a(+/+)	PLA$_2$g4a(−/−)	PLA$_2$g4a(+/+)	PLA$_2$g4a(−/−)
Experiment	1	1	2	2
Vehicle	7[a]	9	6	5
OZ	31	10	45	7

[a] fmol of arachidonic acid/10^6 neutrophils/10 min. Estimated detection limit for arachidonate is 2 fmol. Results from two independent experiments are shown. ANOVA, $p < 0.01$, *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−).

mine whether the lack of cPLA$_2$-α caused a complete *versus* a near-complete reduction in the amount of OZ-stimulated neutrophil PAF biosynthesis. Collectively, our pharmacological inhibition and gene inactivation studies provide the first direct evidence that cPLA$_2$-α activity is necessary for PAF biosynthesis by neutrophils in response to a phagocytic stimulus.

cPLA$_2$-α Gene Inactivation Inhibits Pulmonary PAF Biosynthesis and E. coli Clearance in Vivo—The generation of cPLA$_2$-α catalyzed bioactive lipids such as arachidonic acid, LTB$_4$ (6), and PAF is requisite for an effective innate immune response to microbicidal infection. To begin to evaluate the role of cPLA$_2$-α in innate immunity, we utilized a murine model of Gram-negative bacterial pulmonary infection (29). For these experiments, live *E. coli* (10^9 colony-forming units) were injected into the trachea of anesthetized *PLA$_2$g4a*(−/−) mice or their wild type littermates and pulmonary bacterial clearance was assessed by quantitative culture of BAL fluid and lung homogenates. Three hours after intratracheal inoculation, significantly more viable *E. coli* were recovered in the BAL fluid, lung parenchyma, and whole lung homogenates (*i.e.* lungs prior to lavage) of *PLA$_2$g4a*(−/−) mice compared with *PLA$_2$g4a*(+/+) mice (Fig. 3, *A–C*). No bacteria were identified in blood samples obtained before or 3 h after intratracheal administration of live *E. coli* in either *PLA$_2$g4a*(+/+) or *PLA$_2$g4a*(−/−) mice. Therefore, in comparison with wild type mice, bacterial clearance in the lungs of mice lacking cPLA$_2$-α was impaired.

Three hours after intratracheal inoculation of live *E. coli*, significantly higher levels of PAF and inflammatory cells and significantly lower levels of total protein were present in the BAL fluid obtained from wild type mice compared with *PLA$_2$g4a*(−/−) mice (Table III). In contrast, no significant differences in BAL levels of a variety of cytokines and chemokines including TNFα, interferon-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, or MCP-1 were identified following intratracheal inoculation of live *E. coli* in *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice (Table III). Arachidonic acid levels in the BAL fluid obtained from *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice were below the level of detection of the gas chromatography/mass spectrometry assay used in this study. In concert, these studies provide the first direct evidence that cPLA$_2$-α regulates PAF biosynthesis and the innate immune response to pulmonary infection with *E. coli in vivo*.

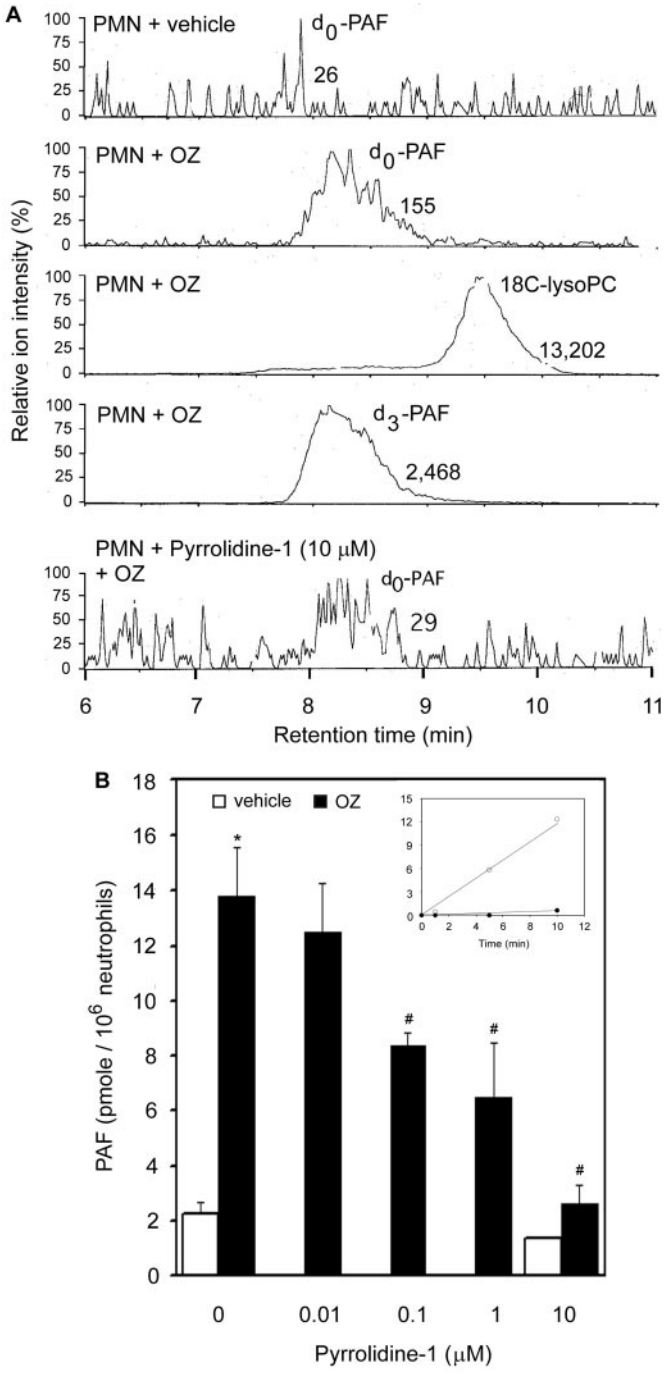

FIG. 2. **cPLA$_2$-α regulates PAF synthesis by human neutrophils.** *A*, neutrophils were treated with vehicle or Pyrrolidine-1 (10 μM) for 5 min. Cells were then treated with vehicle or OZ (5 mg/ml) for 10 min as indicated in the figure. Following Bligh and Dyer extraction (24), d_0-PAF, C_{18}-lyso-PC, and d_3-PAF mass were measured by HPLC-tandem mass spectrometry. In each case, the peak ion intensity is normalized to 100% and the area under the curve for each major peak is presented numerically (*i.e.* PMN + vehicle; d_0-PAF = 26). Representative results from > 20 experiments are shown. Note that C_{18}-lyso-PC and C_{16}-PAF have the same mass and are not fully resolved during HPLC but that C_{16}-PAF is selectively detected by tandem mass spectrometry as described under "Experimental Procedures." *PMN*, polymorphonuclear leukocyte. *B*, neutrophils were preincubated with increasing concentrations of Pyrrolidine-1 (5 min) and then treated with vehicle (*open bars*) or OZ (5 mg/ml, *filled bars*) for 10 min. *Insert*, time course of PAF synthesis after treatment with OZ (*open circles*) or Pyrrolidine-1 (10 μM, 5 min) and then OZ (5 mg/ml, *filled circles*). Data represent the mean ± S.D. of $n = 6–8$ experiments for each group. ANOVA, $p < 0.001$; *, $p < 0.01$, vehicle *versus* OZ; #, $p < 0.01$, OZ *versus* Pyrrolidine-1 and subsequent treatment with OZ.

cPLA$_2$-α Does Not Regulate Neutrophil NADPH Oxidase Activity—The generation of cytotoxic oxidants by the membrane-bound phagocytic NADPH oxidase is required for efficient bacterial killing by neutrophils, and cPLA$_2$-α has been implicated in the assembly of the NADPH oxidase in human and murine neutrophils and in a granulocyte cell line (8, 9). Therefore, we tested the hypothesis that the defect in *E. coli* killing observed in the murine *E. coli* pneumonia model was due to impaired activation of the neutrophil NADPH oxidase in *PLA$_2$g4a*(−/−) mice. To evaluate this hypothesis, cPLA$_2$-α activity was inhibited by incubation with Pyrrolidine-1 (human neutrophils) or by gene inactivation (murine neutrophils) and NADPH oxidase activity was assessed with three independent assays. The cytochrome *c* reduction assay measures extracellular superoxide production, the luminol chemiluminescence assay measures extracellular and intracellular oxidant production, and the dihydrorhodamine reduction assay measures cellular H_2O_2 biosynthesis.

