Roles of Cytoplasmic Phospholipase in Expression of the Antimicrobial Activity of Host Macrophages against *Mycobacterium tuberculosis* Infection

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We studied roles of phospholipase A_2 (PLA_2) isozymes, including type IIA secretory PLA_2 ($sPLA_2$-IIA), type IV cytosolic, Ca^{2+}-dependent PLA_2 ($cPLA_2$), type V secretory PLA_2 ($sPLA_2$-V), and type VI cytosolic, Ca^{2+}-independent PLA_2 ($iPLA_2$), in macrophage (Mϕ) antimicrobial activity against *Mycobacterium tuberculosis* (Mtb) H37Ra (avirulent) strain and Mϕ mRNA expression of these PLA_2 isotypes in response to infection with the microorganisms. First, a $cPLA_2$ inhibitor arachidonyl trifluoromethylketone mildly reduced Mϕ anti-Mtb activity, while the other PLA_2 inhibitors did not significantly block the Mϕ antimycobacterial function, if any. Second, Mϕ expression of $cPLA_2$ and $sPLA_2$-V mRNAs was up-regulated during 6 to 12 h after infection with Mtb H37Ra strain. These findings suggest that $cPLA_2$ plays a role in cellular mechanisms participating in the expression of Mϕ anti-Mtb activity.

Key words: *Mycobacterium tuberculosis*, macrophages, phospholipase A_2, antimicrobial activity

INTRODUCTION

Macrophages (Mϕs) play a central role as antimicrobial effector cells in the expression of host resistance to *Mycobacterium tuberculosis* (Mtb). Reactive nitrogen intermediates (RNIs) have been demonstrated to play an important role in the activity of Mϕs against Mtb (1, 2, 3). Studies employing inducible nitric oxide synthase (iNOS) gene-disrupted mice indicated that RNIs were required for the activity of Mϕs against Mtb (4, 5). With respect to the role of reactive oxygen intermediates (ROIs), it has been reported that ROIs are insufficient to inhibit and/or kill Mtb (3, 4, 6, 7), although some investigators including us indicated possible roles of ROIs, such as H_2O_2-mediated halogenation system, in intramacrophage killing of Mtb (8, 9).

We previously found that there was no relationship between the degree of susceptibility of a given *Mycobacterium avium* complex (MAC) strain to RNIs and ROIs and its virulence in mice (10). Thus, RNIs and ROIs each alone are not decisive as effector components of the host defence mechanism against MAC, and alternative antimicrobial effectors may be involved in the antimycobacterial activity of Mϕs. We also found that unsaturated long-chain free fatty acids, such as oleic acid, linoleic acid, linolenic acid, and arachidonic acid, exhibited strong antimycobacterial activity (11). In addition, intramacrophage growth of Mtb was accelerated by an inhibitor of type IV cytosolic phospholipase A_2 ($cPLA_2$), arachidonyl trifluoromethylketone (a-TFMK) (12). These findings support the concept that free fatty acids, especially arachidonic acid, may play important roles in Mϕ antimicrobial function against mycobacterial pathogens including Mtb and MAC. In this study, to determine the roles of other PLA_2 isozymes than $cPLA_2$, such as type IIA secretory PLA_2 ($sPLA_2$-IIA), type V secretory PLA_2 ($sPLA_2$-V), and type VI cytosolic, Ca^{2+}-independent PLA_2 ($iPLA_2$) (13) in Mϕ anti-Mtb antimicrobial function, we examined the effects of various PLA_2 inhibitors on Mϕ antimicrobial activity against Mtb. In addition, we studied profiles of intracellular expression of these PLA_2 isotypes in Mtb-infected Mϕs.

MATERIALS AND METHODS

Microoganisms

Attenuated Mtb H37Ra strain (ATCC 25177) was

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used. The organisms were cultured in Middlebrook 7H9 broth and bacterial suspension prepared with phosphate-buffered saline (PBS) containing 1% bovine serum albumin was used as an inoculum.

