INFECTION AND IMMUNITY, Mar. 2006, p. 1751–1756 Vol. 74, No. 3
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.3.1751–1756.2006
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Cytosolic Phospholipase A_2 Enzymes Are Not Required by Mouse Bone Marrow-Derived Macrophages for the Control of *Mycobacterium tuberculosis* In Vitro

Omar H. Vandal,[1] Michael H. Gelb,[2] Sabine Ehrt,[1] and Carl F. Nathan[1]*

Department of Microbiology and Immunology, Weill Medical College of Cornell University, and Programs in Immunology and Microbial Pathogenesis and in Molecular Biology, Weill Graduate School of Medical Sciences of Cornell University, New York, New York 10021,[1] and Departments of Chemistry and Biochemistry, University of Washington, Seattle, Washington 98195[2]

Received 7 September 2005/Returned for modification 14 October 2005/Accepted 20 December 2005

During the course of infection *Mycobacterium tuberculosis* predominantly resides within macrophages, where it encounters and is often able to resist the antibacterial mechanisms of the host. In this study, we assessed the role of macrophage phospholipases A_2 (PLA_2s) in defense against *M. tuberculosis*. Mouse bone marrow-derived macrophages (BMDMs) expressed $cPLA_2$-IVA, $cPLA_2$-IVB, $iPLA_2$-VI, $sPLA_2$-IIE, and $sPLA_2$-XIIA. The expression of $cPLA_2$-IVA was increased in response to *M. tuberculosis*, gamma interferon, or their combination, and $cPLA_2$-IVA mediated the release of arachidonic acid, which was stimulated by *M. tuberculosis* in activated, but not unactivated, macrophages. We confirmed that arachidonic acid is highly mycobactericidal in a concentration- and pH-dependent manner in vitro. However, when *M. tuberculosis*-infected macrophages were treated with PLA_2 inhibitors, intracellular survival of *M. tuberculosis* was not affected, even in inducible nitric oxide synthase-deficient macrophages, in which a major bactericidal mechanism is removed. Moreover, intracellular survival of *M. tuberculosis* was similar in $cPLA_2$-IVA-deficient and wild-type macrophages. Our results demonstrate that the cytosolic PLA_2s are not required by murine BMDMs to kill *M. tuberculosis*.

Mycobacterium tuberculosis is an intracellular pathogen that primarily inhabits macrophage phagosomes. In response to *M. tuberculosis*, macrophages activate antimicrobial pathways which control bacterial replication but are unable to achieve sterilization in vitro (32).

Only three gamma interferon (IFN-γ)-induced pathways of defense against *M. tuberculosis* have been defined. These involve inducible nitric oxide synthase (iNOS), phagocyte oxidase (Phox), and the predicted guanosine triphosphatase, LRG-47. Macrophages deficient in iNOS or LRG-47 are defective in their ability to control infection by *M. tuberculosis* (5, 11, 26, 27). On the other hand, Phox-deficient macrophages are able to control *M. tuberculosis* (11, 18, 19). However, a *katG* knockout of *M. tuberculosis*, which is attenuated in wild-type macrophages, returns to virulence in macrophages lacking Phox, indicating that potential antimicrobial mechanisms may be masked by the capacity of *M. tuberculosis* to detoxify defenses of the host (33). Previous work from our laboratory has shown that bone marrow derived-macrophages (BMDMs) are able to restrict the growth of *M. tuberculosis* in an IFN-γ-, iNOS-, and Phox-independent manner (11). This illustrates that unidentified pathways of host defense against *M. tuberculosis* are operative in these cells.

Transcriptome analysis of *M. tuberculosis* within the macrophage phagosome revealed that SigE-dependent genes, involved with the breakdown of fatty acids and resynthesis of cell envelope lipids, were induced in intraphagosomal bacteria (37). This transcriptional profile could be simulated by the treatment of *M. tuberculosis* with the cell wall-damaging detergent sodium dodecyl sulfate. Thus, within the phagosome, *M. tuberculosis* may experience a cell wall-perturbing stress. This observation and others showing that mycobacterial lipids are released within macrophages (3) suggest that macrophages may exert a damaging effect on the lipid-rich *M. tuberculosis* cell wall. Additionally, several studies have noted that the integrity of the *M. tuberculosis* cell wall is important for bacterial virulence (6, 9, 16, 35). This cell wall is likely to be critical in protecting the bacterium against innate defenses.

Phospholipase A_2 (PLA_2) enzymes hydrolyze the *sn*-2 bond of phospholipids to release a free fatty acid and a lysophospholipid. Three classes of PLA_2s exist in mammals: secreted PLA_2s ($sPLA_2$s), cytosolic PLA_2s ($cPLA_2$s) that are calcium dependent, and cytosolic PLA_2s that are calcium independent ($iPLA_2$s). The $cPLA_2$s and $iPLA_2$ are large (~80 kDa) proteins that reside within the cytosol, whereas $sPLA_2$s are smaller (~14 kDa), positively charged proteins that are found in many body tissues and fluids. The $sPLA_2$s display potent bactericidal activity (22, 47), which is dependent on their ability to penetrate the bacterial envelope and hydrolyze phospholipids in the cell membrane (4). Additionally, free fatty acid products of PLA_2s may be toxic to bacteria or may influence immunity of host cells.

One study concluded that free fatty acid released by $cPLA_2$-IVA-killed *M. tuberculosis* and PLA_2 inhibitors enhanced *M. tuberculosis* survival in murine peritoneal macrophages (1). In human-monocyte-derived macrophages, release of arachidonic acid (AA) by $cPLA_2$s promoted macrophage apoptosis and consequent killing of *M. tuberculosis* (8).

In the present study we reexamined the role of $cPLA_2$ enzymes as mediators of macrophage defense against *M. tuber-*

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Weill Medical College of Cornell University, 1300 York Avenue, Box 57, New York, NY 10021. Phone: (212) 746-6505. Fax: (212) 746-8587. E-mail: cnathan@med.cornell.edu.

culosis. We show that C57BL/6 mouse BMDMs express five PLA$_2$ enzymes. Only cPLA$_2$-IVA expression is increased by *M. tuberculosis*, IFN-γ, and their combination. Additionally, *M. tuberculosis* stimulated activated but not unactivated macrophages to release AA, and reagent AA was potently mycobactericidal. However, PLA$_2$ inhibitors did not alter intracellular viability of *M. tuberculosis* in macrophages. Further, cPLA$_2$-IVA null macrophages did not demonstrate a defect in restricting the growth of *M. tuberculosis*. Our results indicate that mouse BMDMs do not require cPLA$_2$s for defense against *M. tuberculosis* in vitro.