Incubation with Pyrrolidine-1 (10 μM, sufficient to fully block arachidonic acid release and PAF production) had no effect on the extent or rate of cytochrome *c* reduction by human neutrophils following exposure to OZ, PMA, or PAF and OZ compared with vehicle-treated cells (Fig. 4, *A–D*). Similarly, exposure to OZ (Fig. 4*E*) or to PMA or PAF and OZ (Fig. 4*F*) resulted in comparable levels of chemiluminescence in vehicle and pyrollidine-1 (10 μM) pretreated human neutrophils. Preincubation with Me-Indoxam also failed to inhibit neutrophil cytochrome *c* reduction (data not shown) or cellular chemiluminescence after exposure to OZ (Fig. 4*G*) or to PMA or PAF and OZ (Fig. 4*H*). Finally, treatment with OZ (Fig. 4*I*) or with fMLP or PMA (Fig. 4*J*) resulted in similar levels of dihydrorhodamine reduction by neutrophils harvested from the bone marrow of *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice. Taken together, the results of our pharmacological and gene inactivation studies rule out a role for cPLA$_2$-α in the activation of the NADPH oxidase in human or murine neutrophils. Therefore, the defect in bacterial killing following intratracheal inoculation of live *E. coli* in *PLA$_2$g4a*(−/−) mice (*cf* Fig. 3) could not be explained by deficient neutrophil oxidant production.

cPLA$_2$-α-mediated Arachidonic Acid Release Is Necessary for Efficient E. coli Killing by Neutrophils in Vitro—The killing of bacteria such as *E. coli* by neutrophils is a complex process that involves multiple sequential steps including the expression of specific surface receptors, secretion of granule contents, binding of bacteria to neutrophil surface receptors, internalization (phagocytosis), and intracellular killing (33). Neutrophil extracellular traps may also degrade virulence factors and kill extracellular bacteria (34). To define the role of cPLA$_2$-α in bacterial killing by neutrophils *in vitro*, neutrophils from *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice or human neutrophils treated with Pyrrolidine-1 were studied.

A lack of functional cPLA$_2$-α had no effect on neutrophil FcγII/III surface expression or on fMLP-, PMA-, or OZ-stimulated surface expression of CD11b, two key phagocytic receptors (Table IV). Similarly, preincubation of human neutrophils with Pyrrolidine-1 had no effect on the surface expression of CD63 or CD66b, markers for primary (azurophilic) and secondary (specific) granules, respectively, by neutrophils treated with ionomycin or cytochalasin D and aggregated IgG, which are potent stimulators of neutrophil granule secretion (Fig. 5, *A* and *B*). Functional cPLA$_2$-α deletion also had no effect on phagocytosis of *E. coli* by murine neutrophils *in vitro* (Table IV). In contrast, neutrophils harvested from the bone marrow of *PLA$_2$g4a*(−/−) mice exhibited a 42% decrease in killing of live *E. coli in vitro* compared with neutrophils from *PLA$_2$g4a*(+/+) mice (Table IV).

TABLE II
PAF biosynthesis by OZ-stimulated mouse neutrophils
Results of three independent experiments are shown. ANOVA, p 0.005, neutrophils from *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−) mice.

Genotype[a]	*PLA$_2$g4a*(+/+)	*PLA$_2$g4a*(−/−)	*PLA$_2$g4a*(+/+)	*PLA$_2$g4a*(−/−)	*PLA$_2$g4a*(+/+)	*PLA$_2$g4a*(−/−)
Experiment	1	1	2	2	3	3
Vehicle	ND[b]	ND	ND	ND	ND	ND
OZ	170[a]	ND	410	ND	570	ND

[a] fmol of PAF/10^6 neutrophils/10 min.
[b] ND, not detected. Estimated detection limit for PAF is 100 fmol.

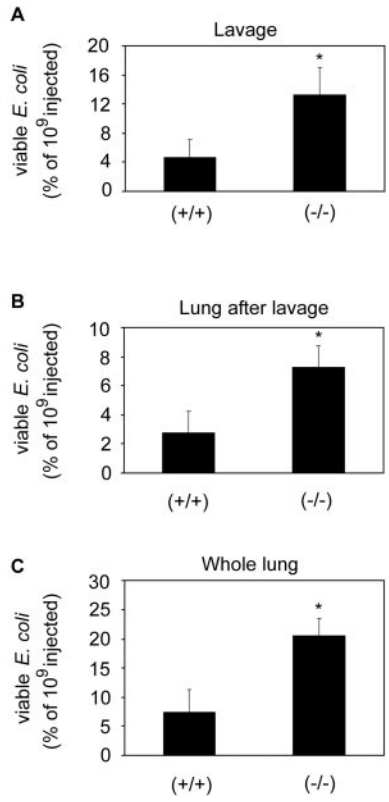

FIG. 3. **Inactivation of the gene encoding cPLA$_2$-α inhibits pulmonary *E. coli* clearance *in vivo*.** Following induction of anesthesia, live *E. coli* (10^9 colony-forming units) were injected into the lungs of *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice. After 3 h, *E. coli* levels in BAL fluid (*A*), lung parenchyma (*B*) and whole lungs (*C*) were measured by serial dilution culture. Data represent the mean ± S.D. from three *PLA$_2$g4a*(+/+) and three *PLA$_2$g4a*(−/−) mice analyzed on the same day and are representative of three sets of experiments carried out on different days. *, $p < 0.01$, *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−) mice.

To study the potential role of cPLA$_2$-α-dependent arachidonic acid release and PAF generation on neutrophil-mediated *E. coli* killing, human neutrophils treated with Pyrrolidine-1 (10 μM) or neutrophils from the bone marrow of *PLA$_2$g4a*(+/+) or *PLA$_2$g4a*(−/−) mice were treated with exogenous arachidonic acid or PAF prior to assessment of neutrophil-mediated *E. coli* killing *in vitro*. The inhibition of cPLA$_2$-α in human neutrophils with Pyrrolidine-1 was associated with a decrease in neutrophil-mediated *E. coli* killing *in vitro* from 63 ± 7 to 45 ± 1% ($p = 0.0008$) (Fig. 6*A*), an observation consistent with the results of our cPLA$_2$-α gene inactivation studies with mouse neutrophils (Table IV). The addition of exogenous PAF increased *E. coli* killing by untreated neutrophils and partially reversed the defect in *E. coli* killing observed in Pyrrolidine-1-treated human neutrophils (Fig. 6*A*). Preincubation with arachidonic acid had no effect on *E. coli* killing by untreated neutrophils but reversed the defect in *E. coli* killing observed in Pyrrolidine-1-treated cells (63 ± 7 *versus* 59 ± 3%, vehicle *versus* Pyrrolidine-1 plus exogenous arachidonate, $p = 0.28$) (Fig. 6*A*).

