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**Anthrax lethal toxin down-regulates type-IIA secreted phospholipase A₂ expression through
MAPK/NF- κ B inactivation**

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Abstract

Bacillus anthracis, the etiological agent of anthrax, produces lethal toxin (LT) that displays a metallo-proteolytic activity toward the *N*-terminus of the MAPK-kinases. We have previously shown that secreted type-IIA phospholipase A₂ (sPLA₂-IIA) exhibits potent anthracidal activity. *In vitro* expression of sPLA₂-IIA in guinea pig alveolar macrophages (AMs), the major source of this enzyme in lung tissues, is inhibited by LT. Here, we examined the mechanisms involved in sPLA₂-IIA inhibition by LT. We first showed that chemical inhibitors of p-38 and ERK MAPKs reduced PLA₂-IIA expression in AMs indicating that these kinases play a role in PLA₂-IIA expression. LT inhibited IL-1 β -induced p-38 phosphorylation as well as sPLA₂-IIA promoter activity in CHO cells. Inhibition of sPLA₂-IIA promoter activity was mimicked by co-transfection with dominant negative construct of p38 (DN-p38) and reversed by the active form of p38 MAPK (AC-p38). Both LT and DN-p38 decreased IL-1 β -induced NF- κ B luciferase activity. This contrasted with the effect of AC-p38, which enhanced this activity. However, neither LT nor specific p-38 inhibitor interfered with LPS induced I κ B α degradation or NF- κ B nuclear translocation in AMs. Subcutaneous administration of LT to guinea pig before LPS challenge reduced sPLA₂-IIA levels in broncho-alveolar lavages and ears. We conclude that sPLA₂-IIA expression is induced via a sequential MAPK-NF- κ B activation and that LT inhibits this expression likely by interfering with the transactivation of NF- κ B in the nucleus. This inhibition, which is operating both *in vitro* and *in vivo*, may represent a mechanism by which *B. anthracis* subvert host defense.

1. Introduction

Anthrax is primarily a disease of herbivores due to *Bacillus anthracis* infection, but humans are also susceptible (1). *B. anthracis*, the ethiological agent of anthrax disease, is a gram-positive, spore-forming bacterium. Although infection by this bacterium frequently occurs *via* the cutaneous route, both gastrointestinal and pulmonary infections cause the highest mortality in human. Macrophages and/or dendritic cells take up inhaled spores leading to their germination. This is followed by the migration of these cells to the draining lymph nodes (2). Encapsulated bacilli then enter the blood compartment and disseminate in the whole organism. Despite appropriate therapy, all these forms of infection may progress to fatal systemic anthrax, leading to shock-like symptoms and respiratory failure (3).

Studies have suggested that type-IIA secreted phospholipase A₂ (sPLA₂-IIA) may represent an effector involved in host defense against invading bacteria. Indeed, sPLA₂-IIA is a highly bactericidal enzyme, especially toward Gram positive bacteria (4-6) and is found at high levels in airways of humans (7,8) and various animal species (9-11). sPLA₂-IIA belongs to a family of enzymes that catalyzes the hydrolysis of phospholipids leading to the generation of lysophospholipids and free fatty acids (12). We have recently shown that sPLA₂-IIA is highly bactericidal towards *B. anthracis* (13) and protects infected mice against *B. anthracis* infection (14). These findings suggest that sPLA₂-IIA may represent an efficient weapon participating in host defense against anthrax.

However, a number of studies have reported the ability of *B. anthracis* to subvert immune response (15-18). This inhibition mainly results from the action of specific *B. anthracis* toxins. Indeed, *B. anthracis* carry two plasmids, pXO1 (19) and pXO2 (20), which encode the primary virulence factors: lethal and edema toxins, and the proteins required for capsule synthesis, respectively. The toxins are composed of three secreted proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins act in pairs (21) leading to the lethal toxin (LT) and the edema toxin (ET), composed of the association of LF/PA and EF/PA respectively. Uptake of LT by the target cells is followed by the release of LF within the cytoplasm of these cells. There, LT displays its metallo-proteolytic activity toward the *N*-terminus of the MAPK-kinase (MAPKK), thereby interfering with the MAPK pathway

(22). We have recently reported that LT was able to inhibit sPLA₂-IIA expression by guinea pig AM (13), the main source of pulmonary sPLA₂-IIA in guinea pig (9). However, the mechanisms involved in this inhibition have not been examined. The present study has been addressed to identify the signaling pathways involved in this modulation and whether LT is able to interfere with sPLA₂-IIA production *in vivo*.