MATERIALS AND METHODS

Bacteria. *Mycobacterium tuberculosis* (strain H37Rv) was grown at 37°C in Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, 0.5% bovine serum albumin (BSA), 0.2% dextrose, and 0.085% NaCl. In all experiments early-log-phase *M. tuberculosis* was used (optical density at 600 nm, 0.2 to 0.4).

Macrophages. Femoral bone marrow cells from 8- to 10-week-old C57BL/6, C3H/HeN, or cPLA$_2$-IVA$^{-/-}$ (34) mice were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.58 g/liter L-glutamine, 1 mM Na-pyruvate, 10 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20% L929 cell-conditioned medium for 6 to 8 days to produce nearly pure cultures of macrophages by morphology and cell surface staining of macrophage markers. Greater than 90% of macrophages were CD14, F4/80, and FcγRII/III positive and upregulated major histocompatibility complex class II after IFN-γ activation. For infection with *M. tuberculosis*, macrophages were maintained in DMEM supplemented with 10% FBS, 0.58 g/liter L-glutamine, 1 mM Na-pyruvate, 10 mM HEPES, and 10% L929 cell-conditioned medium. The cPLA$_2$-IVA$^{-/-}$ mice (45) are on the C3H/HeN background, and therefore BMDMs from C3H/HeN littermates were used as wild-type controls in those experiments.

RT-PCR. For reverse transcription-PCRs (RT-PCRs) the following primer sets were used: sPLA$_2$-IB forward, 5′CAGACTCATGACCACTGCTACAGTC3′, reverse, 5′TGTATTCCTTGTTGTACGGGACCT3′; sPLA$_2$-IIC forward, 5′TTC ATCTTCTACTGGACAACCTCCACCC3′, reverse, 5′CCATATTCCTTACGA TTGTGGTAGCA3′; sPLA$_2$-IID forward, 5′CTCCTGAACCTGAACAAGATG GTC3′, reverse, 5′GCTGTATTTGTAGTTGTCTCTCAGGC3′; sPLA$_2$-IIE forward, 5′AGTTTGGAGTGATGATTGAGAGAATG3′, reverse, 5′CACAGA AGATGTTGTCTCGAGTGATA3′; sPLA$_2$-IIF forward, 5′ATCACACACAGA AACTCCATCCTG3′, reverse, 5′GTAGACGTTGAAGTAGCCTCGGTAC3′; sPLA$_2$-V forward, 5′TTGCTAGAACTCAAGTCCATGATTG3′, reverse, 5′AGA TGACTAGGCCATTTGTGTATCTG3′; sPLA$_2$-X forward, 5′GAACTATGG CTGTTATTGTGGCCT3′, reverse, 5′GAAGAGGTATTTCAGGTGGTACTCG3′; sPLA$_2$-XIIA forward, 5′TCCACAAGATAGACACGTACCTCAAC3′, reverse, 5′TGGACGTTCTGAGATAGTCCGAG3′; sPLA$_2$-XIIB forward, 5′AGAAGA AGGTCTCAGGATTGGAAGAT3′, reverse, 5′TGGACGTTCTGAGATAGT CCGAG3′; cPLA$_2$-IVA forward, 5′AAAATATTACAGCAAAGCACATCG TG3′, reverse, 5′CAGGTTAAATGTGAGCCCACTATCT3′; cPLA$_2$-IVB forward, 5′GTGGTCCCTGTCCTACTTAAGAGC3′, reverse, 5′GGGTGGATG TAACAAGAAGTGTTTC3′; and iPLA$_2$-VI forward, 5′GTGTGTACTTCC GTATGAAGGACG3′, reverse, 5′TCTACACAGGTTACAGGCACTTGAG3′. PCR amplification conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s. As positive controls for RT-PCR the following tissue samples were used: heart (for sPLA$_2$-V), spleen (for sPLA$_2$-IB, sPLA$_2$-IID, sPLA$_2$-X, and sPLA$_2$-XIIB), and testis (for sPLA$_2$-IIC, sPLA$_2$-IIF).

Real-time quantitative RT-PCR (qRT-PCR). BMDMs were seeded at 3 × 10^6 cells/flask in a T75 and were left untreated or were infected with *M. tuberculosis* at a multiplicity of infection (MOI) of 4:1 for 24 h and activated with 10 ng/ml IFN-γ for 40 h or with 10 ng/ml IFN-γ for 16 h followed by infection with *M. tuberculosis* at 4:1 for 24 h (total time in IFN-γ was 40 h). The monolayers were then lysed with Trizol (GIBCO BRL) and total RNA was isolated. After treatment with DNase I (Ambion) and purification (QIAGEN RNeasy), 100 ng of RNA was reverse transcribed into cDNA using gene-specific primers and analyzed by PCR on the ABI PRISM 7900HT sequence detection system (Perkin-Elmer). Primers and probes for qRT-PCR were synthesized by Biosearch Technologies. The probes were labeled with the reporter dye FAM at the 5′ end and Black Hole Quencher at the 3′ end. The following primer/probe sets were used: sPLA$_2$-IIE forward, 5′GGATTGGTG TTGTCATGCCC3′, reverse, 5′GGGTCACAGCCCAGCTTCT3′, probe, 5′TGA CTGCTGCTATGGCCGCCTG3′; sPLA$_2$-XIIA forward, 5′TAGACACGTACC TCAACGCCG3′, reverse, 5′TATCCATAGCGTGGAACAGGC3′, probe, 5′TGC CAGTACAAGTGCAGCGACG3′; cPLA$_2$-IVA forward, 5′CAGCAAAGC ACATCGTGAGTAA3′, reverse, 5′TTCATTCTCGGTGCCTTTGG3′, probe, 5′CAGCTCCGACAGTGATGATGAGGCTC3′; cPLA$_2$-IVB forward, 5′AACCTGCCCACTGAGCTGC3′, reverse, 5′GTGACTCAGAGGCCCAGG G3′, probe, 5′CCAGCTTCTGTCTGACATTGAGTCCCATG3′; iPLA$_2$-VI forward, 5′GACAGGGACACTGTCTGACCG3′, reverse, 5′GGCTTCGGGAGC ATCGTAA3′, probe, 5′CCAGCAGAGCTCCACCTATTCCG3′.