Special agents

Special agents used in this study were as follows: manoalide (Wako Pure Chemical Industries, Ltd., Osaka Japan), cyclic (2-NaphthylAla-Leu-Ser-2-NaphthylAla-Arg) trifluoroacetate salt, GIIA-Inh (SPI525145) (Calbiochem, San Diego, CA), arachidonyl trifluoromethylketone (a-TFMK) (Sigma Chemicals Co., St. Louis, MO), N-{(2S,4R) -4- (Biphenyl-2-ylmethyl-isobutyl-amino) -1- [2- (2,4-difluorobenzoyl) -benzoyl] -pyrrolidin-2-ylmethyl}-3- [4- (2,4-dioxothiazolidin-5-ylidenemethyl) -phenyl] acrylamide (CPI525143) (Calbiochem), 12-episcalaradial (EPR) (Wako), and bromoenol lactone (BEL) (Sigma). These agents were initially dissolved in dimethyl-sulfoxide (DMSO) at 50 mM.

Fig. 1 shows chemical structures of various PLAs inhibitors. IC50 values against PLA_2 are as follows. Manoalide: 4.7 μM against human recombinant $sPLA_2$, SPI525145: 12.8 μM against human $sPLA_2$-ⅡA, a-TFMK: 0.3 μM against U937 $cPLA_2$, CPI525143: 1.8 nM against $cPLA_2\alpha$, EPR: 5.4 μM against human recombinant $sPLA_2$, BEL: 0.03μM against myocardial $iPLA_2$.

Macrophage monolayer culture

Peritoneal cells collected from 8-12-week-old BALB/c mouse given an i.p. injection 1mg zymozan A (Sigma) 4 days before. Mϕ monolayer cultures were prepared either by seeding 1.5 x 10^5 of zymosan A-induced peritoneal exudate cells in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and 25 mM HEPES (designated RPMI medium) on a 96-well flat-bottomed microculture well. In some experiments, mouse macrophage cell lines RAW 264.7 (ATCC TIB-78, derived from BALB/c) were used. 1 x 10^6 of RAW 264.7 Mϕs seeded on a 60-mm culture dish followed by subsequent 3-day cultivation in 5 ml of 5% FBS-RPMI medium containing 500 units/ml IFN-γ.