2. Materials and Methods

2.1. Animals and reagents

Male Hartley guinea pigs were purchased from Charles River Laboratories (L'Arbresle, France). RPMI 1640 cell culture medium was obtained from Invitrogen (Cergy-Pontoise, France) and Fetal calf serum (FCS) from Hyclone (Logan, USA). Chinese hamster ovary (CHO) cells were purchased from ATCC (Manassas, USA). SB 203580 and PD 98059 were purchased from Biomol (Le Perray-en-Yvelines, France). MG-132 and *Pseudomonas aeruginosa* LPS were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies directed against, IκB-α (sc-371), MEK-3 (sc-959) and β-actin (sc-81178) were from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies directed against p38-MAPK (9212), phospho-p38-MAPK (p38pT180-Y182, 9211), ERK (9102) and phospho-ERK (ERKpT202-Y204, 9101) were from Cell Signaling (Danvers, USA). Recombinant LF and PA from *B. anthracis* were purchased from List Biological Laboratory (Campbell, USA). Dominant negative and active form constructs of p38 MAPK are a gift of Dr. Jacques Pouyssegur (Institute of Developmental Biology and Cancer, CNRS, Nice, France).

2.2. Preparation of AMs and CHO cells and incubation procedures

Guinea pig broncho alveolar lavages (BALs) were performed with PBS and AMs were isolated as previously described (23). AMs, adjusted at 2×10^6 cells/mL in RPMI 1640 with 3% FCS, were pretreated either with LT, MAPK inhibitors or NF-κB inhibitor 1 h before LPS stimulation. Chinese hamster ovary (CHO) adjusted at 2×10^4 cells/ml (24-well plates) in HAM F12 with 10% FCS, 4 mM glutamine, 100 units/mL penicillin and 100 mg/ml streptomycin were incubated with LT for 1 h before

stimulation with IL-1 β . After stimulation, media and cell lysis were collected for sPLA₂-IIA activity, EMSA and western blot analyses were performed as described below.

2.3. Assay of sPLA₂-IIA activity

sPLA₂-IIA activity was assayed using [³H]-oleic acid labeled membranes of *Escherichia coli*, following the method of Franson *et al.* (24) modified by Paya *et al.* (25). Briefly, the *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.6% sodium dihydrogen orthophosphate, pH 5.0 and grown for 6-8 h at 37 °C in the presence of 5 μ Ci/mL [³H]-oleic acid (specific activity 10 Ci/mmol) until growth approached the end of the logarithmic phase. After centrifugation at 1,800 x *g* for 10 min at 4 °C, the membranes were washed in buffer (0.7 M Tris-HCl, 10 mM CaCl₂, 0.1% bovine serum albumin, pH 8.0), re-suspended in saline and autoclaved for 30-45 min. The membranes were then washed, centrifuged again and frozen at -70 °C. At least, 95% of the radioactivity was incorporated into the phospholipid fraction.

Aliquots (5-20 μ L) of AM supernatants, broncho alveolar lavage fluids (BALF) or ear homogenates were incubated with 250 μ L of buffer A (100 mM Tris-HCl, 1 mM CaCl₂, pH 7.5), for 15 min 37 °C in the presence of 10 μ L autoclaved [³H]-oleic acid-labeled membranes. The reactions were stopped by addition of 100 μ L ice-cold stop solution (0.25% BSA in 100 mM Tris-HCl, 1 mM CaCl₂, pH 7.5). After centrifugation at 1,800 x *g* for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting. BALF and ear homogenates were treated with the sPLA₂-IIA inhibitor, LY311727 to check that hydrolysis is due to sPLA₂-IIA activity. The latter was expressed as pmoles hydrolyzed substrate per min per mg ear homogenates or per ml BALF or AM supernatants.