Arachidonic acid release. BMDMs were seeded at 1.5 × 10^5 cells/well in 48-well plates and loaded for 14 to 20 h with 0.1 μCi/ml of [^{3}H]arachidonate (Perkin-Elmer). Cells were then washed 3 times with DMEM containing 10% FBS to remove unincorporated arachidonate, and 0.4 ml fresh medium was replaced. Macrophages were then stimulated with phorbol myristate acetate (PMA) (100 nM) and A23187 (2 μM) or *M. tuberculosis* at an MOI of 8:1 with pyrrolidine-2 (10 μM) or indoxam (10 μM) as indicated. After 6 h the medium was removed and centrifuged to pellet any dislodged cells. An aliquot (0.2 ml) of this medium was submitted to scintillation counting. The cell monolayers were lysed with 0.4 ml H$_2$0 containing 0.5% Triton X-100, and 0.2 ml of this was submitted to scintillation counting. The percentage of arachidonate release was calculated as 100 × (disintegrations per minute [dpm] in medium)/(dpm in medium + cell-associated dpm).

Macrophage infections. BMDMs were seeded at 1.5 × 10^5 cells/well in 48-well plates with or without 10 ng/ml murine IFN-γ (R&D) and 10 ng/ml lipopolysaccharide (LPS) (Sigma). Sixteen hours later, macrophages were infected at an MOI of 4:1. Four hours after infection, extracellular *M. tuberculosis* was washed out with 1× phosphate-buffered saline; fresh medium was replaced every 36 h. At the time points indicated, macrophages were lysed with H$_2$O containing 0.5% Triton X-100, and intracellular bacteria were enumerated by plating serial dilutions of the lysates on agar (Middlebrook 7H11, 10% oleic acid-albumin-dextrose-catalase enrichment; Difco). Inhibitors were added 30 min prior to infection and replaced when fresh medium was added to the wells.

PLA$_2$ inhibitors. Pyrrolidine-2 (pyrrophenone) and Me-indoxam were prepared as described previously (39, 41). Arachidonyl trifluoromethyl ketone (ATFMK), bromoenol lactone, and methyl arachidonyl fluorophosphonate (MAFP) were purchased from Cayman Chemical. Quinacrine was purchased from Sigma.

Arachidonic acid treatment of *M. tuberculosis*. To test the toxicity of AA on *M. tuberculosis*, early-log-phase *M. tuberculosis* was diluted to an optical density of 0.05 and exposed in triplicate for 4 h to various concentrations of AA (Sigma) in Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol, 0.2% dextrose, and 0.085% NaCl, with or without 0.5% BSA. After exposure, serial dilutions were plated on agar plates (Middlebrook 7H11, 10% oleic acid-albumin-dextrose-catalase enrichment; Difco).

RESULTS

Expression of PLA$_2$s in macrophages. We tested for expression of the known 13 PLA$_2$s (cPLA$_2$-IVA, cPLA$_2$-IVB, iPLA$_2$-VI, sPLA$_2$-IB, sPLA$_2$-IIA, sPLA$_2$-IIC, sPLA$_2$-IID, sPLA$_2$-IIE, sPLA$_2$-IIF, sPLA$_2$-V, sPLA$_2$-X, sPLA$_2$-XIIA, and sPLA$_2$-XIIB) in BMDMs using RT-PCR. Expression of the following five PLA$_2$s was detected: cPLA$_2$-IVA, cPLA$_2$-IVB, iPLA$_2$-VI, sPLA$_2$-IIE, and sPLA$_2$-XIIA (data not shown). This expression pattern did not alter after treatment of BMDMs with *M. tuberculosis*, IFN-γ, or the combination of *M. tuberculosis* and IFN-γ. The remaining eight PLA$_2$s that were not found in BMDMs were detected in tissue samples known to express these PLA$_2$s (see Materials and Methods), indicating that the PCR primers were functional. A recent study detected sPLA$_2$-V protein in peritoneal macrophages by immunohistochemistry, but we did not detect sPLA$_2$-V in BMDMs by RT-PCR (36).

Next, real-time qRT-PCR was performed to study the regulation of PLA$_2$s in response to *M. tuberculosis*, IFN-γ, and their combination. The expression of cPLA$_2$-IVA was increased by more than 3-fold after treatment with *M. tuberculosis*, 8-fold after IFN-γ, and 12-fold after a combination of *M. tuberculosis* and IFN-γ (Fig. 1). The expression of sPLA$_2$-IIE was upregulated 4-fold in response to *M. tuberculosis*, downregulated 100-

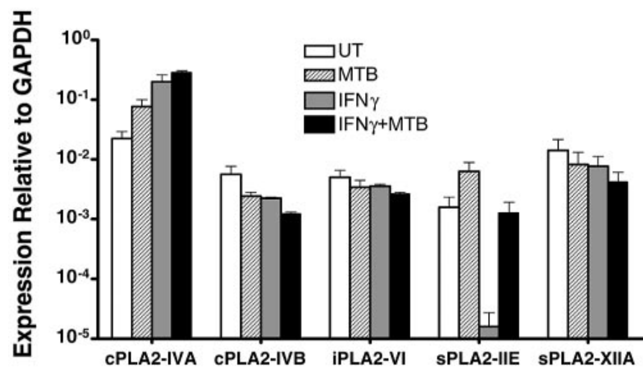


FIG. 1. Transcriptional regulation of macrophage PLA$_2$s. Expression of BMDM-expressed PLA$_2$s was measured in response to *M. tuberculosis*, IFN-γ, or the combination of *M. tuberculosis* and IFN-γ. BMDMs were untreated (UT) or treated with *M. tuberculosis* for 24 h, IFN-γ for 40 h, or IFN-γ for 16 h followed by *M. tuberculosis* for 24 h (total time in IFN-γ was 40 h), and mRNA was extracted for quantification by qRT-PCR. Data shown are means ± standard errors of three independent experiments with triplicates per experiment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. MTB, *M. tuberculosis.*

fold in response to IFN-γ, and restored by the combination of both stimuli (Fig. 1). No increase in expression of the remaining three PLA$_2$s was observed in response to *M. tuberculosis*, IFN-γ, or the combination (Fig. 1).