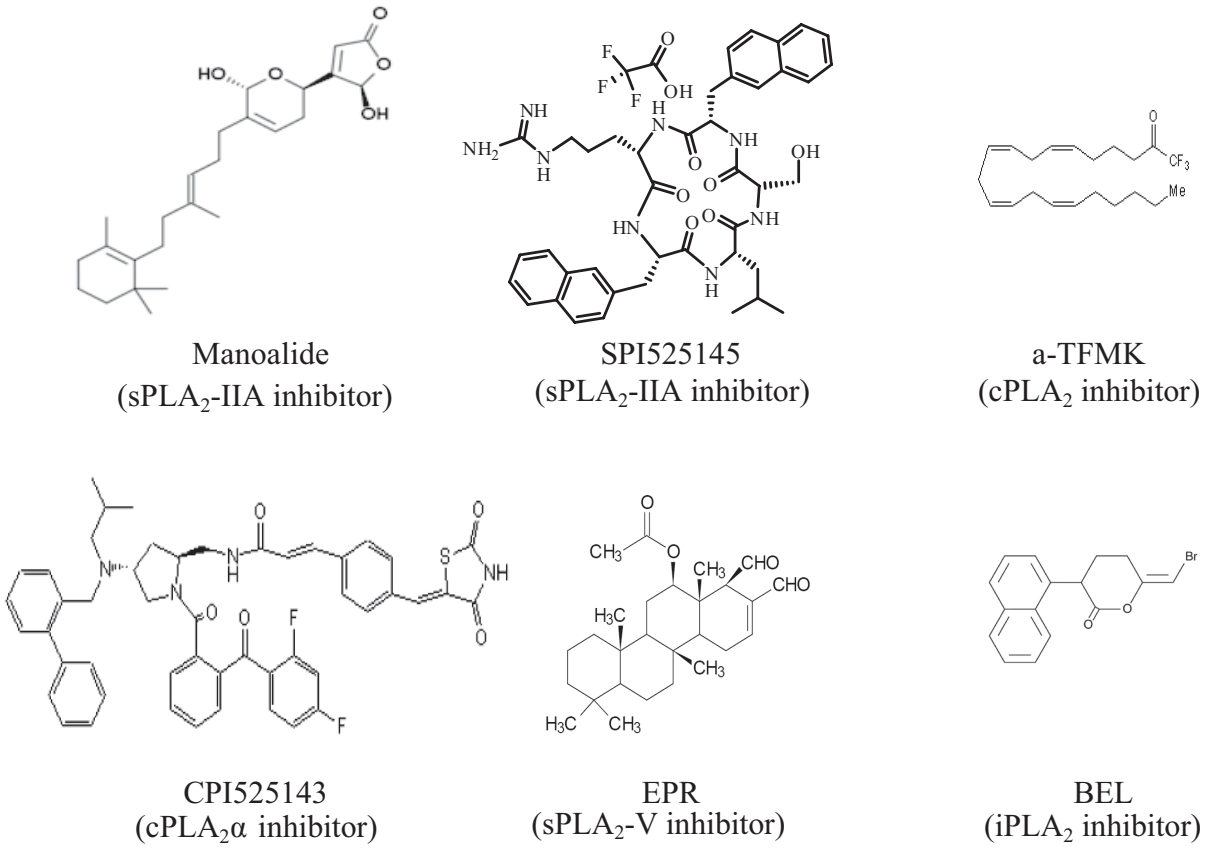

Fig. 1. Chemical structures of various PLA_2 inhibitors in this study.

MTT cytotoxicity assay

Monolayer cultures of mouse peritoneal Mϕs were cultured in 0.2 ml of 5% FBS-RPMI medium with or without test agents for 24 h. After medium change with test drug-free fresh 5% FBS-RPMI medium containing 5 mg/ml of MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide, a tetrazole)(Dojindo, Tokyo, Japan), the Mϕs were incubated for 3 h, cell-associated MTT was extracted with DMSO, and measured for the optical density at 550nm.

Macrophage antimicrobial activity against Mtb

For measurement of Mϕ antimicrobial activity, the monolayer cultures of mouse peritoneal Mϕs were incubated in 0.1 ml of RPMI medium containing 7.5 x 10^4 CFU/ml of Mtb H37Ra in a CO_2 incubator for 2 h. Infected Mϕs were then washed with Hanks' balanced salt solution containing 2% FBS to remove extracellular organisms, and thereafter cultivated in 0.2 ml of RPMI medium with or without the addition of test agents. After 3- and 5-day cultivation, the Mϕs were lysed with 0.07% SDS and the number of CFU in resulting cell lysate was counted on 7H11 agar plates.

Measurement of expression of PLA_2 and iNOS mRNAs

RT-PCR analysis of PLA_2 and iNOS mRNAs in Mtb-phagocytizing Mϕs was performed as follows. Monolayer cultures of RAW264.7 Mϕs were incubated in 5 ml of RPMI medium containing 500 units/ml IFN-γ and 1 x 10^6 CFU/ml of UV-killed Mtb for up to 12 h. At intervals, total RNA was isolated from cultured Mϕs using the ISOGEN kit (Nippon Gene Co., Toyama, Japan). After DNase-I treatment, the resultant RNA samples were reverse transcribed using random hexamer primers and 200 units Superscript II reverse transcriptase. One μl of aliquots of resultant cDNA was amplified by PCR in the standard reaction mixture containing 1 x PCR buffer, 0.2 mM of each dNTP, 1 unit of Taq polymerase, and 20 pmoles of sense and antisense primers for test PLA_2 (synthesized by Greiner Labortechnik Co., Tokyo, Japan) as follows (sense/antisense): $sPLA_2$-IIA (CGGCTTAAGACAGGAAAGAGAG/TGCAAAACATGTTGGGGTAGAA), $cPLA_2$ (CTTACACCACAGAAAGTTAAAAGAT/AAATAGGTCAGGAGCCATAAA), $sPLA_2$-V (CAGGGGGCTTGCTAGAACTCAA/AAGAGGGTTGTAAGTCCAGAGG), iNOS (CCTGCTCACTCAGCCAAG/AGTCATGGAGCCGCTGCT) β-actin (TGGAATCCTGCGGCATCCATGAAAC/TAAAACGCAGCTCAGTAACAGTCCG). Reactions were carried out in a DNA thermal cycler for 30 cycles including denaturing at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min for each cycle. PCR products were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels. Intensity of the PLA_2 bands were determined with public software NIH image. $iPLA_2$ mRNA was not done, since an appropriate primer set could not be provided.