Incubation of *E. coli* with neutrophils from *PLA$_2$g4a*(+/+) mice resulted in significantly more bacterial killing than when *E. coli* were co-incubated with neutrophils from *PLA$_2$g4a*(−/−) mice *in vitro* (61 ± 3 *versus* 42 ± 6%, respectively, $p = 0.004$) (Fig. 6*B*). Exogenous addition of either PAF or arachidonic acid both significantly increased *E. coli* killing by neutrophils from *PLA$_2$g4a*(+/+) mice (Fig. 6*B*). Preincubation with arachidonic acid, but not PAF, reversed the defect in *E. coli* killing observed in neutrophils from *PLA$_2$g4a*(−/−) mice (61 ± 3 *versus* 59 ± 7%, neutrophils from *PLA$_2$g4a*(+/+) mice *versus* neutrophils from *PLA$_2$g4a*(−/−) mice plus exogenous arachidonate, $p = 0.59$) (Fig. 6*B*). Taken together, our pharmacological inhibition and gene inactivation studies provide direct evidence that cPLA$_2$-α-catalyzed arachidonic acid release and, to a lesser extent, cPLA$_2$-α-dependent PAF biosynthesis are necessary for efficient neutrophil-mediated *E. coli* killing *in vitro*.

In addition to arachidonic acid release (Fig. 1) and PAF biosynthesis (Fig. 2), cPLA$_2$-α also regulates LTB$_4$ production by human neutrophils (6). Intratracheal challenge with *Klebsiella pneumoniae* markedly increased lung leukotriene levels in wild type mice, and mice with targeted disruption of the 5-lipoxygenase (5-LO) gene manifest a greater degree of bacteremia and lethality following intratracheal *K. pneumoniae* injection than wild type littermates (35). In addition, alveolar macrophages from 5-LO(−/−) mice exhibit impaired bacterial phagocytosis and killing *in vitro* in comparison with macrophages from 5-LO(+/+) mice, defects that are reversed by addition of exogenous LTB$_4$ (35, 36). Therefore, in addition to arachidonic acid and PAF, LTB$_4$ may also participate in the regulation of neutrophil-mediated *E. coli* killing *in vitro*.

To assess the potential role of LTB$_4$ in neutrophil-mediated *E. coli* killing, neutrophils from wild type mice were incubated with the 5-LO inhibitor, MK886, or the LTB$_4$ receptor antagonist, U75302, prior to co-incubation with live *E. coli*. Controls included cells pretreated with vehicle (Me_2SO) or LTB$_4$. As shown in Fig. 5*C*, pretreatment with MK886 or U75302 or exogenous addition of LTB$_4$ had no effect on *E. coli* killing by neutrophils from wild type mice *in vitro*. Furthermore, exogenous administration of LTB$_4$ failed to reverse the killing defect observed in neutrophils from cPLA$_2$-α(−/−) mice. Taken together, these results do not support a role for LTB$_4$ in the pathophysiology of neutrophil-mediated *E. coli* killing *in vitro*.

DISCUSSION

Our studies demonstrate the importance of cPLA$_2$-α in neutrophil arachidonic acid release, PAF biosynthesis, bacterial killing, and the innate immune response to bacterial infection *in vivo*. Importantly, we provide direct evidence that synthesis of arachidonic acid by cPLA$_2$-α plays a role in neutrophil-mediated bacterial killing. These observations document a novel and physiologically important role for cPLA$_2$-α in host defense against bacterial infection.

Role of cPLA$_2$-α in Neutrophil PAF Biosynthesis—PAF is a potent pro-inflammatory mediator with diverse biological functions. Neutrophils are a major source of PAF *in vivo*, and two biosynthetic pathways have been proposed for PAF biosynthesis in these cells (37). In the first pathway, a PLA$_2$ catalyzes the

TABLE III
Composition of BAL fluid 3 h after intratracheal inoculation of live E. coli in PLA$_2$g4a(+/+) and PLA$_2$g4a(−/−) mice in vivo

Bronchoalveolar lavage fluid	*PLA$_2$g4a*(+/+)	*PLA$_2$g4a*(−/−)	*n*	*p* value[a]
PAF (fmol)	670 ± 170	40 ± 80	5	0.002
Cell count (×10^5)	3.42 ± 1.09	1.33 ± 0.77	6	0.0003
Total protein (mg/liter)	0.17 ± 0.04	0.29 ± 0.08	6	0.013
TNFα (units/ml)	1,181 ± 1,188	1,459 ± 1,401	9	0.66
Interferon-γ (units/ml)	1.64 ± 0.86	1.43 ± 1.19	9	0.67
IL-2 (units/ml)	1.33 ± 0.84	0.73 ± 0.78	9	0.14
IL-4 (units/ml)	3.83 ± 2.36	4.03 ± 0.67	9	0.81
IL-5 (units/ml)	2.68 ± 0.55	2.26 ± 1.41	9	0.42
IL-6 (units/ml)	3.46 ± 0.84	11.57 ± 13.38	9	0.11
IL-10 (units/ml)	77.60 ± 15.28	76.73 ± 27.97	9	0.94
IL-12p70 (units/ml)	8.31 ± 7.01	8.14 ± 4.85	9	0.95
MCP-1 (units/ml)	15.53 ± 5.94	13.66 ± 7.90	9	0.58

[a] *PLA$_2$g4a*(+/+) versus *PLA$_2$g4a*(−/−) mice, paired Student's *t* test (two-tailed and unequal variance).

hydrolysis of 1-*O*-alkyl-2-acyl-phosphatidylcholine yielding lyso-PAF, which is acetylated by acetyl-CoA:1-*O*-alkyl-2-lyso-phosphatidylcholine acetyltransferase to produce PAF. In the second pathway, lyso-PAF is formed by a CoA-independent transacylase, which transfers the *sn*-2 fatty acyl group from 1-*O*-alkyl-2-acyl-phosphatidylcholine to a 1-acyl-lyso-phospholipid. PAF may also be generated *de novo* by acetylation of 1-alkyl-2-lyso-*sn*-glycerophosphate, the removal of the phosphate group and transfer of phosphocholine from CDP-choline (38). The pathway that mediates PAF biosynthesis in neutrophils has not been conclusively identified. In the current study, using two complementary approaches, the pharmacological inhibition with the highly potent and selective cPLA$_2$-α inhibitor Pyrrolidine-1 (19) in human neutrophils and cPLA$_2$-α gene deletion in murine neutrophils, we clearly document a role for cPLA$_2$-α in PAF biosynthesis by neutrophils in response to the physiologically relevant phagocytic stimulus OZ *in vitro* and during the inflammatory response induced by pulmonary inoculation of live *E. coli in vivo*. Earlier attempts to explore the role of cPLA$_2$-α in PAF production by neutrophils and basophils using the cPLA$_2$-α inhibitors AACOCF$_3$ or methylarachidonyl fluorophosphonate are complicated by the demonstration that these compounds also inhibit CoA-independent transacylase (39) and PAF-acetylhydrolase (40), respectively. Our findings are consistent with recent studies of the effect of mitogen-activated protein kinase inhibitors on cPLA$_2$-α activity, which indirectly suggest that the PLA$_2$ pathway is the major route of PAF synthesis in fMLP- and calcium ionophore-stimulated neutrophils (41). Although a role for CoA-independent transacylase in neutrophil PAF biosynthesis is still postulated (41), our data suggest that cPLA$_2$-α provides the lyso-phospholipid substrate for the transacylase. Taken together, our studies are the first to document a central role for cPLA$_2$-α in PAF biosynthesis by neutrophils *in vitro* and the innate immune response to bacterial infection *in vivo*. In this regard, the production of PAF by the first wave of neutrophils infiltrating infected tissues may serve to recruit additional inflammatory cells to combat invading microbial pathogens. In support of this notion, PAF levels and the number of inflammatory cells in BAL fluid were ~10- and 3-fold lower, respectively, in *PLA$_2$g4a*(−/−) mice compared with wild type mice following pulmonary infection with *E. coli*. The attenuated inflammatory response to pulmonary infection with *E. coli* may explain why decreased clearance of *E. coli* was observed in *PLA$_2$g4a*(−/−) mice compared with *PLA$_2$g4a*(+/+) mice.

