Platelet-activating Factor Contributes to Bacillus anthracis Lethal Toxin-associated Damage*

Received for publication, October 4, 2013, and in revised form, January 27, 2014. Published, JBC Papers in Press, January 29, 2014, DOI 10.1074/jbc.M113.524900

Johanna Rivera‡, Rani S. Sellers‡, Wangyong Zeng‡, Nico van Rooijen§, Arturo Casadevall§*, and David L. Goldman‡**1,2

From the Departments of‡ Microbiology and Immunology, ‡Pediatrics, Albert Einstein College of Medicine, Bronx, New York 10461 and the§ Department of Molecular Cell Biology, Vrije Universiteit, 1081 BT Amsterdam, The Netherlands

Background: PAF has been implicated as a potent lipid mediator of endotoxin-induced sepsis, but its role in anthrax-associated shock is unknown.

Results: Increased serum levels of PAF were present in LeTx-challenged mice. Inhibition of PAF activity prolonged survival and decreased levels of PAF-AH have been noted in patients of anthrax in animal models, and the administration of toxin specific antibody significantly reduces the mortality of experimentally B. anthracis-infected animals (5–7).

Significance: PAF antagonists may be helpful as adjunctive therapy for anthrax-associated shock.

The lethal toxin (LeTx) of Bacillus anthracis plays a central role in the pathogenesis of anthrax-associated shock. Platelet-activating factor (PAF) is a potent lipid mediator that has been implicated in endotoxin-associated shock. In this study, we examined the contribution of PAF to the manifestations of lethal toxin challenge in WT mice. LeTx challenge resulted in transient increase in serum PAF levels and a concurrent decrease in PAF acetylhydrolase activity. Inhibition of PAF activity using PAF antagonists or toxin challenge of PAF receptor negative mice reversed or ameliorated many of the pathologic features of LeTx-induced damage, including changes in vascular permeability, hepatic necrosis, and cellular apoptosis. In contrast, PAF inhibition had minimal effects on cytokine levels. Findings from these studies support the continued study of PAF antagonists as potential adjunctive agents in the treatment of anthrax-associated shock.

Severe respiratory distress, shock, multiorgan dysfunction, and bleeding are characteristics of anthrax. Despite the availability of effective anti-microbial therapy, the morbidity and mortality of this disease remains exceedingly high (1), possibly reflecting the action of tissue-damaging toxins. Lethal toxin (LeTx),3 edema toxin, and anthrolysin O have each been shown to contribute to Bacillus anthracis virulence, though LeTx is considered particularly important (reviewed in Ref. 2). LeTx is a Zn2+-dependent endoprotease that cleaves MAPK kinases and alters cell signaling. In vitro, LeTx challenge induces rapid lysis of macrophages from susceptible mouse strains (3, 4). Furthermore, LeTx challenge reproduces many of the clinical features of anthrax in animal models, and the administration of toxin specific antibody significantly reduces the mortality of experimentally B. anthracis-infected animals (5–7).

Platelet-activating factor (PAF) is a potent lipid mediator that was originally described in the context of its ability to alter platelet function (8). PAF is produced in response to stimuli by a variety of cell types, including monocytes/macrophages, polymorphonuclear leukocytes, eosinophils, basophils, platelets, mast cells, vascular endothelial cells, and lymphocytes. This lipid mediator exerts diverse biologic effects and has been implicated in several pathologic conditions including systemic inflammatory response and shock. Elevated serum PAF levels have been reported in septic patients (9, 10), and administration of PAF to animals reproduces many features of shock (11). PAF is rapidly inactivated by serum PAF acetylhydrolase (PAF-AH), and decreased levels of PAF-AH have been noted in patients with anaphylactic and septic shock (12–14). PAF antagonists have been studied as potential therapeutics in endotoxin-mediated shock (reviewed in Ref. 15), although initial results have been equivocal (16). Given the overlap in the clinical syndromes induced by LeTx and PAF, we sought to determine whether PAF contributes to the pathologic disturbances induced by LeTx.

EXPERIMENTAL PROCEDURES

Mice—Wild type female BALB/c (WT) (6–8 weeks old) mice were obtained from NCI (Bethesda, MD). Heterozygous breeding pairs of PAF receptor deficient BALB/c mice (PAFr−/−) were a gift from Dr. Peter Murray (St. Jude Children’s Hospital) (17). Mice were bred to obtain homozygous PAFr−/− mice in a specific pathogen-free barrier facility at the Animal Institute of Albert Einstein College of Medicine. Genotypes were determined by PCR using tail DNA with the following primers: AMS060, CAGCGGACACATAATGGGTCTGT; AMS061, TTTTGTTGGATTCTGAGTTT; and AMS062, CAGCGGATGTTCTGTTGTC. Briefly, a sample of genomic tail DNA was...
Platelet-activating Factor and Anthrax

used in PCR with 2.5 mM deoxynucleoside triphosphate and 20 μM each primer under the following conditions with Taq polymerase Gold (Applied Biosystems, Foster City, CA): 95°C for 10 min, 95°C for 1 min, 55°C for 1 min, and 72°C for 2.5 min for 33 cycles. All animal studies were carried out with protocols approved by the Albert Einstein College of Medicine Animal Care and Use Committee.