RESULTS AND DISCUSSION

Fig. 2 shows the cytotoxic effects of various PLA_2 inhibitors, including manoalide ($sPLA_2$-IIA inhibitor), SPI525145 ($sPLA_2$-IIA inhibitor), a-TFMK ($cPLA_2$ inhibitor), CPI525143 ($cPLA_2\alpha$ inhibitor), 12-episcalaradial (EPR)($sPLA_2$-V inhibitor), and

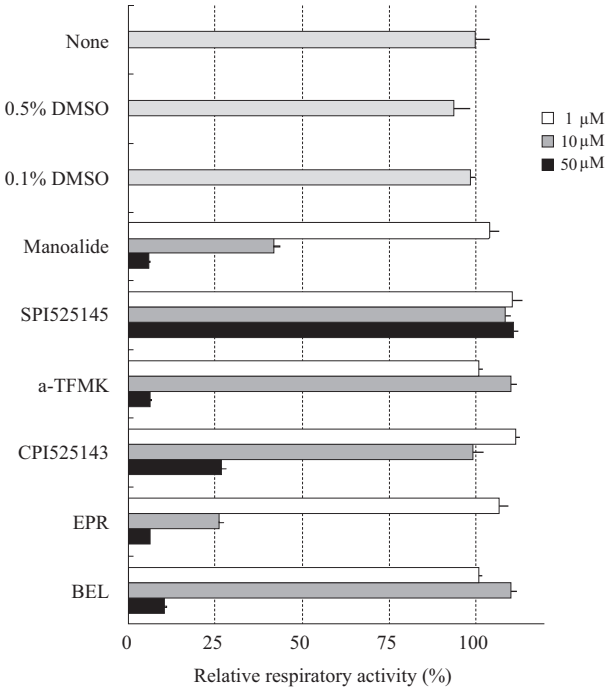

Fig. 2. Cytotoxic effects of various PLA_2 inhibitors. Cytotoxicity of test agents added at 1 μM (open bar), 10 μM (gray bar), and 50 μM (shaded bar) was measured by the MTT assay.

bromoenol lactone (BEL) (iPLA$_2$ inhibitor) on mouse peritoneal macrophages. All the tested PLA$_2$ inhibitors showed no cytotoxic effect when added at 1 μM. These drugs except SPI525145 exerted strong cytotoxicity when added at 50 μM. Manoalide and EPR but not the other inhibitors including a-TFMK, CPI525143, and BEL exhibited partial levels of cytotoxicity at 10 μM. Based on these findings, we then examined the effects of these PLA$_2$ inhibitors on Mϕ antimicrobial activity against Mtb H37Ra strain. In this experiment, zymosan A-activated mouse peritoneal Mϕs were used, because it has been indicated that their cytoplasmic PLA$_2$ activity is significantly increased in response to mycobacterial infection (14). Notably, a-TFMK suppressed the bactericidal activity of Mϕs against Mtb organisms residing in Mϕs but the effect was non-significant (Fig. 3). The other tested agents failed to exert such a blocking activity. Therefore, it appears that type IV cPLA$_2$ plays some roles in Mϕ killing activity against Mtb H37Ra strain. Notably, in the present experiment, the number of intramacrophage Mtb was decreased during the course of 5-day cultivation in a drug-free medium, presumably due to the Mϕ antimycobacterial ability connected with Mϕ apoptosis induced by mycobacterial infection (15, 16). We previously found that a-TFMK exhibited a blocking activity against the anti-Mtb antimicrobial activity of IFN-γ-activated mouse peritoneal Mϕs (12). In this case, intramacrophage Mtb showed one-log growth during the chase cultivation of infected Mϕs in a drug-free medium. As recently reported by Garedew *et al*., IFN-γ-activated Mϕs prevent apoptotic cell death by utilizing glycolytic ATP to maintain mitochondrial membrane potential, differing from the case of mildly activated Mϕs, such as zymosan A-stimulated peritoneal Mϕs (17). This may be the reason that in IFN-γ-treated Mϕs, intracellular bacterial growth of infected Mtb was seen without causing Mϕ apoptosis-associated bacterial killing, as in the case of zymosan A-induced Mϕs indicated in Fig. 3.