2.4. Nuclear protein extraction and Electrophoretic Mobility Shift Assays

Nuclear proteins were extracted from 2.10⁶ AMs as previously described (26). The NF- κ B double-stranded oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, USA) consensus sequence 5'-AGT TGA GGG GAC TTTT CCC AGG C-3' was γ -³²P-labeled with T4 polynucleotide kinase (New

England Biolabs, Ipswich, USA) on the overhanging ends. Protein concentrations were determined by using Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Binding reactions were performed as follow: briefly, incubations were performed by adding 5 μ g of nuclear extract, 10 μ L of 2 X binding buffer (40 mM HEPES, pH 7, 140 mM KCl, 4 mM DTT, 0.02 % Nonidet P-40, 8% Ficoll, 200 μ g/ml BSA, 1 μ g of poly(dI:dC), and 1 μ L of γ -³²P-labeled probe, in a total volume of 20 μ L for 20 min at room temperature. The reaction mixtures were separated on a 5% polyacrylamide gel in 0.5% Tris / borate / EDTA buffer at 150 V for 2 h. Gels were dried and exposed for 2 to 12 h. We have previously shown, using supershift analysis, that antibodies directed against NF- κ B's p50 and p65 subunits shift the NF- κ B band in LPS-stimulated AMs, thus confirming that the observed complexes belong to the NF- κ B family (27).

2.5. Protein extraction and Western blot analyses

Proteins from AMs and CHO cells were extracted in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 3 mM EDTA, 100 μ M leupeptin, 100 μ M aprotinin, 1 μ M soybean trypsin inhibitor, 5 mM NEM, 1 mM PMSF, 5 mM benzamidin and 1% Triton X-100, pH 7.4) and electrophoresed under reducing conditions. Semidry transferred proteins were applied onto polyvinylidene difluoride membranes. Nonpecific binding sites were blocked overnight with 5% BSA in 20 mM Tris-HCl, pH 7.6, 140 mM NaCl and 0.1% Tween 20. Blots were probed for 1 h with indicated antibodies. After washing, the immunoreactive bands were visualized using the specifics peroxidase-conjugated anti-IgG and the ECL Plus Western Blotting Detecting System (Amersham Biosciences, Orsay, France).

2.6. Plasmid constructions and transfection

CHO cells, adjusted at 2×10^4 cells/ml (24-well plates) in HAM F12 with 10% FCS, 4 mM glutamine, 100 units/mL penicillin and 100 mg/ml streptomycin were transfected or cotransfected with the Ig- κ B luciferase plasmid (28), [-1153; +46]-sPLA₂-IIA luciferase plasmid (28), dominant negative construct of p-38 (DN-p38) or active form of p38 MAPK (AC-p38) using LIPOFECTAMINE™ Plus (Invitrogen, Carisbad, USA). After 24 h of transfection the cells were pretreated with LT (PA + LF) 1

h before LPS (1 $\mu\text{g/mL}$) or IL-1 β (10 ng/mL) stimulation. 24 h after stimulation luciferase activity was determined in duplicate using a luciferase reporter assay kit (Promega, Madison, USA), with signal detection for 12 s by a luminometer (Berthold, Pforzheim, Germany), and normalized by dividing the relative light units by β -galactosidase activity.

2.7. *In vivo experiments*

Guinea pigs weighing 500-600 g were anesthetized by i.m. injection of ketamine hydrochloride (50 mg/kg body weight; Parke Davis, Courbevoie, France) and xylazine (2 mg/kg body weight; Bayer, Leverkusen, Germany). LT (PA + LF) and LPS were injected subcutaneously in each ear. A first injection (PA 10 μg + LF 5 μg in 20 μl of sterile PBS) was administrated 1 h before a second injection (PA 5 μg + LF 2.5 μg + 100 μg of LPS in 20 μL of sterile PBS). Control experiments were performed using PBS instead of LT. After overnight treatment with LPS the animals were euthanized with 40 $\mu\text{g/kg}$ pentobarbital sodium (Sanofi, Libourne, France) and broncho-alveolar lavages (BALs) and ear homogenates were performed as described below.