B. anthracis and Toxin Components—B. anthracis Sterne 34F2 (pXO1 +, pXO2 −) was obtained from Dr. Alex Hoffmaster at the Centers for Disease Control (Atlanta, GA). Bacterial cultures were grown from frozen stock in brain-heart infusion broth (Difco, Detroit, MI) at 37°C for 18 h with shaking. Recombinant protective antigen (PA) and lethal factor (LF) proteins and endotoxin-reduced PA and LF were obtained from the Northeast Biodefense Center Expression Core of the NYS Department of Health (Albany, NY). Briefly, histidine-tagged PA and LF were expressed in Escherichia coli and purified by affinity chromatography using a ready to use column prepacked with precharched high performance nickel-Sepharose (HisTrap HP) (GE Life Sciences). Proteins were further purified by ion exchange (Mono Q) chromatography (GE Life Sciences). LPS measurements on these preparations revealed levels of <12.3 endotoxin units/ml. To further reduce endotoxin, proteins were purified by affinity chromatography using Endotrap Blue resin (Hyglos, Chandler, NC), which significantly reduced LPS levels (0.023 enzyme unit/mg). Studies done with the endotoxin reduced and nonendotoxin reduced preparations gave comparable results. All proteins were quantitated using the colorimetric Bradford reagent (ThermoScientific Pierce). SDS-PAGE analysis revealed more than 95% of the protein in one band at molecular masses of 83 kDa (PA) and 89 kDa (LF). Recombinant protective antigen (PA) and lethal factor (LF) proteins and endotoxin-reduced PA and LF were obtained from the Northeast Biodefense Center Expression Core of the NYS Department of Health (Albany, NY). Briefly, histidine-tagged PA and LF were expressed in Escherichia coli and purified by affinity chromatography using a ready to use column prepacked with precharched high performance nickel-Sepharose (HisTrap HP) (GE Life Sciences). Proteins were further purified by ion exchange (Mono Q) chromatography (GE Life Sciences). LPS measurements on these preparations revealed levels of <12.3 endotoxin units/ml. To further reduce endotoxin, proteins were purified by affinity chromatography using Endotrap Blue resin (Hyglos, Chandler, NC), which significantly reduced LPS levels (0.023 enzyme unit/mg). Studies done with the endotoxin reduced and nonendotoxin reduced preparations gave comparable results. All proteins were quantitated using the colorimetric Bradford reagent (ThermoScientific Pierce). SDS-PAGE analysis revealed more than 95% of the protein in one band at molecular masses of 83 kDa (PA) and 89 kDa (LF).

PAF Antagonists—CV3988, WEB 2086, and quinacrine were solubilized in ethanol and diluted in either PBS or normal saline and administered at doses of 3 and 5 mg/kg. Ginkgolide B was solubilized in DMSO, diluted in PBS, and administered at a dose of 5 mg/kg. CV3988 and WEB 2086 are competitive PAF receptor antagonists. Ginkgolide B accelerates PAF degradation by promoting PAF-AH I α2 homodimer activity and quinacrine inhibits PAF synthesis. For hematocrit studies, PAF antagonists were administered at 5 mg/kg intravenously 1 h prior to toxin challenge. All antagonists except quinacrine (Sigma) were obtained from Enzo Life Science (Farmingdale, NY).

Macrophage Depletion—Dichloromethylene bisphosphonate (CL2MBP), also known as clodronate, was a gift from Roche and was encapsulated in liposomes as previously described (41). Liposome clodronate selectively depletes macrophages after intravenous administration (5, 41). Clodronate liposomes and PBS liposomes were given to WT mice (n = 6 per group) ~48 h prior to toxin challenge. To confirm macrophage depletion, mice (n = 3 per group) were given 0.1 ml of clodronate liposomes or PBS liposomes intravenously. Two days later, the mice were sacrificed, the spleens and livers were removed, and cells were prepared for FACS analysis. Briefly, the cells (106) were stained for 30 min on ice with 100 μl of the following antibodies diluted in staining buffer (1% FCS/PBS): 2 μg/ml of R-phycocerythrin-labeled anti-CD45 and 5 μg/ml of FITC-labeled anti-mouse MAC-3 (Pharmingen, San Diego, CA). The samples were washed twice in staining buffer and fixed in 1% paraformaldehyde. Stained samples were stored in the dark at 4°C overnight and analyzed on a Calibur FACScan flow cytometer (Becton Dickinson, Mountainview CA) using the CELLQuest (Becton Dickinson) software. Live cells were gated as judged from forward and side laser scatter and CD45+ cells. Controls consisted of isotype-matched irrelevant antibodies.

Survival Studies—WT and PAFr−/− mice (n = 10 per group) were injected into the tail vein with 120 μg of PA and 50 μg of LF in 100 μl of PBS as described (7). For some experiments, mice (n = 10 per group) were infected intravenously with 106 B. anthracis Sterne bacterial cells. For some experiments, WT mice were treated with 3 mg/kg CV3988 or WEB 2086 (n = 5 per group) 2 h prior to LeTx injection. Control mice received PBS (n = 5 per group). The mice were monitored daily for mortality.

PAF Measurements—WT mice were challenged with LeTx (120 μg of PA and 50 μg of LF) intravenously and euthanized at 30 min, 2 h, and 16 h. Mice were bled from the retroorbital sinus, and serum was collected and stored at −20°C until tested. The mice were then sacrificed, and the liver was removed and homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Roche Applied Science). Homogenates were centrifuged at 2000 × g for 10 min to remove cell debris, and the supernatant was frozen at −80°C until tested. PAF measurements were using ELISA kit for PAF (Cedarlane Laboratories (USCN Life Science), Burlington, NC) as per the manufacturer’s instructions. Briefly, samples were added into 96-well plate, 50 μl of detection reagent A was then added, and the plate was incubated for 1 h at 37°C. ELISA plate was then washed 3× with wash solution, and 100 μl of detection reagent B was added. The plate was then incubated for 30 min at 37°C, washed 3× with wash solution, and 90 μl of substrate solution was then added. The plates were then incubated for 25 min at 37°C. Stop solution (50 μl) was added to each well and immediately read at 450 nm (Labsystems Multiskan, Franklin, MA). Average values were obtained, and calculations of results were done based on the standard curve.

PAF Acetylhydrolase Measurements—PAF-AH activity was measured as per the manufacturer’s instructions (Cayman, Ann Arbor, MI). Briefly, 10 μl of 5,5′-dithiobis(nitrobenzoic acid), 10 μl of sample, and 5 μl of PAF-AH assay buffer were added to a 96-well plate. The reactions were initiated by adding 200 μl of 2-thio PAF substrate solution. Absorbance was read every minute for 10 min at 405 nm (Labsystems Multiskan, Franklin, MA) to obtain 10 time points.