Role of cPLA$_2$-α in Neutrophil Arachidonic Acid Release but Not NADPH Oxidase Activation—Previous work has suggested that arachidonic acid, specifically cPLA$_2$-α-generated arachidonic acid, is required for activation of the granulocytic NADPH oxidase (9, 10). Furthermore, the addition of micromolar amounts of arachidonic acid to cells and cell-free suspensions containing NADPH oxidase components leads to oxidase activation (42). However, the physiological relevance of these findings is called into question by the observation that a variety of anionic amphiphiles including SDS can activate NADPH oxidase *in vitro* by inducing a structural change in the oxidase complex (43) and it has been suggested that arachidonic acid may produce nonspecific effects in neutrophils (44). Other studies suggesting a PLA$_2$-NADPH oxidase connection are based on the use of PLA$_2$ inhibitors of low potency and/or imperfect specificity (45). In contrast, the reduction of cPLA$_2$-α levels using antisense technology led to the suppression of superoxide production in the granulocyte cell line PLB-985 (8), a finding consistent with a role for cPLA$_2$-α in NADPH oxidase activation. To resolve the role of cPLA$_2$-α in neutrophil arachidonic acid release and NADPH oxidase activation, we studied human neutrophils treated with the highly potent and specific cPLA$_2$-α inhibitor Pyrrolidine-1 as well as neutrophils from *PLA$_2$g4a*(−/−) mice. The results unequivocally show that cPLA$_2$-α is responsible for all of the detectable arachidonic acid release in agonist-stimulated human and murine neutrophils. However, neutrophil NADPH oxidase activation, measured by three independent assays, is unaffected by inhibition or genetic deletion of cPLA$_2$-α (*cf* Fig. 4), thereby ruling out a role for cPLA$_2$-α in neutrophil oxidant production. Similarly, recent studies have shown that superoxide production is normal in zymosan-stimulated peritoneal macrophages isolated from *PLA$_2$g4a*(−/−) mice (46). Based on antisense knockdown of cPLA$_2$-α, it has been suggested that this enzyme is required for superoxide production in monocytes (10). Further study of superoxide production in monocytes is warranted in light of the studies with neutrophils (c. f. Fig. 4) and macrophages (46). It is possible that earlier findings suggesting a role for cPLA$_2$-α in NADPH oxidase activation using antisense technology may be due to nonspecific off-target effects, which have recently been well documented (47).

Role of cPLA$_2$ in Neutrophil-mediated Bacterial Killing and Innate Immunity—The process of neutrophil-mediated bacterial killing follows a specific sequence of events that includes recognition and binding of bacteria to surface receptors, transportation of new membrane to the plasma membrane of nascent phagosomes, and internalization (phagocytosis) of bacteria. Subsequent maturation of developing phagosomes proceeds by complex processes that include recruitment and activation of the NADPH oxidase, fusion of phagosomes with intracellular lysosomal granules, and acidification of the phagolysosome (33). A potential role for cPLA$_2$-α in neutrophil-bacterial killing is suggested by the translocation of cPLA$_2$-α from the cytosol to phagosomes during the early stages of phagocytosis (5). Our studies document for the first time that pharmacological inhibition or genetic deletion of cPLA$_2$-α attenuates neutrophil-mediated bacterial killing *in vitro* and im-