2.8 *Preparation of ear homogenates*

Guinea pigs were anesthetized and then 12 mm sections of ears were punched after euthanasia. Then, frozen tissues were suspended in 0.25 mM sucrose solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 2 $\mu\text{g/mL}$ leupeptin, and 2 $\mu\text{g/mL}$ aprotinin to yield a 10% (w/v) homogenate. The tissues were disrupted with a mixer Ultra-Turrax T-25 (Janke and Kunkel, Staufen, Germany) at 4 °C and then centrifuged for 5 min at 1,000 $\times$ g. The resulting supernatant was centrifuged at 20,000 $\times$ g for 20 min and supernatants were used for sPLA₂-IIA activity analyses as described above.

2.9. *Control of cell viability*

Cell viability was checked by the trypan blue dye exclusion test. Cell lysis was controlled by measuring the release of lactate dehydrogenase activity using a commercial kit from Boehringer

(Mannheim, Germany). No changes of cell viability or lysis were observed in AMs or CHO cells after addition of LT or LPS.

2.10. Statistical analysis

The results are presented as mean $\pm$ SEM; and are represented of at least 3 independent experiments.

The level of statistical significance was determined by two-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

3. Results

3.1. A role for MAPK in the inhibition of sPLA₂-IIA expression by LT

Our previous report showed that LT inhibited LPS-induced sPLA₂-IIA synthesis by guinea pig AMs (13). LT was able to cleave MEK-3 in AMs (data not shown), which is in agreement with previous reports in various cell types (22). Thus, it is likely that inhibition of MAPK activation by LT may play a key role in the inhibition of sPLA₂-IIA expression in AMs. We first examined the effect of known inhibitors of MAPK cascade on sPLA₂-IIA production by AMs. Pre-incubation of AMs with PD 98059 and SB 203580 (respectively inhibitors of ERK and p38 MAPK), reduced LPS-induced sPLA₂-IIA secretion (Fig. 1A and B) and inhibited LPS-induced phosphorylation of ERK or p38 MAPK (Fig. 1C and D) as expected. We also showed that LT inhibits LPS-induced p38 and ERK phosphorylations in AMs (Fig 1E and F).

We then investigated the impact of LT on the activation of sPLA₂-IIA promoter construct transfected in CHO cells. Pretreatment of CHO cells with LT inhibited IL-1 β -induced sPLA₂-IIA promoter activity (Fig. 2A). Similar results were observed when the cells were stimulated with LPS instead of IL-1 β (data not shown). Co-transfection of CHO cells with the dominant negative form of p38 (DN-p38) construct inhibited IL-1 β -induced sPLA₂-IIA promoter activity (Fig. 2B). In contrast, co-transfection with the active form of p38 MAPK (AC-p38) construct significantly increased sPLA₂-IIA transcriptional activity and reversed the inhibitory effect of LT on this activity (Fig. 2C).

3.2. Inhibition of NF- κ B transactivation but not on its translocation by LT: implication of MAPK

Because NF- κ B is known to play a pivotal role in the induction of sPLA₂-IIA expression (29) we examined the effect of LT on NF- κ B activation in AMs. Indeed, we have previously shown that MG-132 prevents LPS-induced NF- κ B translocation as well as sPLA₂-IIA expression in AMs (27). This compound is a proteasome inhibitor that prevents I κ B α degradation which leads to inhibition of NF- κ B translocation. EMSA analysis showed that LT failed to interfere with LPS-induced NF- κ B nuclear translocation in