AA release. Previous studies have shown that cPLA$_2$-IVA has high specificity for AA-containing phospholipids and is the primary PLA$_2$ involved in the release of AA (42). Consistent with this, we observed that the cPLA$_2$-IVA-specific inhibitor pyrrolidine-2 (14) blocked PMA/A23187-induced AA release, whereas the sPLA$_2$ inhibitor indoxam did not (Fig. 2A). We next sought to determine whether BMDMs released AA after stimulation with *M. tuberculosis*. Release of AA by unactivated or IFN-γ-activated macrophages in response to *M. tuberculosis* was not detectable (data not shown). However, a modest statistically significant release of AA in response to *M. tuberculosis* was observed in macrophages activated with both LPS and IFN-γ (Fig. 2B). This release was inhibited by pyrrolidine-2 (Fig. 2B), indicating that AA release in response to *M. tuberculosis* is primarily mediated by cPLA$_2$-IVA.

Toxicity of AA towards *M. tuberculosis*. We next tested whether AA was toxic to *M. tuberculosis*. Long-chain, unsaturated free fatty acids are known to be potently mycobactericidal (10, 23–25). We confirmed that exposure of *M. tuberculosis* to AA in the absence of BSA resulted in significant killing and showed that this was dependent not only on the concentration of AA but also on the pH of the medium (Fig. 3). BSA binds free fatty acids. Consistent with this, no killing of *M. tuberculosis* was observed in the presence of 0.5% BSA (Fig. 3).

Cytosolic PLA$_2$s are not required to control *M. tuberculosis* survival. We next examined whether cPLA$_2$-IVA was required for control of *M. tuberculosis* in BMDMs. Macrophages treated with PLA$_2$ inhibitors were infected with *M. tuberculosis*, and the intracellular survival of *M. tuberculosis* was monitored. The use of the cPLA$_2$ inhibitor ATFMK or the cPLA$_2$-IVA-specific inhibitor pyrrolidine-2 did not affect the survival of *M. tuberculosis* within unactivated or LPS/IFN-γ-activated macrophages (Fig. 4). Additionally, no impact on survival of intracellular *M. tuberculosis* was seen in macrophages treated with the cPLA$_2$ inhibitor MAFP (10 μM) or the sPLA$_2$-specific inhibitor Me-indoxam (10 μM) (data not shown). The PLA$_2$ inhibitor quinacrine (5 μM) and the iPLA$_2$-VI inhibitor bromoenol lactone (10 μM) were also tested, but these proved to be toxic to the cells as they drastically altered cellular morphology and disrupted the cell monolayer. Because iNOS-mediated killing of *M. tuberculosis* might mask a possible role for PLA$_2$s, we also tested the PLA$_2$ inhibitors in iNOS-deficient macrophages (11). The use of ATFMK, MAFP, pyrrolidine-2, or Me-indoxam did not impact the survival of *M. tuberculosis* in macrophages lacking iNOS (data not shown).

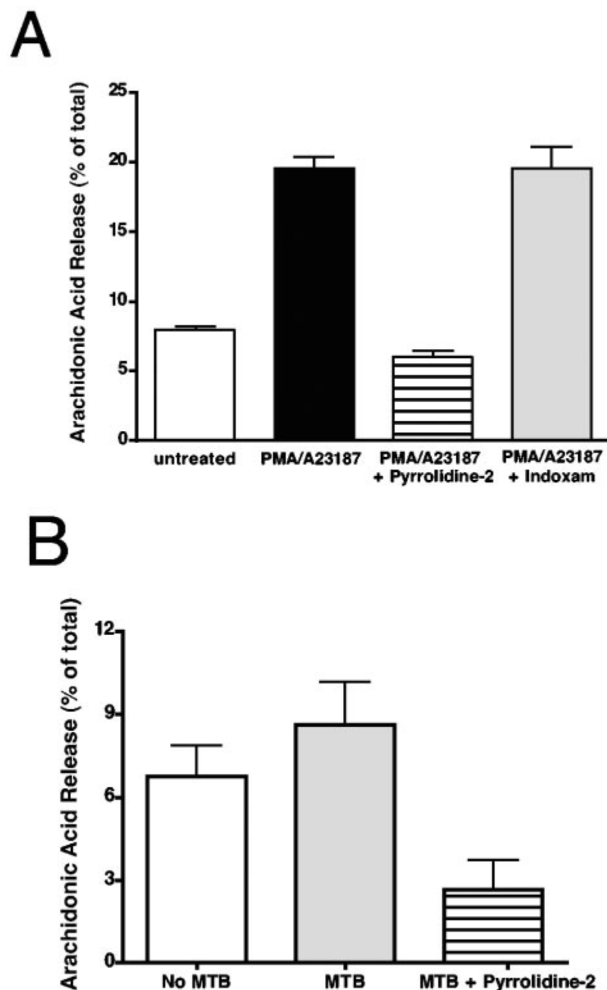


FIG. 2. Arachidonic acid release by macrophages. (A) Percent release of AA was measured in unactivated BMDMs after stimulation with PMA (100 nM) and A23187 (2 μM) alone or in the presence of the cPLA$_2$-IVA inhibitor pyrrolidine-2 (10 μM) or the sPLA$_2$ inhibitor Me-indoxam (10 μM). (B) Percent release of AA by activated BMDMs in response to *M. tuberculosis*. Macrophages were activated by exposure to LPS (10 ng/ml) and IFN-γ (10 ng/ml). AA release in response to *M. tuberculosis* was blocked by pyrrolidine-2 (10 μM). Groups are significantly different ($P < 0.05$) by repeated-measures one-way analysis of variance followed by Bonferroni's multiple comparison test using Prism software (GraphPad Software, Inc.). Data shown are means ± standard errors of four independent experiments with triplicates per experiment. MTB, *M. tuberculosis.*