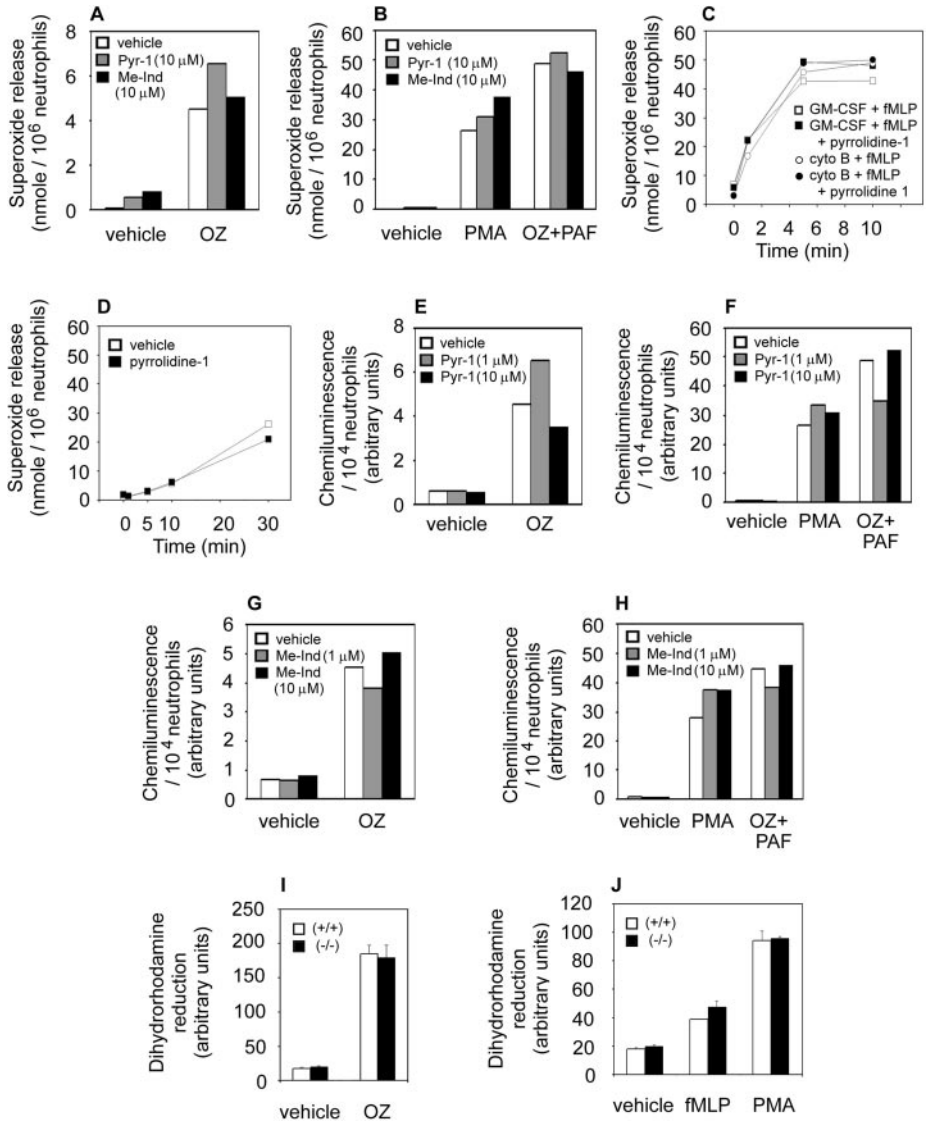

FIG. 4. **cPLA$_2$-α does not regulate neutrophil NADPH oxidase activity.** Human neutrophils were incubated with vehicle (*open bars*), Pyrrolidine-1 (*Pyr-1*, 10 μM, *gray bars*), or Me-Indoxam (*Me-Ind*, 10 μM, *filled bars*) for 5 min. Cytochrome *c* reduction was measured after treatment with vehicle or OZ (1 mg/ml) for 30 min (*A*) or vehicle, PMA (100 nM), or PAF (12.5 nM) and OZ (1 mg/ml) for 30 min (*B*). *C*, time course of cytochrome *c* reduction after pretreatment with GM-CSF (1 nM, 60 min) and then fMLP (1 μM, *open square*); pretreatment with Pyrrolidine-1 (10 μM, 5 min) and GM-CSF (1 nM, 60 min) and then fMLP (1 μM, *filled square*); pretreatment with cytochalasin B (5 μM, 2 min) and then fMLP (1 μM, *open circle*); or pretreatment with Pyrollidine-1 (10 μM, 5 min) and cytochalasin B (5 μM, 2 min) and then fMLP (1 μM, *filled circle*). *D*, time course of cytochrome *c* reduction after treatment with OZ (1 mg/ml, *open square*) with or without pretreatment with Pyrollidine-1 (10 μM, 5 min). Human neutrophils were incubated with vehicle (*open bars*), 1 μM Pyrrolidine-1 (*gray bars*), or 10 μM Pyrrolidine (*filled bars*) for 5 min. Cellular chemiluminescence was then measured after treatment with vehicle or OZ (1 mg/ml) for 15 min (*E*) or vehicle, PMA (100 nM), or PAF (12.5 nM) and OZ (0.3 mg/ml) for 15 min (*F*). Human neutrophils were incubated with vehicle (*open bars*), 1 μM Me-Indoxam (*gray bars*), or 10 μM Me-Indoxam (*filled bars*) for 5 min. Cellular chemiluminescence was then measured after treatment with vehicle or OZ (1 mg/ml) for 15 min (*G*) or vehicle, PMA (100 nM), or PAF (12.5 nM) and OZ (0.3 mg/ml) for 15 min (*H*). In *panels A–H*, representative data from 3–5 separate experiments, each done in triplicate using neutrophils obtained from different blood donors, are shown. Neutrophils were harvested from the bone marrow of *PLA$_2$g4a*(+/+) and *PLA$_2$g4a*(−/−) mice. Following treatment with vehicle or OZ (1 mg/ml) for 10 min (*I*) or vehicle, fMLP (1 μM, 2 min), or PMA (100 nM, 10 min) (*J*), dihydrorhodamine reduction was measured by flow cytometry. Data in *panels I* and *J* represent the mean ± S.D. of one experiment done in triplicate and are representative of five independent experiments.

TABLE IV

cPLA$_2$ partially regulates E. coli killing in vitro but has no effect on neutrophil-mediated E. coli phagocytosis or neutrophil FcγII/III or CD11b surface expression

	FcγII/III surface expression	CD11b surface expression				*E. coli* phagocytosis by neutrophils *in vitro*	*E. coli* killing by neutrophils *in vitro*
		Control	fMLP	PMA	OZ		
		arbitrary units				%	
PLA$_2$g4a(+/+)	26 ± 2	63 ± 34	136 ± 81	413 ± 196	85 ± 34	28 ± 16	59 ± 9.4
PLA$_2$g4a(−/−)	25 ± 2	68 ± 29	171 ± 141	353 ± 141	94 ± 38	34 ± 6	34 ± 15
n	6	5	5	5	5	5	7
p Value[a]	0.18	0.94	0.63	0.55	0.63	0.49	0.005

[a] Neutrophils from *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−) mice, paired Student's *t* tests (two-tailed and unequal variance).

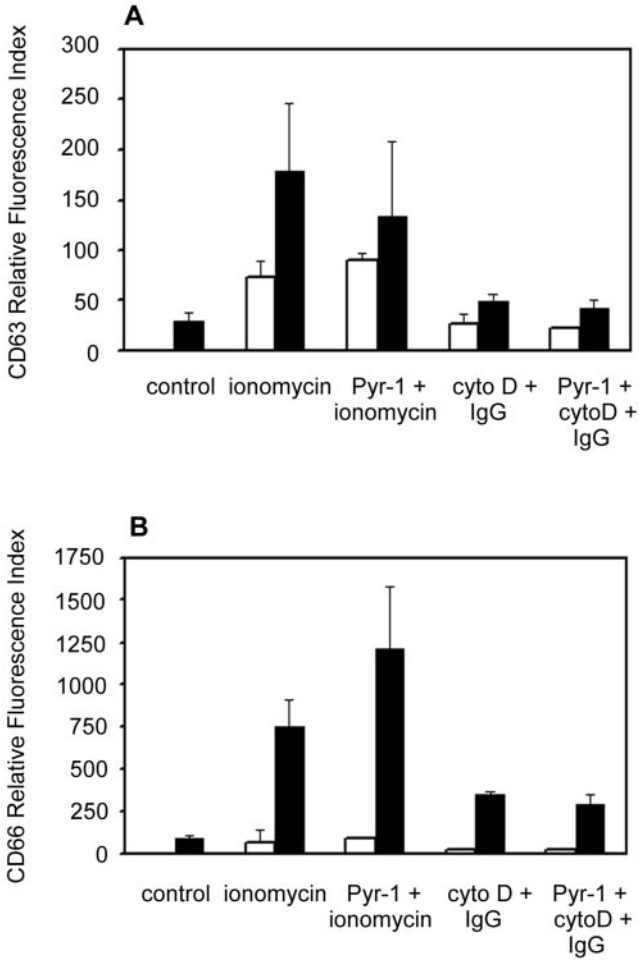

FIG. 5. **cPLA$_2$-α does not regulate the secretion of primary or secondary neutrophil granules.** Human neutrophils were incubated with Me_2SO (0.1%) or Pyrrolidine-1 (*Pyr-1*, 10 μM) for 10 min at 37 °C. Cells were then washed and incubated with ionomycin (2 μM) or cytochalasin D (10 μM) and aggregated human IgG for 10 min at 37 °C as indicated in the figure legends. The surface expression of αCD63 (*A*, *filled bars*) and αCD66b (*B*, *filled bars*) was then measured by fluorescence-activated cell sorter analysis. Results are reported as relative fluorescence index. In *panels A* and *B*, the *open bars* represent fluorescence-activated cell sorter analysis of cells incubated with anti αN-RAS antisera (N-RAS, a small GTPase, was used as a control for studies evaluating the surface expression of αCD63 and αCD66b on human neutrophils). Results are the mean ± S.D. of three independent experiments done in triplicate.

pairs pulmonary bacterial clearance *in vivo*. The critical role of cPLA$_2$-α activity in bacterial killing was confirmed by demonstrating that exogenous addition of arachidonic acid partially reverses the defect in neutrophil-bacterial killing induced by inhibition or genetic deletion of cPLA$_2$-α. In this regard, it is noteworthy that cPLA$_2$-α gene disruption diminishes neutrophil-dependent bacterial killing by 42%, a substantial effect that is comparable to the effect of disruption of the granule enzyme elastase (48) on neutrophil-mediated *E. coli* killing *in vitro*. In contrast, the deletion of other phagocyte genes, such as the gp47^{phox} or the gp91^{phox} components of the NADPH oxidase (49), had a more pronounced effect on pulmonary *E. coli* clearance *in vivo* than the deletion of *PLA$_2$g4a*.