1 AMs (Fig. 3A). The use of the p38 MAPK inhibitor SB 203580 also failed to interfere with LPS-
2 induced NF- κ B translocation in AMs. Pretreatment of AMs with LPS induced a transient degradation
3 of the NF- κ B inhibitor I κ B α (Fig. 3B). LPS-induced I κ B α degradation was not altered neither by LT
4 (Fig 3C) nor by SB 203580 (Fig 3D). As expected, this degradation was abolished by MG-132 (Fig.
5 3C).
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10 We then examined the effect of LT on NF- κ B activation using CHO cells. Pretreatment of CHO cells
11 with LT abolished the IL-1 β -induced p38-MAPK phosphorylation (Fig 4D). CHO cells were then
12 transfected with Ig-(κ B) luciferase plasmid followed by pre-incubation with LT 1 h before addition
13 of IL-1 β . As shown in Figure 4A, IL-1 β induced a significant increase in luciferase activity, which
14 was abrogated by LT. Similar results were observed when cells were stimulated with LPS (data not
15 shown). Co-transfection of CHO cells with DN-p38 reduced IL-1 β -induced NF- κ B luciferase activity
16 (Fig. 4B). In contrast, co-transfection with AC-p38 significantly increased this activity (Fig. 4C).
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29 3.3. *In vivo effects of LT on sPLA₂-IIA production*

30 These findings prompted us to examine whether the inhibitory effect of LT on sPLA₂-IIA expression
31 is operating *in vivo*. Guinea pigs received ear subcutaneous administration of LT before a second
32 inoculation of both LT and LPS. Our results showed that LPS enhanced the levels of sPLA₂-IIA
33 activity, both in ears (Fig. 5B) and BALF (Fig. 5A). The levels of sPLA₂-IIA activity were lowered
34 either in BALF and ears in animals pretreated with LT before LPS instillation.
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4. Discussion

Our previous studies suggested that sPLA₂-IIA, a component of lung innate immunity present in airways of human and various animal species, plays a role in host defense against *B. anthracis* (14). However, despite the ability of sPLA₂-IIA to kill this bacterium, pulmonary anthrax has been shown to cause near 100% mortality. This suggests that *B. anthracis* probably affects sPLA₂-IIA expression and/or activity in lung tissues. Our previous studies showed that LT was indeed able to inhibit sPLA₂-IIA synthesis in AMs (13), the main pulmonary source of this enzyme in guinea pig (9), but the mechanisms involved in this inhibition have not been investigated. This inhibitory effect was not due to a cytotoxic action of LT, because guinea pig AMs were found to be resistant to the lethal effect of this toxin. Thus, guinea pig AMs belong to the group of macrophages that are resistant to the lytic action of LT, but responsive to its action, as detected by the cleavage of MEK-3 (data not shown) and inhibition of sPLA₂-IIA production (13). The definition of resistance and susceptibility to LT relies on its ability to exert a lethal cytotoxic effect on certain types of cells from given species. However, the PA receptor has been observed on all cell types studied to date and it has been shown that these cells are sensitive to the modifications of the intracellular pathways induced by the enzymatic activity of LF. Thus, guinea pig AMs are indeed resistant to the lethal effect of LT, but are sensitive to the MAPKK pathway disruption triggered by LF. This suggests that cleavage of MAPKK by LT and subsequent alteration of MAPK cascade have not effect on cell viability on these cells.

LT is known to cleave MAPKK thereby interfering with the activation of p38 and ERK pathways (22). Our results clearly showed that both p38 and ERK (whose phosphorylations are inhibited by LT) play a role in the induction of sPLA₂-IIA expression in AMs as specific inhibitors of these kinases significantly reduced sPLA₂-IIA levels. This finding, *per se*, is of great interest, as it reveals an unrecognized role of these kinases in the modulation of sPLA₂-IIA expression. Remarkably, our studies showed that LT inhibited sPLA₂-IIA expression by interfering with p38 MAPK activation. We also demonstrated that this kinase mediates NF- κ B activation, a major transcription factor involved in sPLA₂-IIA expression in guinea pig AMs (29), and that this activation is abrogated by LT. Although