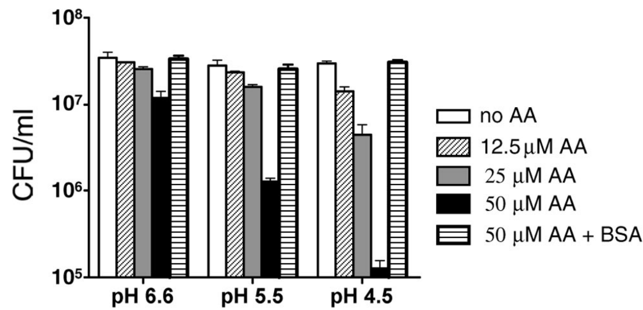
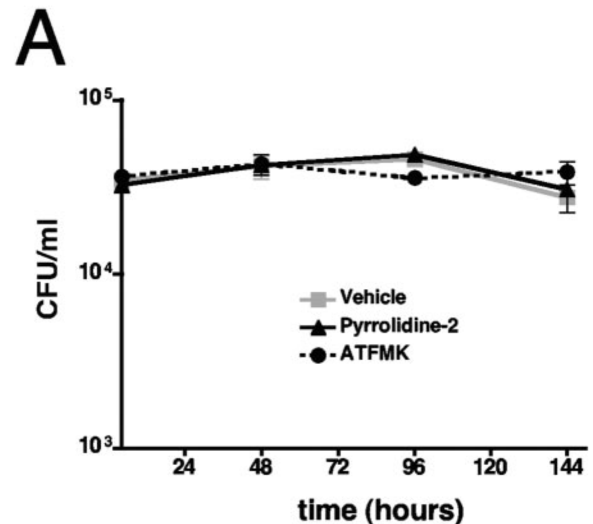


FIG. 3. Mycobactericidal activity of arachidonic acid. *M. tuberculosis* was treated with increasing concentrations of AA in 7H9 medium in the absence of BSA at a pH of 6.6, 5.5, or 4.5 for 4 h, after which serial dilutions were plated on agar plates for enumeration. No killing was observed by AA in the presence of BSA. Data shown are means ± standard deviations of one experiment done in triplicate and are representative of three independent experiments, each done in triplicate.



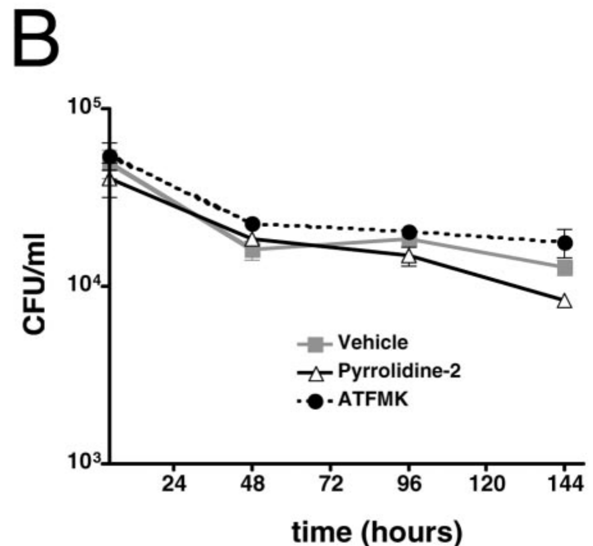


FIG. 4. Survival of *M. tuberculosis* in macrophages treated with PLA_2 inhibitors. (A) Unactivated BMDMs or (B) BMDMs activated with LPS (10 ng/ml) and IFN-γ (10 ng/ml) were infected with *M. tuberculosis* and treated with the inhibitor ATFMK (10 μM) or pyrrolidine-2 (10 μM). Intracellular *M. tuberculosis* was determined at the indicated time points by plating serial dilutions of cell lysates on agar plates. Data shown are means ± standard deviations of one experiment done in triplicate and are representative of two independent experiments, each done in triplicate.

As the $cPLA_2$ inhibitors may have resulted in incomplete inhibition, macrophages from $cPLA_2$-IVA-deficient mice were infected with *M. tuberculosis*. Unactivated or LPS- and IFN-γ-activated $cPLA_2$-IVA-deficient macrophages were able to control *M. tuberculosis* as well as wild-type macrophages (Fig. 5). These results indicate that $cPLA_2$-IVA is dispensable for BMDM defense against *M. tuberculosis*.

DISCUSSION

PLA_2s hydrolyze the sn-2 bond of phospholipids, resulting in the release of free fatty acids and lysophospholipids. PLA_2s are involved in a range of biological processes, including homeostasis of cell membranes, lipid metabolism, signaling, production of eicosanoids, and host defense (46). In this study, we observed that BMDMs enhanced the expression of $cPLA_2$-IVA in response to *M. tuberculosis*, IFN-γ, and their combination. $cPLA_2$-IVA has a marked specificity for phospholipids with AA at the *sn*-2 position, and accordingly we were able to block AA release with the $cPLA_2$-IVA-specific inhibitor pyrrolidine-2. In addition, $sPLA_2$-IIE was upregulated in BMDMs in response to *M. tuberculosis*, significantly downregulated by IFN-γ, and restored by the combination of both stimuli, a pattern of gene regulation observed for four other genes by microarray analysis (40). The $sPLA_2$s possess potent bactericidal activity, particularly against gram-positive microbes (22). LPS induced the elevation of $sPLA_2$-IIE in alveolar macrophages, suggesting that $sPLA_2$-IIE may play an important role in defense against airway pathogens (43). As alveolar macrophages are one of the first cell types to encounter *M. tuberculosis* and serve as a bacterial reservoir during the course of the infection, $sPLA_2$-IIE may have a function against *M. tuberculosis* in these cells. In our studies, the $sPLA_2$ inhibitor indoxam had no impact on survival of *M. tuberculosis* in BMDMs. This may have been due to poor permeability of the inhibitor toward the macrophages, as seen with an epithelial cell line (30). In macrophages, however, membrane-impermeant molecules are delivered to phagosomes by pinosomes that fuse with the phagosomes (7). Moreover, indoxam may also be taken up by macrophages during phagocytosis of *M. tuberculosis*. Upon delivery by either or both routes, indoxam may be able to inhibit phagosomal $sPLA_2$s.