Abolishing cPLA$_2$-α activity had no effect on neutrophil FcγII/III or CD11b surface receptor expression, primary or secondary granule secretion, bacterial phagocytosis by neutrophils or activation of the neutrophil NADPH oxidase *in vitro*, or the generation of selected pro-inflammatory cytokines or chemokines following tracheal inoculation with live *E. coli in vivo*. Thus, the defects in these processes cannot explain the defects in neutrophil-mediated bacterial killing and pulmonary bacterial clearance associated with inhibition or deletion of cPLA$_2$-α. As arachidonate promotes the fusion of complex liposomes with each other as well as with specific granules isolated from human neutrophils (50), it is possible that the defect in bacterial killing induced by the inhibition of cPLA$_2$-α activity is due to impaired phagolysosome maturation and failure to deliver antimicrobial factors such as proteolytic enzymes, antimicrobial peptides, and proteins such as defensins and cathelicidins in high concentration to the compartmentalized pathogen. Alternatively, the impaired bacterial killing associated with inhibition or deletion of cPLA$_2$-α may be due to defective or suboptimal activation of signaling pathways modulated by arachidonic acid, such as 3-phosphoinositide-dependent protein kinase-mediated activation of protein kinase C-ζ (51), whereas protein kinase C-ζ is expressed in neutrophils and has been implicated in phagocytosis (52, 53). However, because bacterial phagocytosis was not impaired in neutrophils derived from cPLA$_2$-α-deficient mice or in macrophages treated with Pyrrolidine-1 (5), it is possible that cPLA$_2$-α participates in the regulation of phagocytosis-independent bacterial killing such as the process mediated by neutrophil extracellular traps (34). The defect in bacterial killing observed with neutrophils from cPLA$_2$-α-deficient mice was not reversed by exogenous addition of LTB$_4$. In addition, the inhibition of 5-lipoxygenase activity or treatment with a LTB$_4$ receptor antagonist had no effect on *E. coli* killing by neutrophils from cPLA$_2$-α(+/+) mice. Therefore, in contrast to the clear role of LTB$_4$ in macrophage-mediated phagocytosis and killing of *K. pneumoniae* (35, 36), LTB$_4$ does not appear to participate in the regulation of neutrophil-mediated *E. coli* killing *in vitro*.

We noted that protein levels were significantly higher in the BAL fluid obtained from *PLA$_2$g4a*(−/−) than from *PLA$_2$g4a*(+/+) mice following pulmonary infection with *E. coli*. In contrast, higher BAL protein levels were identified in the BAL fluid obtained from *PLA$_2$g4a*(+/+) than from *PLA$_2$g4a*(−/−) mice following acid aspiration or infusion of lipopolysaccharide-zymosan (17). These finding are consistent with differential roles for cPLA$_2$-α in infectious and inflammatory pulmonary diseases. It is possible that the increase in BAL protein in the *PLA$_2$g4a*(−/−) mice observed after intratracheal inoculation of live *E. coli* is due to higher concentrations of intrapulmonary *E. coli* compared with *PLA$_2$g4a*(+/+) mice and a proportional increase in *E. coli* protein synthesis. Alternatively, increased production of *E. coli* toxins that can alter pulmonary microvascular permeability and facilitate the transfer of proteins from blood to alveoli could account for the increased protein levels in the BAL fluid from *PLA$_2$g4a*(−/−) mice after pulmonary infection with *E. coli*.

Therapeutic Implications of Inhibiting cPLA$_2$ Activity, Susceptibility to E. coli Infection—Deletion of a functional cPLA$_2$-α gene attenuates the development of arthritis (54) and bone resorption (55) in experimental models. In addition, inactivation of the gene encoding cPLA$_2$-α decreases the pulmonary dysfunction induced by acid aspiration or the intravenous infusion of lipopolysaccharide-zymosan and attenuates bleomycin-induced pulmonary fibrosis (17, 18). Based on these studies, the inhibition of cPLA$_2$-α has been proposed as a potential therapeutic strategy for the management of arthritis, bone resorption, and pulmonary inflammation.

Our data demonstrate that cPLA$_2$-α activity is necessary for efficient neutrophil-mediated *E. coli* killing and eradication of pulmonary *E. coli in vivo*. Similarly, genetic defects in or pharmacological interference with other aspects of the innate immune system, such as the blockade of TNFα (56), deletion of