Histology—WT and PAFr−/− mice were evaluated histologically after intravenous PBS or LeTx treatment at 2 h (n = 3 per group) and 24 or 48 h (n = 5 per group). The mice were euthanized at 2, 24, or 48 h post-LeTx injection. Lung, liver, and spleen were isolated and fixed in 10% neutral buffered formalin (Fisher Scientific). One animal from each the 24-h and 48-h dose groups had a full tissue evaluation (liver, kidney, spleen, heart, lungs, adrenal glands, bone marrow, thymus, brain, skeletal muscle, nerve, small and large intestines, bladder, pancreas, submandibular salivary glands, and lymph nodes). Tissues were processed for paraffin embedding, and histological sections of 5...
µm were stained with hematoxylin and eosin. Sections were evaluated by a board-certified veterinary pathologist in a blinded manner.

**Apoptosis Studies**—Apoptosis was studied by examination of nuclear and cellular morphology in tissue sections stained with hematoxylin and eosin. Additional tissue samples were stained for cleaved caspase 3. Briefly, 5-µm sections were deparaffinized in xylene followed by graded alcohols. Antigen retrieval was performed by incubating sections in 10 mM sodium citrate buffer (pH 6.0) and heated to 96 °C for 20 min. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in PBS for 10 min. Sections were blocked with 5% normal donkey serum and 2% BSA in PBS for 1 h. The primary antibody to cleaved caspase 3 (Cell Signaling, Danvers, MA) was used at 1:50 for 1 h at room temperature. The primary species (rabbit IgG1) was substituted for the primary antibody to serve as a negative control. Sections were stained by routine immunohistochemistry methods using HRP polymer conjugate (Invitrogen) to localize the antibody bound to antigen with diaminobenzidine as the final chromogen. All immunostained sections were lightly counterstained with hematoxylin.

**Pulmonary Function Analysis**—Whole body plethysmography (Buxco Electronics, Inc., Wilmington, NC) was used to measure respiratory parameters including tidal volume, respiratory rate, inspiratory, and expiratory time by monitoring the box flow pattern created by the animal’s respiration (18–20). A respiratory rate, inspiratory, and expiratory time by monitoring the box flow pattern created by the animal’s respiration (18–20).

**Hematocrit Measurements**—WT and PAFr−/− mice (n = 5–6 per group) were challenged with LeTx or PBS intravenously and then bled from the retroorbital sinus using heparinized microhematocrit capillary tubes (Fisher Scientific). Tubes were sealed with Hemato-Seal (Fisher Scientific) and spun in microcapillary centrifuge (Fisher Scientific) for 5 min. Hematocrits were measured by determining the red blood cell volume relative to the volume of whole blood.

**Evans Blue Extravasation**—A 100 µl of intraperitoneal injection of 0.22-µm filtered 10 mg/ml Evans blue PBS was administered 45 min prior to LeTx injection. For some experiments, WEB 2086 (3 mg/kg) was administered 2 h prior to LeTx injection. The mice were sacrificed and perfused with 30 ml PBS. Lungs were removed and homogenized in 1.5 ml of PBS. To extract the dye, TCA (60%) was added to each sample, vortexed, and centrifuged (1000 × g) for 30 min at 4 °C. Optical densities of the supernatants were measured at 620 nm (Labsystem Miltiskan, Franklin, MA).

**Biochemical Profile**—After challenge with LeTx (24 h postchallenge), WT and PAFr−/− mice (n = 5 per group) were bled from the retroorbital sinus by use of heparinized microhematocrit capillary tubes (Fisher Scientific). Serum was then separated and sent to a commercial veterinary laboratory (Antech Diagnostics, Lake Success, NY) for standard mammalian chemistry and liver function tests.

**Cytokine and Chemokine Studies**—WT and PAFr−/− mice (n = 6 per group) were sacrificed 2 and 24 h post-intravenous injection of LeTx or PBS. The mice were sacrificed, and the lungs were homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Roche Applied Science). Homogenates were centrifuged at 2000 × g for 10 min to remove cell debris, and the supernatant was frozen at −80 °C until tested. Supernatants were assayed using mouse cytokine protein array I (Ray Biotech, Norcross, GA) as per the manufacturer’s instructions. Briefly, membranes were blocked with 1× blocking buffer, washed three times, and then incubated with samples. Membranes were washed again and incubated for 1 h with biotin-conjugated cytokines, which were detected by incubation with HRP-conjugated streptavidin. All incubations were done at 37 °C for 1 h. Unbound reagents were removed by washing and the membranes developed. This kit assays for the following cytokines: GCSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, SCF, sTNFRI, TNF-α, and thrombopoietin. Supernatants were also assayed for IL-2, IL-4, IL-6, IL-10, MCP-1, and MIP-1α concentrations using ELISA kits (Pharmingen, San Diego, CA and R&D Systems Inc., Minneapolis, MN). The detection limits of cytokine assays are 3.1 pg/ml for IL-2, 7.8 pg/ml for IL-4, 15.6 pg/ml for IL-6 and TNF-α, and 31.3 pg/ml for IL-10 and IFN-α as stated by the manufacturer. The detection limits of the chemokine assays are 4.7 pg/ml for MIP-1α and 15.6 pg/ml for MCP-1 as determined by the manufacturer.