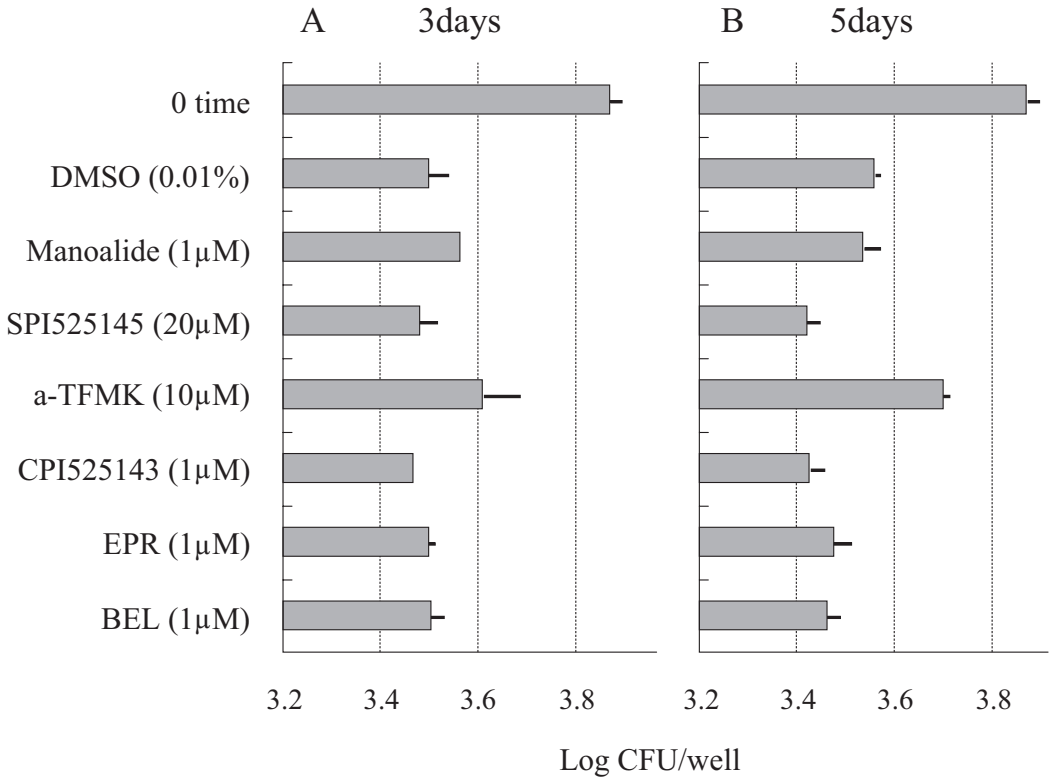

Fig. 3. Effects of various PLA$_2$ inhibitors on Mϕ anti-Mtb antimicrobial activity. Mtb H37Ra-infected Mϕs were cultured in 5% FBS-RPMI medium in the presence or absence of the PLA$_2$ inhibitors at indicated concentrations. After 3-day (A) or 5-day (B) cultivation, the number of residual CFU of intramacrophage Mtb was counted on 7H11 agar plates. For solute control, infected Mϕs were cultured RPMI medium containing 0.01% DMSO. Each bar indicates the mean + SEM (n=3).

In separate experiments where Mϕs were infected with Mtb at a higher dose (1.5 x 10^5 CFU/well), 1μM manoalide, 0.05 μM CPI525143, 6 μM EPR, and 1 μM BEL also attenuated the Mϕ bacterial killing activity (data not shown). However, the reproducibility of this finding was somewhat low. In this context, it should be noted that the specificity of these PLA_2 inhibitors are not perfectly strict. For instance, it has been demonstrated that manoalide at 10 μM concentration actually inhibited $sPLA_2$-V activity (18). Moreover, it has been reported that manoalide weakly inhibits $cPLA_2$ activity (19). Therefore, although the above PLA_2 inhibitors, especially a-TFMK, are capable of blocking Mϕ antimicrobial activity against Mtb, it is concluded that their effects are mild, indicating the participation of PLA_2, particularly type IV $cPLA_2$, in Mϕ antimicrobial activity against intramacrophage mycobacteria is partial but not crucial as we previously proposed (8, 10, 12).