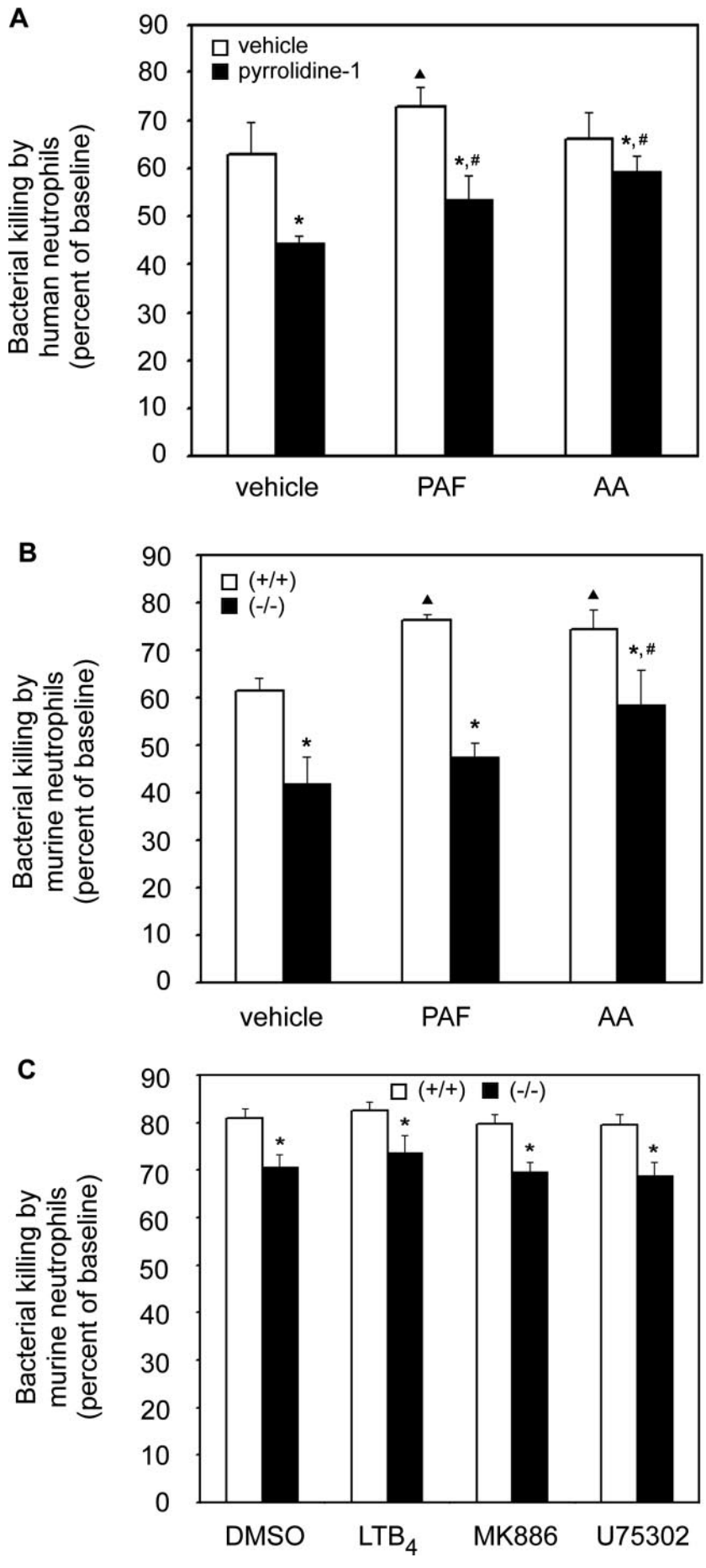

FIG. 6. **Exogenous arachidonic acid partially reverses the defect in neutrophil-mediated *E. coli* killing induced by inhibition or genetic deletion of cPLA$_2$-α.** *A*, human neutrophils were incubated with vehicle (*open bars*) or Pyrrolidine-1 (10 μM, 5 min, *filled bars*). Cells were then treated with vehicle, PAF (500 nM, 5 min), or arachidonic acid (5 μM, 5 min) prior to co-culture with live *E. coli* at a bacteria to neutrophil ratio of 3:1 for 60 min at 37 °C. Bacterial killing was measured by serial dilution culture as described under "Experimental Procedures." Data represent the mean ± S.D. of one experiment done at both 100- and 1,000-fold dilutions (each in triplicate) and are representative of five independent experiments using neutrophils obtained from different blood donors. ANOVA, $p < 2 \times 10^{-10}$, all Me_2SO (*DMSO*) *versus* all Pyrrolidine-1-treated samples. *, $p < 0.02$, vehicle *versus* Pyrrolidine-1, PAF *versus* Pyrrolidine plus PAF, arachidonic acid *versus* Pyrrolidine plus arachidonic acid; ▲, $p < 0.02$, vehicle *versus* vehicle plus PAF; #, $p < 0.005$, Pyrrolidine-1 *versus* Pyrrolidine-1 plus arachidonic acid (*AA*) or PAF. *B*, neutrophils from *PLA$_2$g4a*(−/−) mice or their wild type littermates were treated with vehicle, PAF (500 nM, 5 min), or arachidonic acid (5 μM, 5 min) prior to co-culture with live *E. coli* at a bacteria to neutrophil ratio of 3:1 for 60 min at 37 °C. Bacterial killing was measured by serial dilution culture. Data represent the mean ± S.D. of one experiment done at both 100- and 1,000-fold dilutions (each in triplicate) and are representative of four independent experiments using neutrophils obtained from different mice. ANOVA, $p < 3 \times 10^{-6}$, all *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−) samples. *, $p < 0.01$, *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−), *PLA$_2$g4a*(+/+) plus PAF *versus* *PLA$_2$g4a*(−/−) plus PAF, *PLA$_2$g4a*(+/+) plus arachidonic acid *versus* *PLA$_2$g4a*(−/−) plus arachidonic acid; ▲, $p < 0.01$, *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(+/+) plus arachidonic acid or PAF; #, $p < 0.02$, *PLA$_2$g4a*(−/−) *versus* *PLA$_2$g4a*(−/−) plus arachidonic acid. *C*, neutrophils from *PLA$_2$g4a*(−/−) mice (*filled bars*) or their wild type littermates (*open bars*) were treated with vehicle (DMSO, 0.1%), MK886 (1 μM), or U75302 (10 μM) for 5 min at 37 °C. Cells were then treated with DMSO or LTB_4 (10 nM) for 5 min at 37 °C and subsequently co-cultured with live *E. coli* at a bacteria to neutrophil ratio of 3:1 for 60 min at 37 °C. Bacterial killing was measured by serial dilution culture. Data represent the mean ± S.D. of three independent experiments done at both 100 and 1,000-fold dilutions (each in triplicate). ANOVA, *p*, 0.0002, all *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−) samples; *, $p < 0.05$, *PLA$_2$g4a*(+/+) *versus* *PLA$_2$g4a*(−/−), *PLA$_2$g4a*(+/+) plus LTB_4 *versus* *PLA$_2$g4a*(−/−) plus LTB_4, *PLA$_2$g4a*(+/+) plus MK866 *versus* *PLA$_2$g4a*(−/−) plus MK866, *PLA$_2$g4a*(+/+) plus U75302 *versus* *PLA$_2$g4a*(−/−) plus U75302.

C5a receptors (57), or inactivation of elastase, cathepsin G (58), or phosphatidylinositol-3 kinase (59), have all been shown to diminish inflammatory tissue damage at the price of compromised host defense against microbial pathogens. Therefore, in addition to defining a novel role for cPLA$_2$-α in host defense against bacterial infection, our study suggests that the inhibition of cPLA$_2$-α, while potentially beneficial to patients with a diverse array of inflammatory pathologies, may be associated with a diminished capacity to eradicate Gram-negative pulmonary infections.