**Macrophage and Hepatocyte Isolation**—Peritoneal macrophages were isolated from mice (PAFr−/− and WT) by peritoneal lavage without prior peritoneal irritation. Briefly, the abdominal cavity was washed five times with sterile Hanks balanced salt solution, 1% penicillin-streptomycin, 0.1 mM EGTA (Fisher Scientific) using a sterile Pasteur pipette. Following centrifugation, erythrocytes were lysed by resuspension in ice-cold 0.17 M NH4Cl for 10 min. A 10-fold excess of DMEM solution was then added to make the solution isotonic, the cells were collected by centrifugation, and live cells (trypan blue exclusion and morphological examination) were counted in a hemocytometer chamber. The cells were suspended in DMEM (Invitrogen), 10% NCTC-109 medium, 1% penicillin-streptomycin, and 1% nonessential amino acids (Mediatech, Inc, Manassas, VA). Cells were plated at a density of 1 × 10^6 cells/well in a 96-well tissue culture plate and incubated overnight at 37 °C. Primary mouse hepatocytes were isolated from WT and PAFr−/− mice as described with minor modifications (21).

**MTT Assay**—MTT (Sigma) assay was used to determine LeTx toxicity to peritoneal macrophages and primary hepatocytes as described (22). Macrophages (10^6) and hepatocytes (5 × 10^6) per well were incubated in a 96-well plate with LeTx for 4 h at 37 °C. Dose-response experiments were done. All wells were plated in duplicates. Macrophage supernatants were removed for PAF measurements as described above. A 25-µl volume of 5 mg/ml stock-solution of MTT (in sterile PBS) was then added to each well and after 2 h of incubation of 37 °C, 100 µl of the extraction buffer (12.5% SDS, 45% DMF) was added, and cells were incubated overnight at 37 °C. Optical densities were measured at 570 nm (Labsystem Miltiskan, Franklin, MA).

**Statistics**—For parametric data, individual comparisons were done using a Student’s t test. Multiple comparisons were done
with an ordinary one-way analysis of variance, and post hoc analyses were done with a Dunnett multiple comparison test. For nonparametric data, individual comparisons were done with a Mann-Whitney test. Multiple comparisons were done using the Kruskal-Wallis test, and post hoc analyses were done with the Dunn test. Survival curves were compared by log rank analysis (Graphpad Prism; GraphPad Software, Inc., La Jolla, CA). All variance listed is standard deviation.

RESULTS

**PAF and PAF-AH Activity**—Serum and liver levels of PAF were measured several times after injection of LeTx. At 30 min, serum from WT mice challenged with LeTx contained higher amounts of PAF (48 ng/ml ± 30.2 ng/ml) compared with PBS-treated mice (15 ng/ml ± 2.7 ng/ml) (p < 0.05). In addition, serum PAF levels at 2 and 16 h were not significantly different between mice treated with LeTx and controls (data not shown). Administration of LeTx did not affect PAF levels in the liver (Fig. 1B). Serum PAF-AH activity was significantly lower in LeTx-treated mice (129 ± 24 μmol/min/ml) compared with PBS-treated (155 ± 22 μmol/min/ml) and untreated mice (178 ± 18 μmol/min/ml) (p < 0.05) (Fig. 1C). There were no differences in serum PAF-AH activity between PBS-treated and PA-treated mice (data not shown).

PAF levels were measured in the supernatants of WT peritoneal macrophages at several times (15, 30, 60, 90, and 120 min). LeTx (100 ng of PA + 100 ng of LF) increased PAF levels as early as 15 min (271.7 ± 57 pg/ml), after exposure when compared with untreated macrophages (191.3 ± 12.5 pg/ml, p < 0.05). PAF levels remained elevated at later times relative to untreated macrophages: 30 min (294.5 ± 4.04 pg/ml), 60 min (308.7 ± 4.37 pg/ml), 90 min (228.6 ± 10.8 pg/ml), and 120 min (290.6 ± 25.3 pg/ml) (p < 0.05). Additionally, the increases in PAF levels were independent of cell death (data not shown).

**Survival and Illness**—WT but not PAFr−/− mice, injected with LeTx were ill (rough hair coat, dehydrated, depressed, and hunched posture) by 24 h. PAFr−/− mice injected with LeTx survived longer (median survival time, 4 days) than WT mice (median survival time, 1 day) but still exhibited significant mortality over a 10-day period (Fig. 2A). Additionally, PAFr−/− mice infected with *B. anthracis* Sterne bacterial cells survived longer (median survival time, 10.5 days) compared with WT mice (median survival time, 6 days) (Fig. 2B). PAFr antagonists, CV3988 (median survival time, 5 days), and WEB 2086 (only 2 deaths in an 11-day period) provided a survival benefit to WT challenged with LeTx compared with PBS-treated WT mouse (median survival time, 2 days) (Fig. 2C).

Because macrophages play a central role in LeTx pathogenesis and produce PAF (23, 24), we investigated the possibility that macrophage depletion would provide protection against lethal toxicity. Macrophage depletion by administration of clodronate liposomes provided a survival benefit to WT mice (median survival, 6 days) from LeTx-induced death compared with WT mice treated with PBS liposomes (median survival, 1 day) (p < 0.05) (Fig. 2D).

**Hematocrit**—Administration of LeTx to WT mice resulted in an average 11% increase in HCT compared with PBS-treated mice, 2 h following LeTx injection (Fig. 3A). This increase in HCT was still present at 6 h following LeTx injection. However, at 24 h WT mice manifested a 42% decrease in HCT compared with mice injected with PBS (Fig. 3B). Administration of some PAF antagonists (CV3988 and WEB 2086), but not others (quinacrine and ginkgolide B) ameliorated, but did not entirely prevent, the increase in HCT produced by LeTx injection at 2 h (Fig. 3C). No effects on HCT by antagonists in the absence of LeTx were observed (data not shown). In addition, PAFr−/− mice experienced smaller changes in HCT at 2 and 24 h in response to LeTx when compared with WT mice (Fig. 3, A and B).