In the present study, macrophages activated by LPS and IFN-γ, but not resting macrophages, released AA in response to *M. tuberculosis*. Macrophage activation may be required to induce signaling pathways that lead to $cPLA_2$ activity and AA release in culture supernatants. However, it is possible that AA release occurs locally in phagosomes, with only minimal amounts being measured in culture supernatants. In support of this, one study demonstrated that phospholipase activity was enriched in the phagosomes of alveolar macrophages after treatment with *Mycobacterium bovis* BCG (13). More recently

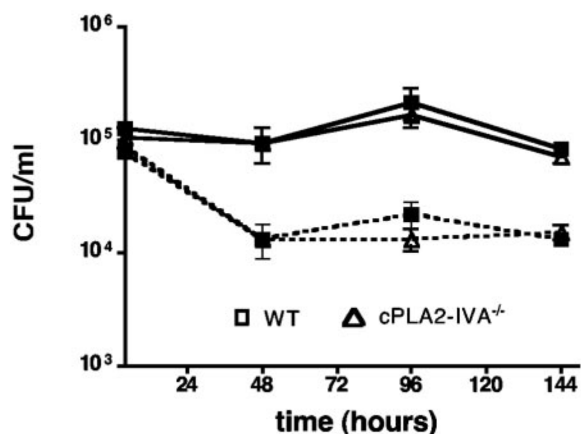


FIG. 5. Survival of *M. tuberculosis* in wild-type and cPLA$_2$-IVA$^{-/-}$ macrophages. Unactivated BMDMs (solid lines) or BMDMs activated with LPS (10 ng/ml) and IFN-γ (10 ng/ml) (dashed lines) from wild-type or cPLA$_2$-IVA$^{-/-}$ mice were infected with *M. tuberculosis*, and the number of intracellular *M. tuberculosis* organisms was determined at the indicated time points by plating serial dilutions of the cell lysates on agar plates. Data shown are means ± standard deviations of one experiment done in triplicate and are representative of two independent experiments, each done in triplicate. WT, wild type.

it was shown that cPLA$_2$-IVA translocates to phagosomes upon macrophage uptake of yeast particles (15).

Work from the 1940s onwards clearly demonstrated that free fatty acids are highly toxic to mycobacteria (10, 23–25). Here, we confirmed that AA is potently mycobactericidal. AA was most toxic at an acidic pH, and it is possible that acidification of the phagosome in activated macrophages may synergize with free fatty acids to kill *M. tuberculosis*. Free fatty acids possibly embed themselves in the lipid-rich membrane of *M. tuberculosis*, thereby perturbing its structure and function. Akaki et al. showed that AA released by zymosan-elicited peritoneal macrophages can associate with *M. tuberculosis* (1). Additional studies have also demonstrated that macrophages secrete free fatty acids that have mycobactericidal activity (17, 21). It was speculated that this release might occur via the action of macrophage lipases and could potentially serve as a pathway of mycobacterial control. Along these lines, one study showed that snake venom PLA$_2$ in combination with macrophage membrane fractions killed *M. tuberculosis*, indicating that PLA$_2$s could release mycobactericidal free fatty acids from membranes (20). The authors proposed that by localizing to the phagosome, PLA$_2$s could hydrolyze toxic free fatty acids from phagosomal membranes and thereby restrict mycobacterial growth. Alternatively, PLA$_2$s may directly degrade bacterial membrane phospholipids. For example, a macrophage lysosomal PLA$_2$ cleaved *M. tuberculosis* cardiolipin to lysocardiolipin (12).

Free fatty acids or their downstream products can impact the immune status of host cells. For instance, AA is metabolized into eicosanoids, which serve as important lipid mediators of inflammation, and it is likely that the eicosanoids are instrumental in regulating host immunity during the course of *M. tuberculosis* infection. Moreover, AA itself promotes phagosome-lysosome fusion, which in turn promotes killing of mycobacteria in macrophages (2). Administration of AA to *M. tuberculosis*-infected human macrophages promoted host cell apoptosis and led to a reduction in the mycobacterial burden (8). Additionally, PLA$_2$ inhibitors blocked apoptosis of human macrophages and enhanced intracellular survival of the mycobacteria (8). In our study, we did not observe enhanced viability of *M. tuberculosis* in macrophages treated with PLA$_2$ inhibitors or in cPLA$_2$-IVA-deficient macrophages. This may be due to different antimycobacterial mechanisms in the human and mouse cells tested, including differences between bacteriostatic and bactericidal effects.

It is possible that the other macrophage-expressed PLA$_2$s act redundantly with cPLA$_2$-IVA, masking its function. Alternatively, *M. tuberculosis* may have mechanisms to resist damage induced directly by PLA$_2$s or by free fatty acids released by PLA$_2$s. Fatty acids may be the primary carbon source for *M. tuberculosis* during infection (38). *M. tuberculosis* within macrophages and mice expressed β-oxidation genes involved in fatty acid breakdown and resynthesis (37, 44). Acetyl coenzyme A utilization is essential for *M. tuberculosis* pathogenesis, as a mutant lacking the glyoxylate shunt enzymes isocitrate lyase-1 and -2 is attenuated in macrophages and is unable to persist in mice (31). In addition, *M. tuberculosis* deficient in SigE, which is required for the transcription of genes involved in fatty acid metabolism, has a reduced level of virulence in both macrophages and mice (28, 29). The induction of genes involved in fatty acid degradation may not only allow *M. tuberculosis* to use free fatty acids for energy but may also help detoxify potentially lethal free fatty acids (29). Thus, β-oxidation may serve as a bacterial counterdefense against free fatty acids produced by host cells.

Here we explored the role macrophage PLA$_2$s play in host defense against *M. tuberculosis*. Expression of cPLA$_2$IV-A was enhanced in response to *M. tuberculosis*, IFN-γ, and the combination of *M. tuberculosis* and IFN-γ, activated macrophages released AA in response to *M. tuberculosis*, and AA was highly mycobactericidal. Nonetheless, PLA$_2$ inhibitors did not influence intracellular survival of the bacterium in BMDMs. In addition, cPLA$_2$ IV-A-deficient BMDMs were able to control *M. tuberculosis* replication as well as wild-type cells. Our results indicate that cPLA$_2$s are not required by mouse BMDMs to kill *M. tuberculosis* in vitro. Further studies with macrophages deficient in other PLA$_2$s or inhibitors specifically targeting other PLA$_2$s are needed to explore further the function of these enzymes in host defense against *M. tuberculosis*.