Next, we examined profiles of the expression of $sPLA_2$-IIA, $cPLA_2$, $sPLA_2$-V, and iNOS mRNAs in Mtb-phagocytizing Mϕs during chase cultivation after bacterial phagocytosis. The following results were obtained. First, even in the control Mϕs before bacterial contact, $cPLA_2$, $sPLA_2$-V, and iNOS mRNAs were constitutively expressed (Fig. 4, lane 1). In this case, $cPLA_2$ mRNA expression was much weaker than that of $sPLA_2$-V mRNA. Notably, the expression of $sPLA_2$-IIA mRNA was not detected in control Mϕs. Second, in Mϕs stimulated by phagocytosis of Mtb H37Ra strain, $cPLA_2$ mRNA expression was temporarily reduced for 3 to 6 h after bacterial phagocytosis (Fig. 4, lanes 2, 3). In this case, $sPLA_2$-V and iNOS mRNA expression were also decreased 3 h after bacterial internalization (Fig. 4, lane 2). At later stages, especially 6 to 12 h after bacterial phagocytosis (only 12 h in the case of $cPLA_2$), the expression of all these mRNAs increased to some extent compared to those of control Mϕs (lanes 3 and 4). Concerning the temporary reduction of the mRNA expression of $cPLA_2$ and $sPLA_2$-V seen around 3 to 6 h after phagocytosis of Mtb, the following speculation may be provided. Mϕ stimulation in response to triggering of CD14 and Toll-like receptor (TLRs) molecules, such as TLR2-triggering with mycobacterial lipoproteins and

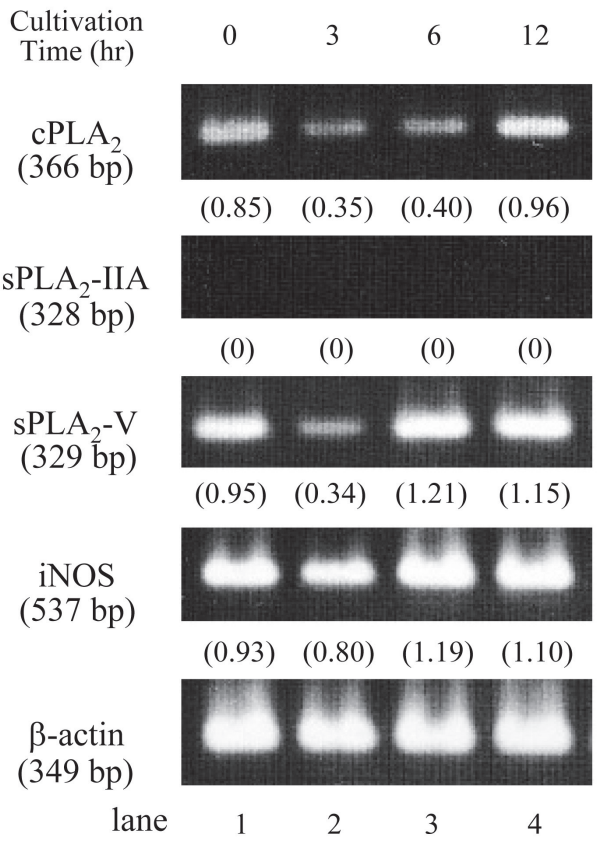

Fig. 4. Profiles of mRNA expression of $cPLA_2$, $sPLA_2$-IIA, $sPLA_2$-V, and iNOS by Mϕs after stimulation due to phagocytosis of Mtb organisms. Test Mϕs, which phagocytosed UV-killed Mtb H37Ra strain, were subsequently cultivated in RPMI medium for up to 12 h. The values in parentheses are PLA_2 band/β-actin band ratios. The β-actin band intensity indicated as 1.0.

lipoarabinomannan, cause prompt increase in $cPLA_2$ activity within minutes, thereby resulting in the generation of prostaglandins (especially prostaglandin E_2) (20-22) and causes up-regulation of mRNA expression of $cPLA_2$ and presumably $sPLA_2$-V in periods later than several hours after bacterial phagocytosis (20). The mRNA expression of these two PLA_2 enzymes may be temporarily depressed during the first 3 to 6 h due to the down-regulatory effect of prostaglandin E_2, which suppresses Mϕ expression of some types of PLA_2, such as $sPLA_2$-IIA (23). Further studies are necessary to justify this speculation.

Notably, in our RT-PCR system, $sPLA_2$-IIA mRNA was under the detection level in any stages of 3- to 12-h cultivation after phagocytosis of Mtb H37Ra. The same phenomenon was also observed for Mϕs infected with Mtb H37Rv (virulent) strain.