**Respiratory Function**—Within 1.5 h of LeTx injection, WT mice developed labored breathing. Visible changes in breathing correlated with changes in respiratory function as measured by whole body plethysmography, including a greater than 50% decrease in respiratory rate and a 20% decline in tidal volume (Fig. 4A). Inspiratory and expiratory times were also markedly prolonged (Fig. 4B). Mice that showed improved respiratory parameters by 24 h generally recovered, whereas animals with persistent respiratory compromise generally died within 24 h (data not shown). In contrast to hematocrit studies, we were unable to demonstrate an effect of the PAF antagonist WEB 2086 on respiratory parameters (data not shown). Furthermore, PAFr−/− mice still developed alterations in respiratory function in response to LeTx, although tidal volume and...
peak expiratory flow were not as affected compared with WT mice (Fig. 4C).

**Biochemical Profile Studies**—WT mice exhibited a dose-dependent increase in serum glutamic oxaloacetic transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) levels (Fig. 5A) that did not occur in PAFr−/− mice (Fig. 5B) at 24 h post-LeTx challenge. WT mice, but not PAFr−/− mice, injected with LeTx also exhibited a dose-dependent increase in blood urea nitrogen and a decrease in serum albumin concentration, also consistent with volume loss (Fig. 5, C and D).

**Evans Blue Extravasation**—Vessel leakage as manifested by the increase in absorbance readings was present in WT mice treated with LeTx compared with PBS-treated WT mice (p < 0.05) (Fig. 6). This effect was ameliorated by the PAF antagonist, WEB 2086 (p = 0.02) to mice before LeTx injection resulted in prolonged survival compared with PBS-treated mice. D, macrophage depletion of WT mice with clodronate liposomes resulted in prolonged survival compared with controls with median survival times of 6 and 1 days, respectively (p < 0.05). For all experiments, n = 10 per group. Survival curves were compared by log rank analysis.

**LeTx-related Histological Findings**—Two hours after LeTx challenge, there were minimal inflammatory changes. Several histologic findings were common to WT and PAFr−/− mice, including the presence of small cells with condensed nuclei suggestive of apoptotic cells within blood vessels in bone marrow and lung (data not shown). Loss of red blood cells within the red pulp was noted, consistent with splenic contracture (Fig. 7, A and B), which is consistent with a physiologic response to volume loss. The lungs of WT and PAFr−/− mice also had increased alveolar capillary cellularity and minimal acute fibrinous pneumonitis (data not shown).

Twenty-four hours after LeTx challenge, WT mice exhibited hepatic necrosis that was widespread, with a centrilobular to midzonal distribution (Fig. 7C, panel i). In contrast, PAFr−/− mice did not have evidence of liver necrosis (Fig. 7C, panel ii). Other findings were generally similar to those identified at 2 h (circulating micronucleated/apoptotic cells) and splenic contracture without significant differences between WT and PAFr−/− mice. At 24 h, significant lymphocytic apoptosis was evident in the spleen of WT (Fig. 7C, panel iii) and PAFr−/− mice.

**Apoptosis Studies**—Examination of the bone marrow and lung of LeTx-challenged PAFr−/− mice revealed negative staining for cleaved caspase 3 (Fig. 8, A and C). In contrast, numerous cells were positive for cleaved caspase 3 in the bone marrow and lungs of LeTx-challenged WT mice (Fig. 8, B and D). Negative staining for cleaved caspase 3 was noted in PBS-treated WT and PAFr−/− mice (data not shown).

**Cytokine Expression**—Given the role of PAF in systemic inflammatory response, we investigated cytokine/chemokine expression in response to LeTx. Twenty-four hours after LeTx injection, alterations in cytokine/chemokine expression were observed in the lungs of mice (WT and PAFr−/−) by cytokine array (Fig. 9A). At 24 h, WT and PAFr−/− mice challenged with LeTx exhibited increased expression of MCP-1 and RANTES compared with mice injected with PBS (Fig. 9, A and B). This induction was confirmed by ELISA studies (Fig. 9B and data not shown). In addition, WT and PAFr−/− mice injected with LeTx exhibited a small, but statistically significant decrease in soluble TNFR-1 expression relative to PBS-injected counterparts. IL-12 expression was only detected in PAFr−/− WT mice injected with LeTx. MIP-1α induction was not detected following LeTx injection (data not shown).

**Cellular Toxicity**—To determine whether survival differences among WT and KO mice were related to differences in
macrophage susceptibility to LeTx, in vitro macrophage experiments were performed. Peritoneal macrophages isolated from WT and PAFr−/− mice exhibited a dose-dependent decrease in MTT signal in response to LeTx treatment. However, there were no differences between WT and PAFr−/− mouse macrophage susceptibility to LeTx at all concentrations tested (data not shown). At a LeTx concentration of 1 μg/ml, there were 29 and 31% decreases in MTT signal for WT and PAFr−/− mouse macrophages compared with cells treated with media alone (data not shown). In contrast, LeTx did not affect viability of hepatocytes isolated from WT mice (data not shown).

**FIGURE 3. Effects of LeTx on HCT.** A and B, average HCT of WT and PAFr−/− mice 2 h (A) and 24 h (B) after LeTx (120 μg of PA and 50 μg of LF) injection (n = 5 per group). Brackets denote standard deviation. Experiments were done in separate sets of mice and repeated with comparable results. *, p < 0.05 for comparison with control; **, p < 0.05 for comparisons between WT and PAFr−/− mice. C, HCT in WT mice treated with PAF antagonists prior to LeTx injection. HCT were measured 2 h following LeTx injection, *, p < 0.05 for comparison between animals pretreated with PAF antagonist versus PBS prior to LeTx challenge. For individual comparisons (A and B), a Mann-Whitney test was used. For multiple comparisons (C), the Kruskall-Wallis test was used, and post hoc analyses were done using the Dunn multiple comparison test.