ACKNOWLEDGMENTS

This work was funded by NIH grant HL72718 to C.F.N. and a Cancer Research Institute Predoctoral Fellowship to O.H.V. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation.

REFERENCES

1. **Akaki, T., H. Tomioka, T. Shimizu, S. Dekio, and K. Sato.** 2000. Comparative roles of free fatty acids with reactive nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages against Mycobacterium tuberculosis. Clin. Exp. Immunol. **121:**302–310.
2. **Anes, E., M. P. Kuhnel, E. Bos, J. Moniz-Pereira, A. Habermann, and G. Griffiths.** 2003. Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. Nat. Cell Biol. **5:**793–802.
3. **Beatty, W. L., E. R. Rhoades, H. J. Ullrich, D. Chatterjee, J. E. Heuser, and D. G. Russell.** 2000. Trafficking and release of mycobacterial lipids from infected macrophages. Traffic **1:**235–247.
4. **Beers, S. A., A. G. Buckland, R. S. Koduri, W. Cho, M. H. Gelb, and D. C. Wilton.** 2002. The antibacterial properties of secreted phospholipases A2:

a major physiological role for the group IIA enzyme that depends on the very high pI of the enzyme to allow penetration of the bacterial cell wall. J. Biol. Chem. **277:**1788–1793.

5. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
6. **Cox, J. S., B. Chen, M. McNeil, and W. R. Jacobs, Jr.** 1999. Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. Nature **402:**79–83.
7. **Drevets, D. A., B. P. Canono, P. J. M. Leenen, and P. A. Campbell.** 1994. Gentamicin kills intracellular *Listeria monocytogenes*. Infect. Immun. **62:** 2222–2228.
8. **Duan, L., H. Gan, J. Arm, and H. G. Remold.** 2001. Cytosolic phospholipase A2 participates with TNF-alpha in the induction of apoptosis of human macrophages infected with Mycobacterium tuberculosis H37Ra. J. Immunol. **166:**7469–7476.
9. **Dubnau, E., J. Chan, C. Raynaud, V. P. Mohan, M. A. Laneelle, K. Yu, A. Quemard, I. Smith, and M. Daffe.** 2000. Oxygenated mycolic acids are necessary for virulence of Mycobacterium tuberculosis in mice. Mol. Microbiol. **36:**630–637.
10. **Dubos, R.** 1947. The effect of lipids and serum albumin on bacterial growth. J. Exp. Med. **85:**9–22.
11. **Ehrt, S., D. Schnappinger, S. Bekiranov, J. Drenkow, S. Shi, T. R. Gingeras, T. Gaasterland, G. Schoolnik, and C. Nathan.** 2001. Reprogramming of the macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles of nitric oxide synthase-2 and phagocyte oxidase. J. Exp. Med. **194:**1123–1140.
12. **Fischer, K., D. Chatterjee, J. Torrelles, P. J. Brennan, S. H. Kaufmann, and U. E. Schaible.** 2001. Mycobacterial lysocardiolipin is exported from phagosomes upon cleavage of cardiolipin by a macrophage-derived lysosomal phospholipase A2. J. Immunol. **167:**2187–2192.
13. **Franson, R. C., and M. Waite.** 1973. Lysosomal phospholipase A_1 and A_2 of normal and bacillus calmette guerin-induced alveolar macrophages. J. Cell Biol. **56:**621–627.
14. **Ghomashchi, F., A. Stewart, Y. Hefner, S. Ramanadham, J. Turk, C. C. Leslie, and M. H. Gelb.** 2001. A pyrrolidine-based specific inhibitor of cytosolic phospholipase A(2)alpha blocks arachidonic acid release in a variety of mammalian cells. Biochim. Biophys. Acta **1513:**160–166.
15. **Girotti, M., J. H. Evans, D. Burke, and C. C. Leslie.** 2004. Cytosolic phospholipase A2 translocates to forming phagosomes during phagocytosis of zymosan in macrophages. J. Biol. Chem. **279:**19113–19121.
16. **Glickman, M. S., J. S. Cox, and W. R. Jacobs, Jr.** 2000. A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of Mycobacterium tuberculosis. Mol. Cell **5:**717–727.
17. **Hemsworth, G. R., and I. Kochan.** 1978. Secretion of antimycobacterial fatty acids by normal and activated macrophages. Infect. Immun. **19:**170–177.
18. **Hisert, K. B., M. A. Kirksey, J. E. Gomez, A. O. Sousa, J. S. Cox, W. R. Jacobs, Jr., C. F. Nathan, and J. D. McKinney.** 2004. Identification of *Mycobacterium tuberculosis* counterimmune (*cim*) mutants in immunodeficient mice by differential screening. Infect. Immun. **72:**5315–5321.
19. **Jung, Y. J., R. LaCourse, L. Ryan, and R. J. North.** 2002. Virulent but not avirulent Mycobacterium tuberculosis can evade the growth inhibitory action of a T helper 1-dependent, nitric oxide synthase 2-independent defense in mice. J. Exp. Med. **196:**991–998.
20. **Kanai, K., and E. Kondo.** 1980. Phospholipase A2-induced antimycobacterial activity in the membrane fraction obtained from peritoneal exudate cells of guinea pigs. Jpn. J. Med. Sci. Biol. **33:**87–101.
21. **Kochan, I., and C. A. Golden.** 1974. Immunological nature of antimycobacterial phenomenon in macrophages. Infect. Immun. **9:**249–254.
22. **Koduri, R. S., J. O. Gronroos, V. J. Laine, C. Le Calvez, G. Lambeau, T. J. Nevalainen, and M. H. Gelb.** 2002. Bactericidal properties of human and murine groups I, II, V, X, and XII secreted phospholipases A(2). J. Biol. Chem. **277:**5849–5857.
23. **Kondo, E., and K. Kanai.** 1976. Further studies on the lethal effect of long-chain fatty acids on mycobacteria. Jpn. J. Med. Sci. Biol. **29:**25–37.
24. **Kondo, E., and K. Kanai.** 1972. The lethal effect of long-chain fatty acids on mycobacteria. Jpn. J. Med. Sci. Biol. **25:**1–13.
25. **Kondo, E., and K. Kanai.** 1977. The relationship between the chemical structure of fatty acids and their mycobactericidal activity. Jpn. J. Med. Sci. Biol. **30:**171–178.
26. **MacMicking, J. D., R. J. North, R. LaCourse, J. S. Mudgett, S. K. Shah, and C. F. Nathan.** 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc. Natl. Acad. Sci. USA **94:**5243–5248.
27. **MacMicking, J. D., G. A. Taylor, and J. D. McKinney.** 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science **302:**654–659.
28. **Manganelli, R., L. Fattorini, D. Tan, E. Iona, G. Orefici, G. Altavilla, P. Cusatelli, and I. Smith.** 2004. The extra cytoplasmic function sigma factor σ^E is essential for *Mycobacterium tuberculosis* virulence in mice. Infect. Immun. **72:**3038–3041.
29. **Manganelli, R., M. I. Voskuil, G. K. Schoolnik, and I. Smith.** 2001. The Mycobacterium tuberculosis ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol. Microbiol. **41:**423–437.
30. **Mounier, C. M., F. Ghomashchi, M. R. Lindsay, S. James, A. G. Singer, R. G. Parton, and M. H. Gelb.** 2004. Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A(2) occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A(2)-alpha. J. Biol. Chem. **279:**25024–25038.
31. **Munoz-Elias, E. J., and J. D. McKinney.** 2005. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. Nat. Med. **11:**638–644.
32. **Nathan, C. F., and S. Ehrt.** 2003. Nitric oxide in tuberculosis, p. 215–235. *In* W. N. Rom and S. M. Garay (ed.), Tuberculosis, 2nd ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
33. **Ng, V. H., J. S. Cox, A. O. Sousa, J. D. MacMicking, and J. D. McKinney.** 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Mol. Microbiol. **52:**1291–1302.
34. **Rubin, B. B., G. P. Downey, A. Koh, N. Degousee, F. Ghomashchi, L. Nallan, E. Stefanski, D. W. Harkin, C. Sun, B. P. Smart, T. F. Lindsay, V. Cherepanov, E. Vachon, D. Kelvin, M. Sadilek, G. E. Brown, M. B. Yaffe, J. Plumb, S. Grinstein, M. Glogauer, and M. H. Gelb.** 2005. Cytosolic phospholipase A2-alpha is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection: cPLA2-alpha does not regulate neutrophil NADPH oxidase activity. J. Biol. Chem. **280:**7519–7529.
35. **Sassetti, C. M., and E. J. Rubin.** 2003. Genetic requirements for mycobacterial survival during infection. Proc. Natl. Acad. Sci. USA **100:**12989–12994.
36. **Satake, Y., B. L. Diaz, B. Balestrieri, B. K. Lam, Y. Kanaoka, M. J. Grusby, and J. P. Arm.** 2004. Role of group V phospholipase A2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. J. Biol. Chem. **279:**16488–16494.
37. **Schnappinger, D., S. Ehrt, M. I. Voskuil, Y. Liu, J. A. Mangan, I. M. Monahan, G. Dolganov, B. Efron, P. D. Butcher, C. Nathan, and G. K. Schoolnik.** 2003. Transcriptional adaptation of Mycobacterium tuberculosis within macrophages: insights into the phagosomal environment. J. Exp. Med. **198:**693–704.
38. **Segal, W.** 1984. Growth dynamics of in vivo and in vitro grown mycobacterial pathogens, p. 547–573. *In* G. P. Kubica and L. G. Wayne (ed.), The mycobacteria: a sourcebook. Marcel Dekker Inc., New York, N.Y.
39. **Seno, K., T. Okuno, K. Nishi, Y. Murakami, K. Yamada, S. Nakamoto, and T. Ono.** 2001. Pyrrolidine inhibitors of human cytosolic phospholipase A2. Part 2: synthesis of potent and crystallized 4-triphenylmethylthio derivative "pyrrophenone". Bioorg. Med. Chem. Lett. **11:**587–590.
40. **Shi, S., C. Nathan, D. Schnappinger, J. Drenkow, M. Fuortes, E. Block, A. Ding, T. R. Gingeras, G. Schoolnik, S. Akira, K. Takeda, and S. Ehrt.** 2003. MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to Mycobacterium tuberculosis. J. Exp. Med. **198:**987–997.
41. **Singer, A. G., F. Ghomashchi, C. Le Calvez, J. Bollinger, S. Bezzine, M. Rouault, M. Sadilek, E. Nguyen, M. Lazdunski, G. Lambeau, and M. H. Gelb.** 2002. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. J. Biol. Chem. **277:**48535–48549.
42. **Six, D. A., and E. A. Dennis.** 2000. The expanding superfamily of phospholipase A(2) enzymes: classification and characterization. Biochim. Biophys. Acta **1488:**1–19.
43. **Suzuki, N., J. Ishizaki, Y. Yokota, K. Higashino, T. Ono, M. Ikeda, N. Fujii, K. Kawamoto, and K. Hanasaki.** 2000. Structures, enzymatic properties, and expression of novel human and mouse secretory phospholipase A(2)s. J. Biol. Chem. **275:**5785–5793.
44. **Timm, J., F. A. Post, L. G. Bekker, G. B. Walther, H. C. Wainwright, R. Manganelli, W. T. Chan, L. Tsenova, B. Gold, I. Smith, G. Kaplan, and J. D. McKinney.** 2003. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. Proc. Natl. Acad. Sci. USA **100:**14321–14326.
45. **Uozumi, N., K. Kume, T. Nagase, N. Nakatani, S. Ishii, F. Tashiro, Y. Komagata, K. Maki, K. Ikuta, Y. Ouchi, J. Miyazaki, and T. Shimizu.** 1997. Role of cytosolic phospholipase A2 in allergic response and parturition. Nature **390:**618–622.
46. **Valentin, E., and G. Lambeau.** 2000. Increasing molecular diversity of secreted phospholipases A(2) and their receptors and binding proteins. Biochim. Biophys. Acta **1488:**59–70.
47. **Weinrauch, Y., P. Elsbach, L. M. Madsen, A. Foreman, and J. Weiss.** 1996. The potent anti-Staphylococcus aureus activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. J. Clin. Investig. **97:**250–257.

Editor: F. C. Fang