REFERENCES

1. Kochanek, K. D., and Smith, B. L. (2004) *Natl. Vital. Stat. Rep.* **52,** 1–47
2. Baughman, R. P. (1999) *Respiration* **66,** 95–109
3. Zhang, P., Summer, W. R., Bagby, G. J., and Nelson, S. (2000) *Immunol. Rev.* **173,** 39–51
4. Leslie, C. C. (1997) *J. Biol. Chem.* **272,** 16709–16712
5. Girotti, M., Evans, J. H., Burke, D., and Leslie, C. C. (2004) *J. Biol. Chem.* **279,** 19113–19121
6. Degousee, N., Ghomashchi, F., Stefanski, E., Singer, A., Smart, B. P., Borregaard, N., Reithmeier, R., Lindsay, T. F., Lichtenberger, C., Reinisch, W., Lambeau, G., Arm, J., Tischfield, J., Gelb, M. H., and Rubin, B. B. (2002) *J. Biol. Chem.* **277,** 5061–5073
7. Fujishima, H., Sanchez Mejia, R. O., Bingham, C. O., III, Lam, B. K., Sapirstein, A., Bonventre, J. V., Austen, K. F., and Arm, J. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4803–4807
8. Dana, R., Leto, T. L., Malech, H. L., and Levy, R. (1998) *J. Biol. Chem.* **273,** 441–445
9. Shmelzer, Z., Haddad, N., Admon, E., Pessach, I., Leto, T. L., Eitan-Hazan, Z., Hershfinkel, M., and Levy, R. (2003) *J. Cell Biol.* **162,** 683–692
10. Zhao, X., Bey, E. A., Wientjes, F. B., and Cathcart, M. K. (2002) *J. Biol. Chem.* **277,** 25385–25392
11. Goggel, R., Winoto-Morbach, S., Vielhaber, G., Imai, Y., Lindner, K., Brade, L., Brade, H., Ehlers, S., Slutsky, A. S., Schutze, S., Gulbins, E., and Uhlig, S. (2004) *Nat. Med.* **10,** 155–160
12. Miotla, J. M., Jeffery, P. K., and Hellewell, P. G. (1998) *Am. J. Respir. Cell Mol. Biol.* **18,** 197–204
13. Souza, D. G., Pinho, V., Soares, A. C., Shimizu, T., Ishii, S., and Teixeira, M. M. (2003) *Br. J. Pharmacol.* **139,** 733–740
14. Tavares, D. L., Steil, A. A., Russo, M., Starobinas, N., Teixeira, C. F., and Jancar, S. (1998) *Eur. J. Pharmacol.* **358,** 69–75
15. Nagase, T., Ishii, S., Kume, K., Uozumi, N., Izumi, T., Ouchi, Y., and Shimizu, T. (1999) *J. Clin. Investig.* **104,** 1071–1076
16. Bonventre, J. V., Huang, Z., Taheri, M. R., O'Leary, E., Li, E., Moskowitz, M. A., and Sapirstein, A. (1997) *Nature* **390,** 622–625
17. Nagase, T., Uozumi, N., Ishii, S., Kume, K., Izumi, T., Ouchi, Y., and Shimizu, T. (2000) *Nat. Immunol.* **1,** 42–46
18. Nagase, T., Uozumi, N., Ishii, S., Kita, Y., Yamamoto, H., Ohga, E., Ouchi, Y., and Shimizu, T. (2002) *Nat. Med.* **8,** 480–484
19. Ghomashchi, F., Stewart, A., Hefner, Y., Ramanadham, S., Turk, J., Leslie, C. C., and Gelb, M. H. (2001) *Biochim. Biophys. Acta* **1513,** 160–166
20. Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, E., Lazdunski, M., Lambeau, G., and Gelb, M. H. (2002) *J. Biol. Chem.* **277,** 48535–48549
21. Kruger, J., Butler, J. R., Cherapanov, V., Dong, Q., Ginzberg, H., Govindarajan, A., Grinstein, S., Siminovitch, K. A., and Downey, G. P. (2000) *J. Immunol.* **165,** 5847–5859
22. Marshall, J., Krump, E., Lindsay, T., Downey, G., Ford, D. A., Zhu, P., Walker, P., and Rubin, B. (2000) *J. Immunol.* **164,** 2084–2091
23. Waddell, T. K., Fialkow, L., Chan, C. K., Kishimoto, T. K., and Downey, G. P. (1994) *J. Biol. Chem.* **269,** 18485–18491
24. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Med. Sci.* **37,** 911–917
25. Bartoli, F., Lin, H. K., Ghomashchi, F., Gelb, M. H., Jain, M. K., and Apitz-Castro, R. (1994) *J. Biol. Chem.* **269,** 15625–15630
26. Harrison, K. A., Clay, K. L., and Murphy, R. C. (1999) *J. Mass Spectrom.* **34,** 330–335
27. Yaffe, M. B., Xu, J., Burke, P. A., Forse, R. A., and Brown, G. E. (1999) *Surgery* **126,** 248–254
28. Vowells, S. J., Sekhsaria, S., Malech, H. L., Shalit, M., and Fleisher, T. A. (1995) *J. Immunol. Methods* **178,** 89–97
29. Sajjan, U., Thanassoulis, G., Cherapanov, V., Lu, A., Sjolin, C., Steer, B., Wu, Y. J., Rotstein, O. D., Kent, G., McKerlie, C., Forstner, J., and Downey, G. P. (2001) *Infect. Immun.* **69,** 5138–5150
30. Morgan, E., Varro, R., Sepulveda, H., Ember, J. A., Apgar, J., Wilson, J., Lowe, L., Chen, R., Shivraj, L., Agadir, A., Campos, R., Ernst, D., and Gaur, A. (2004) *Clin. Immunol.* **110,** 252–266
31. Stroncek, D. F., Jaszcz, W., Herr, G. P., Clay, M. E., and McCullough, J. (1998) *Transfusion* **38,** 663–668
32. Mounier, C. M., Ghomashchi, F., Lindsay, M. R., James, S., Singer, A. G., Parton, R. G., and Gelb, M. H. (2004) *J. Biol. Chem.* **279,** 25024–25038
33. Lee, W. L., Harrison, R. E., and Grinstein, S. (2003) *Microb. Infect.* **5,** 1299–1306
34. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) *Science* **303,** 1532–1535
35. Bailie, M. B., Standiford, T. J., Laichalk, L. L., Coffey, M. J., Strieter, R., and Peters-Golden, M. (1996) *J. Immunol.* **157,** 5221–5224
36. Mancuso, P., Standiford, T. J., Marshall, T., and Peters-Golden, M. (1998) *Infect. Immun.* **66,** 5140–5146
37. Snyder, F. (1995) *Biochem. J.* **305,** 689–705
38. Woodard, D. S., Lee, T. C., and Snyder, F. (1987) *J. Biol. Chem.* **262,** 2520–2527
39. Fonteh, A. N. (2002) *Eur. J. Biochem.* **269,** 3760–3770
40. Lie, W. J., Homburg, C. H., Kuijpers, T. W., Knol, E. F., Mul, F. P., Roos, D., and Tool, A. T. (2003) *Clin. Exp. Allergy* **33,** 1125–1134
41. Baker, P. R., Owen, J. S., Nixon, A. B., Thomas, L. N., Wooten, R., Daniel, L. W., O'Flaherty, J. T., and Wykle, R. L. (2002) *Biochim. Biophys. Acta* **1592,** 175–184
42. Sakata, A., Ida, E., Tominaga, M., and Onoue, K. (1987) *J. Immunol.* **138,** 4353–4359
43. Foubert, T. R., Burritt, J. B., Taylor, R. M., and Jesaitis, A. J. (2002) *Biochim. Biophys. Acta* **1567,** 221–231
44. Ely, E. W., Seeds, M. C., Chilton, F. H., and Bass, D. A. (1995) *Biochim. Biophys. Acta* **1258,** 135–144
45. Daniels, I., Lindsay, M. A., Keany, C. I., Burden, R. P., Fletcher, J., and Haynes, A. P. (1998) *Clin. Diagn. Lab. Immunol.* **5,** 683–689
46. Gijon, M. A., Spencer, D. M., Siddiqi, A. R., Bonventre, J. V., and Leslie, C. C. (2000) *J. Biol. Chem.* **275,** 20146–20156
47. Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P. S. (2003) *Nat. Biotechnol.* **21,** 635–637
48. Belaaouaj, A., McCarthy, R., Baumann, M., Gao, Z., Ley, T. J., Abraham, S. N., and Shapiro, S. D. (1998) *Nat. Med.* **4,** 615–618
49. Gao, X. P., Standiford, T. J., Rahman, A., Newstead, M., Holland, S. M., Dinauer, M. C., Liu, Q. H., and Malik, A. B. (2002) *J. Immunol.* **168,** 3974–3982
50. Blackwood, R. A., Transue, A. T., Harsh, D. M., Brower, R. C., Zacharek, S. J., Smolen, J. E., and Hessler, R. J. (1996) *J. Leukocyte Biol.* **59,** 663–670
51. Haq, S., Kilter, H., Michael, A., Tao, J., O'Leary, E., Sun, X. M., Walters, B., Bhattacharya, K., Chen, X., Cui, L., Andreucci, M., Rosenzweig, A., Guerrero, J. L., Patten, R., Liao, R., Molkentin, J., Picard, M., Bonventre, J. V., and Force, T. (2003) *Nat. Med.* **9,** 944–951
52. Allen, L. A., and Allgood, J. A. (2002) *Curr. Biol.* **12,** 1762–1766
53. Puri, K. D., Doggett, T. A., Douangpanya, J., Hou, Y., Tino, W. T., Wilson, T., Graf, T., Clayton, E., Turner, M., Hayflick, J. S., and Diacovo, T. G. (2004) *Blood* **103,** 3448–3456
54. Hegen, M., Sun, L., Uozumi, N., Kume, K., Goad, M. E., Nickerson-Nutter, C. L., Shimizu, T., and Clark, J. D. (2003) *J. Exp. Med.* **197,** 1297–1302
55. Miyaura, C., Inada, M., Matsumoto, C., Ohshiba, T., Uozumi, N., Shimizu, T., and Ito, A. (2003) *J. Exp. Med.* **197,** 1303–1310
56. Kolls, J., Peppel, K., Silva, M., and Beutler, B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 215–219
57. Hopken, U. E., Lu, B., Gerard, N. P., and Gerard, C. (1996) *Nature* **383,** 86–89
58. Tkalcevic, J., Novelli, M., Phylactides, M., Iredale, J. P., Segal, A. W., and Roes, J. (2000) *Immunity* **12,** 201–210
59. Yum, H. K., Arcaroli, J., Kupfner, J., Shenkar, R., Penninger, J. M., Sasaki, T., Yang, K. Y., Park, J. S., and Abraham, E. (2001) *J. Immunol.* **167,** 6601–6608