**FIGURE 4. Effects of LeTx injection on pulmonary function.** Respiratory functions were measured using whole body plethysmography in unrestrained mice (n = 4 per group) at baseline and 1.5 h after injection LeTx (120 μg of PA and 50 μg of LF). WT mice challenged with LeTx exhibited shallow and slower breathing compared with baseline. A, bars show the average percent decreases from baseline for different respiratory functions following LeTx injection. B, bars show the average percent increase for inspiratory and expiratory times. Brackets denote standard deviation. All changes shown are statistically significant from baseline. C, WT and PAFr−/− mice developed changes in respiratory function compared with baseline. However, decreases in tidal volume and peak expiratory flow were less prominent for PAFr−/− compared with WT mice. Bars represent the average percent decrease in respiratory rate, tidal volume, peak inspiratory flow, and peak expiratory flow. Bars denote averages of n = 4–5 mice, and brackets denote standard deviation. WT (*, p < 0.05). Frequency; TV, tidal volume; PIF, peak inspiratory flow; PEF, peak expiratory flow; Ti, inspiratory time; Te, expiratory time. Statistical analyses were done using a Mann-Whitney test.

**DISCUSSION**

PAF is induced during inflammation by both exogenous (e.g., LPS, HIV infection) and endogenous stimuli and is an active mediator of inflammation (25–27). In animal models, many of the features of endotoxin-induced shock can be reproduced by PAF injections (28). Our findings demonstrate that serum PAF levels are transiently increased in response to LeTx challenge in WT mice. Our findings are consistent with recent studies that reveal a rapid induction of inflammatory lipid mediators by LeTx-mediated activation of the inflammasome (29). In addition, our studies suggest that PAF contributes to the mortality of LeTx in WT mice because PAFr−/− mice and WT mice treated with PAF receptor antagonists exhibited prolonged survival. We also observed that increased serum PAF levels corre-
lated with a decrease in PAF-AH activity. PAF-AH is the enzyme primarily responsible for the degradation of PAF, normally limiting the half-life of PAF to minutes. Thus, decreased activity could result in increased PAF levels in our experiments. The magnitude of the observed decrease in PAF-AH in response to LeTx in WT mice is similar to that described in endotoxin challenged gerbils and comparable to the decreased activity observed in critically ill patients on presentation (30, 31).

Early studies linked LeTx susceptibility in mice to macrophage toxicity, although more recent studies dispute this association (32). The effects of LeTx on macrophage viability are mediated by the NOD-like receptor sensor, NLRP1, and related to inflammasome assembly and caspase-1 activation (33–35). Our results suggest that PAF produced by macrophages contributes to LeTx-related pathology. In this regard, macrophages from WT and PAFr−/− mice were both susceptible to LeTx-induced death. Furthermore, macrophage depletion provided survival benefits to WT mice from LeTx-induced death. Therefore, we hypothesize that LeTx-induced macrophage damage contributes to increased PAF levels, either directly from macrophages or from other cells in response to macrophage damage.

To understand the contribution of PAF in disease following LeTx challenge in a toxin-susceptible mouse strain, we performed a variety of physiologic and biochemical studies. Several of the clinical features of anthrax (e.g., pleural effusions, hemoconcentration, and bleeding) indicate significant alterations in vascular permeability. Many of these features can be reproduced by LeTx injections in animal models (36, 37). Following LeTx challenge, we observed several features consistent with increased vascular permeability in WT mice including hemoconcentration, respiratory distress, increased serum blood urine nitrogen, and decreased protein levels. Furthermore,
LeTx-challenged mice also exhibited splenic contracture, which is likely a physiologic response to volume loss in this context.

The basis by which LeTx enhances vascular permeability is poorly understood. Incubation of endothelial cells with LeTx resulted in apoptosis and altered function in some studies (38, 39). Furthermore, LeTx-induced macrophage death has been reported to augment apoptotic death of endothelial cells (40). We found that some (e.g., hemoconcentration and increased serum blood urine nitrogen levels) but not all of the changes indicative of vascular permeability in WT mice were partially ameliorated by the administration of PAF antagonists and in PAFr−/− mice. These effects on vascular permeability were confirmed with Evans blue studies. Overall, we interpret these findings as suggestive that PAF contributes to, but is not entirely responsible for, the changes in vascular permeability induced by LeTx. PAF is well known to alter endothelial function, and these effects may be related to direct effects (41) or macrophage and endothelial cell activation (42, 43).

Extensive apoptosis following LeTx was present in a variety of organs and cell types, including endothelial cells. In contrast, apoptosis was dramatically reduced in PAFr−/− mice. The pattern of LeTx-induced macrophage death has been related to polymorphisms in Nalp1b and the macrophage activation state (33). Typically, macrophages from sensitive BALB/c mice undergo lysis and not apoptosis in response to LeTx (35, 44), although apoptosis may occur in the context of sublytic levels of

**FIGURE 7.** Histology of WT and PAFr−/− mice following LeTx injection. A, normal spleen architecture with large numbers of red blood cells was present in the red pulp in mice treated with PBS. B, spleens from mice challenged with LeTx exhibited normal splenic architecture, but loss of red blood cells within the red pulp consistent with splenic contraction. The original magnification for A and B was 10×. C, at 24 h following LeTx (120 µg of PA and 50 µg of LF) challenge, WT (panel i) but not PAFr−/− mice (panel ii) exhibited centrilobular hepatic necrosis following LeTx challenge. The original magnification was 40×. At 24 h following LeTx injection, WT (panel iii) and PAFr−/− mice exhibited apoptotic cells (boxes) within the spleen. The original magnification was 20×. Arrows point to apoptotic cells (fragmented nucleus).