In our previous study using IFN-γ-activated mouse peritoneal Mϕs with or without stimulation with mycobacterial phagocytosis, significant $sPLA_2$-IIA mRNA expression was also under detectable levels (12). However, these results do not necessarily indicate that Mϕs are lacking in the $sPLA_2$-IIA mRNA expression, since a low level expression of $sPLA_2$-IIA mRNA (about 1/10 of $cPLA_2$ mRNA expression) has been reported in certain types of Mϕs, such as human monocyte-derived Mϕs (24). In any case, it can be concluded that the test Mϕs (IFN-γ-treated RAW 267.4 Mϕs) exhibited very low ability in expressing $sPLA_2$-IIA mRNA even after Mtb infection. Therefore, it is likely that $sPLA_2$-IIA plays only a trivial role in the expression of Mϕ antimicrobial activity against Mtb organisms.

It is noted that CPI525143 ($cPLA_2\alpha$ inhibitor) did not suppress Mϕ anti-Mtb activity, although a-TFMK ($cPLA_2$ inhibitor) exhibited a blocking activity of the Mϕ anti-Mtb function (Fig. 3). This suggests that $cPLA_2$ isoform (s) other than the isoform 2α may dominantly participates in the expression of Mϕ antimycobacterial activity. Indeed, the Western blotting analysis by Duan *et al*. indicated that strong expression of $PLA_2\gamma$ isotype but not $PLA_2\alpha$ isotype in human monocyte-derived Mϕs was induced during 6-h cultivation after Mtb infection and that this phenomenon was associated with Mϕ apoptosis and concomitant expression of Mϕ antimycobacterial function (25).

The findings in our previous studies imply that arachidonic acid produced by the enzymatic action of $cPLA_2$ plays an important role as antimycobacterial effectors in the expression of Mϕ antimicrobial activity against mycobacterial pathogens including Mtb and MAC (8, 12, 26). In this context, the recent finding by Vandal *et al*. is noteworthy. Using bone marrow-derived Mϕs obtained from type IV $cPLA_2$-deficient mice, they demonstrated that $cPLA_2$ enzymes are not required by the Mϕs for control of intracellularly growing Mtb organisms (27). While Vandal *et al*. used Mtb H37Rv (virulent strain) in their study (27), we used Mtb H37Ra (avirulent strain) or a low-virulence MAC in our previous studies that demonstrated significant roles of $cPLA_2$-dependent mechanisms in expression of Mϕ antimycobacterial activity in vitro (8, 12, 26). Therefore, it appears that $cPLA_2$ enzymes may be required for intramacrophage killing/inhibition of low-virulence mycobacterial organisms but not for killing/inhibition of high-virulence Mtb. This concept is supported by the finding by Harwig *et al*. that a mouse-avirulent *Salmonella typhimurium* mutant strain was much more susceptible to the bactericidal action of $iPLA_2$ than its isogenic virulent parent *S. typhimurium* strain (28). In this context, another possibility remains that other antimicrobial effectors than arachidonic acid play crucial roles in the expression of Mϕ anti-Mtb activity in the case of $cPLA_2$-deleted Mϕs reported by Vandal *et al.* (27), because they used $cPLA_2$-deficient mice of C3H/HeN background with $Nramp1^{+/+}$ (bcg^r) genotype. Since *Nramp1/Slc11a1* gene encodes a proton-coupled bivalent metal/iron transporter expressed within the late endosomes / lysosomes of Mϕs (29) and iron is important for Fenton reaction that generates highly bactericidal reactive oxygen intermediates (ROIs) from superoxide anion and hydrogen peroxide, consequently enabling host Mϕs to effectively killing invading mycobacterial pathogens (30, 31), $cPLA_2$-deleted Mϕs, which Vandal *et al*. used, are regarded to exhibit sufficiently effective levels of ROI-mediated antimicrobial functions against Mtb organisms. Overall, it can be concluded that the $cPLA_2$-mediated antimicrobial mechanism partially participates in Mϕ's bactericidal and bacteriostatic function against mycobacterial pathogens but such $cPLA_2$-dependent mechanism does not play a central role in the Mϕ antimycobacterial function. It is of interest to examine the therapeutic effects of regimens involving $cPLA_2$ modulator in combination with the first-line antituberculous drugs. As we previously proposed, the collaborating action of arachidonic acid generated by enzymatic action of $cPLA_2$ and other types of antimicrobial effector molecules, such as RNIs and cathepsin D is crucial for the expression of Mϕ antimycobacterial activity.

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