**FIGURE 8.** Cleaved caspase 3 studies of WT and PAFr−/− mice following LeTx injection. WT and PAFr−/− mice were injected intravenously with LeTx (120 µg of PA and 50 µg of LF). Bone marrow (A) and lung (C) of PAFr−/− did not exhibit positive cells for cleaved caspase 3. Examination of bone marrow (B) and lung (D) of WT mice revealed numerous positive cells for cleaved caspase 3. Magnification was 40×.
Platelet-activating Factor and Anthrax

In summary, our results implicate PAF in the toxic effects of B. anthracis LeTx, especially as it relates to alterations in vascular permeability and hepatotoxicity. PAF appears to play a role downstream of macrophage death specifically in BALB/c mice. Extrapolations of findings from mice to humans must be done cautiously and judiciously, especially because human macrophages/moноcytes are more displayed decreased susceptibility to experimental infection. Our findings suggest the possibility that PAF antagonists may be helpful as an adjunctive therapy for anthrax. Given the high mortality associated with anthrax-associated shock despite antimicrobial therapy and supportive therapy, additional study of PAF antagonists is warranted.

Acknowledgment—We thank Dr. David Neufeld for preparation of primary mouse hepatocytes.

REFERENCES

1. Centers for Disease Control (2001) Update. Investigation of bioterrorism-related anthrax and interim guidelines for exposure management and antimicrobial therapy. MMWR Morb. Mortal. Wkly. Rep. 50, 909–919
2. Moayeri, M., and Leppla, S. H. (2009) Cellular and systemic effects of anthrax lethal toxin and edema toxin. Mol. Aspects Med. 30, 439–455
3. Friedlander, A. M. (1986) Macrophages are sensitive to anthrax lethal toxin and edema toxin. Mol. Aspects Med. 30, 439–455
4. Chopra, A. P., Boone, S. A., Liang, X., and Duesbery, N. S. (2003) Anthrax lethal factor proteolysis and inactivation of MAPK kinase. J. Biol. Chem. 278, 9402–9406
5. Brossier, F., Lévy, M., Landier, A., Lafaye, P., and Mock, M. (2004) Functional analysis of Bacillus anthracis protective antigen by using neutralizing monoclonal antibodies. Infect. Immun. 72, 6313–6317
6. Migone, T. S., Subramanian, G. M., Zhong, J., Healey, L. M., Corey, A., Devalaraja, M., Lo, L., Ullrich, S., Zimmerman, J., Chen, A., Lewis, M., Meister, G., Gillum, K., Sanford, D., Mott, J., and Bolmer, S. D. (2009) Raxibacumab for the treatment of inhalational anthrax. N. Engl. J. Med. 361, 135–144
7. Rivera, J., Nakouzi, A., Abboud, N., Revskaya, E., Goldman, D., Collier, R. J., Dadachova, E., and Casadevall, A. (2006) A monoclonal antibody to Bacillus anthracis protective antigen defines a neutralizing epitope in do-
Vincent, J. L., Spapen, H., Bakker, J., Webster, N. R., and Curtis, L. (2000) Platelet-activating factor (PAF) a mediator of endotoxin shock? _Crit. Care Med._ **28**, 209–209

Del Sorbo, L., Arese, M., Giraudo, E., Tizzani, M., Biancone, L., Bussolino, F., and Camussi, G. (2001) Tat-induced platelet-activating factor synthesis and multiple organ failure. _Cell Cycle_ **6**, 758–766

von Moltke, J., Trinidad, N. J., Moayeri, M., Kintzer, A. F., Wang, S. B., van Rooijen, N., Brown, C. R., Krantz, B. A., Leppla, S. H., Gronert, K., and Vance, R. E. (2012) Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. _Nature_ **490**, 107–111

Claus, R. A., Russwurm, S., Dohn, B., Bauer, M., and Lösche, W. (2005) Plasma platelet-activating factor acetylhydrolase activity in critically ill patients. _Crit. Care Med._ **33**, 1416–1419

Yang, J., Xu, J., Chen, X., Zhang, Y., Jiang, X., Guo, X., and Zhao, G. (2010) Decrease of plasma platelet-activating factor acetylhydrolase activity in lipopolysaccharide induced mongolian gerbil sepsis model. _PLoS One_ **5**, e9190

Tera, J. K., Cote, C. K., France, B., Jenkins, A. L., Bouza, J. E., Welkos, S. L., LeVine, S. M., and Bradley, K. A. (2010) Cutting edge. Resistance to _Bacillus anthracis_ infection mediated by a lethal toxin sensitive allele of Nalp1b/Nlrp1b. _J. Immunol._ **184**, 17–20

Boydent, E., and Dietrich, W. F. (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. _Nat. Genet._ **38**, 240–244

Fink, S. L., Bergsbaken, T., and Cookson, B. T. (2008) Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. _Proc. Natl. Acad. Sci. U.S.A._ **105**, 4312–4317

Muehlbauer, S. M., Evering, T. H., Bouucchelli, G., Squires, R. C., Ashton, A. W., Percoll, S. A., Lisanti, M. P., and Brojatsch, J. (2007) Anthrax lethal toxin kills macrophages in a strain-specific manner by apoptosis or caspase-1-mediated necrosis. _Cell Cycle_ **6**, 758–766

Cui, X., Moayeri, M., Li, Y., Li, X., Haley, M., Fitz, Y., Correa-Araujo, R., Banks, S. M., Leppla, S. H., and Eichacker, P. Q. (2004) Lethality during continuous anthrax lethal toxin infusion is associated with circulatory shock but not inflammatory cytokine or nitric oxide release in rats. _Am. J. Physiol. Regul. Integr. Comp. Physiol._ **286**, R699–R709

Kuo, S. R., Willingham, M. C., Bour, S. H., Andreas, E. A., Park, S. K., Jackson, C., Duesbery, N. S., Leppla, S. H., Tang, W. J., and Frankel, A. E. (2008) Anthrax toxin-induced shock in rats is associated with pulmonary edema and hemorrhage. _Microb. Pathol._ **44**, 467–472

Kirby, J. E. (2004) Anthrax lethal toxin induces human endothelial cell apoptosis. _ Infect. Immun._ **72**, 430–439

Warfel, J. M., Steele, A. D., and D’Agnillo, F. (2005) Anthrax lethal toxin induces endothelial barrier dysfunction. _Am. J. Pathol._ **166**, 1871–1881

Pandey, J., and Warburton, D. (2004) Knock-on effect of anthrax lethal toxin on macrophages potentiates cytotoxicity to endothelial cells. _Microbes Infect._ **6**, 835–843

Victorino, G. P., Newton, C. R., and Curran, B. (2004) Modulation of microvascular hydraulic permeability by platelet-activating factor. _J. Trauma_ **56**, 379–384

Montrucchio, G., Lupia, E., De Martino, A., Silvestro, L., Savu, S. R., Caccia, G., De Filippi, P. G., Emanuelli, G., and Camussi, G. (1996) Plasmin promotes an endothelium-dependent adhesion of neutrophils. Involvement of platelet activating factor and P-selectin. _Circulation_ **93**, 2152–2160

Silvestro, L., Ruikun, C., Sommer, F., Duc, T. M., Biancone, L., Montrucchio, G., and Camussi, G. (1994) Platelet-activating factor-induced endothelial cell expression of adhesion molecules and modulation of surface glycolcalyx, evaluated by electron spectroscopy chemical analysis. _Semin. Thromb. Hemost._ **20**, 214–222

Muehlbauer, S. M., Lima, H., Jr., Goldman, D. L., Jacobson, L. S., Rivera, J., Goldberg, M. F., Palladino, M. A., Casadevall, A., and Brojatsch, J. (2010) Proteasome inhibitors prevent caspase-1-mediated disease in rodents challenged with anthrax lethal toxin. _Am. J. Pathol._ **177**, 735–743

Selles, I. E., Tucker, A. E., Voth, D. E., and Ballard, J. D. (2003) Toll-induced resistance in _Bacillus anthracis_ lethal toxin-treated macropores. _Proc. Natl. Acad. Sci. U.S.A._ **100**, 12426–12431

Huang, D., Ding, Y., Luo, W. M., Bender, S., Qian, C. N., Kort, E., Zhang, Z. F., VandenBeldt, K., Duesbery, N. S., Resau, J. H., and Teh, B. T. (2008) Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. _Cancer Res._ **68**, 81–88

Bachi, A. L., Dos Santos, L. C., Nonogaki, S., Janzar, J., and Sasiulionis, M. G. (2012) Apoptotic cells contribute to melanoma progression and this effect is partially mediated by the platelet-activating factor receptor. _Mediators Inflamm._ **2012**, 610371

Claud, E. C., Lu, J., Wang, X. Q., Abe, M., Petrof, E. O., Sun, J., Nelson, D. J.,...
9. Ma, X., and Bazan, H. E. (2001) Platelet-activating factor (PAF) enhances apoptosis induced by ultraviolet radiation in corneal epithelial cells through cytochrome c-caspase activation. *Curr. Eye Res.* 23, 326–335

50. Ryan, S. D., Harris, C. S., Carswell, C. L., Baenziger, J. E., and Bennett, S. A. (2008) Heterogeneity in the sn-1 carbon chain of platelet-activating factor glycerophospholipids determines pro- or anti-apoptotic signaling in primary neurons. *J. Lipid Res.* 49, 2250–2258

51. Stafforini, D. M., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (2003) Platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Crit. Care Med.* 30, S294–301

52. Zimmerman, G. A., McIntyre, T. M., Prescott, S. M., and Stafforini, D. M. (2002) The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. *Crit. Care Med.* 30, S294–301

53. Moayeri, M., Haines, D., Young, H. A., and Leplla, S. H. (2003) *Bacillus anthracis* lethal toxin induces TNF-α-independent hypoxia-mediated toxicity in mice. *J. Clin. Invest.* 112, 670–682

54. Grinberg, L. M., Abramova, F. A., Yampolskaya, O. V., Walker, D. H., and Smith, J. H. (2001) Quantitative pathology of inhalational anthrax I. Quantitative microscopic findings. *Mod. Pathol.* 14, 482–495

55. Grypioti, A. D., Theocharis, S. E., Demopoulos, C. A., Papadopoulou-Daifoti, Z., Basayiannis, A. C., and Mykoniatis, M. G. (2006) Effect of platelet-activating factor (PAF) receptor antagonist (BN52021) on acetaminophen-induced acute liver injury and regeneration in rats. *Liver Int.* 26, 97–105

56. Murohisa, G., Kobayashi, Y., Kawasaki, T., Nakamura, S., and Nakamura, H. (2002) Involvement of platelet-activating factor in hepatic apoptosis and necrosis in chronic ethanol-fed rats given endotoxin. *Liver Int.* 22, 394–403

57. Serizawa, A., Nakamura, S., Suzuki, Baba, S., and Nakano, M. (1996) Involvement of platelet-activating factor in cytokine production and neutrophil activation after hepatic ischemia-reperfusion. *Hepatology* 23, 1656–1663

58. Kimura, K., Moriyama, M., Nishisako, M., Kannan, Y., Shiota, M., Sakurada, K., Musashi, M., and Sugano, T. (1998) Modulation of platelet activating factor-induced glycogenolysis in the perfused rat liver after administration of endotoxin in vivo. *J. Biochem.* 123, 142–149

59. Kuiper, J., De Rijke, Y. B., Zijlstra, F. J., Van Waas, M. P., and Van Berkel, T. J. (1988) The induction of glycogenolysis in the perfused liver by platelet activating factor is mediated by prostaglandin D2 from Kupffer cells. *Biochem. Biophys. Res. Commun.* 157, 1288–1295

60. Altin, J. G., Dieter, P., and Bygrave, F. L. (1987) Evidence that Ca\(^{2+}\) fluxes and respiratory, glycogenolytic and vasoconstrictive effects induced by the action of platelet-activating factor and L-α-lysophosphatidylcholine in the perfused rat liver are mediated by products of the cyclo-oxygenase pathway. *Biochem. J.* 245, 145–150