the mechanisms by which this kinase stimulates NF- κ B activation in our cell system are still unclear, our findings indicated that this activation might not involve I κ B α degradation or NF- κ B nuclear translocation. It is thus likely that p38 MAPK induces NF- κ B activation at a level located downstream nuclear translocation of this transcription factor. We hypothesize that p38 MAPK induces a nuclear transactivation of NF- κ B through either a phosphorylation of NF- κ B subunits or coactivators and that this process would lead to stimulation of sPLA₂-IIA expression. In this model, the cleavage of MAPKK by LT would result in the inhibition of NF- κ B activation ultimately leading to alteration of sPLA₂-IIA expression. Our recent report showed that LT inhibit IL-8 expression in the pulmonary epithelial cells line Beas-2B in part by altering chromatin accessibility of IL-8 promoter to activated NF- κ B without any effect on NF- κ B activation (15). Whether LT inhibits sPLA₂-IIA expression in AMs by a similar mechanism remains to be investigated. Recent reports showed that epigenetic mechanisms play a key role the modulation of sPLA₂-IIA expression (30, 31).

To confer a pathophysiological relevance to these studies we examined the effect of LT instillation on the production of sPLA₂-IIA *in vivo*. Our results showed that subcutaneous instillation of LPS in ears induced an increased production of sPLA₂-IIA either in ear homogenates and BALF. We suggest that in this model LPS induces locally (in ear tissues) sPLA₂-IIA expression the cell sources of which remains are still to be identified. In parallel, LPS may diffuse into lung tissues where it stimulates sPLA₂-IIA by AMs, a major source of this enzyme in lungs (9). Moreover, a previous study showed that i.p. instillation of LPS induced an increased expression of pulmonary TNF α transcript, suggesting that LPS rapidly entered the blood stream and reached the competent cells within the lung tissues (32). Interestingly, our data revealed that LT instillation decreased the levels of sPLA₂-IIA both in BALF and ears of LPS-challenged guinea pigs. Although the cell targets of LT in lung tissues have not been identified it is likely that LT target AMs leading to sPLA₂-IIA inhibition.

It is clearly established now that i) sPLA₂-IIA exhibits potent anthracidal properties (13), ii) this enzyme is present in airways at sufficient concentrations to kill *B. anthracis*, and iii) LT is synthesized shortly after germination of *B. anthracis* in the host. Thus, the control of sPLA₂-IIA expression could represent an adaptive mechanism that allows *B. anthracis* to escape from the innate immune response.

Our previous report showed that the decrease in sPLA₂-IIA levels was paralleled by a decrease in the anthracidal activity in LT-treated AMs (13). The present study identifies a mechanism by which *B. anthracis* might modulate sPLA₂-IIA expression in AMs, thus opening a new therapeutic strategy for the treatment of this deadly infection.

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6. Legends

Figure 1: MAPK inhibition reduces sPLA₂-IIA secretion by AMs

AMs were pretreated with (A) PD 98059 (100 μ M) or (B) SB 203580 (10 μ M) for 1 h, and then stimulated with LPS (50 ng/mL). After 24 h incubation, the levels of sPLA₂-IIA was measured in the supernatants. In (C) and (D), AMs were incubated with PD 98059 (100 μ M) or SB 203580 (10 μ M) for 1 h and then stimulated with LPS (50 ng/mL) for 30 min. Erk and p-38 phosphorylations were analyzed by western blot. In E and F, AMs were pretreated for 1 h with LT (1 μ g/mL) and then stimulated with LPS (50 ng/ml) for 30 min. Erk (E) and p-38 (F) phosphorylations were analyzed by western blot.

Figure 2: LT inhibits sPLA₂-IIA expression in CHO cells

(A) CHO cells were transfected with the [-1153; +46]-sPLA₂-IIA luciferase construct. The cells were pretreated for 1 h with LT (PA 1 μ g/mL + increasing concentrations of LF) and then stimulated with IL-1 β (10 ng/ml). (B) Co-transfected cells with the [-1153; +46]-sPLA₂IIA luciferase construct plus DN-p38 construct were stimulated with IL-1 β (10 ng/ml). (C) Co-transfected cells with the [-1153; +46]-sPLA₂IIA luciferae construct plus AC-p38 were pretreated for 1 h with LT (500 ng/ml) and then stimulated with IL-1 β (10 ng/ml). Luciferase activity was measured 24 h after the addition of IL-1 β .

Figure 3: LT fails to affect NF- κ B translocation in AMs

(A) AMs were pretreated for 1 h with LT (1 μ g/mL) and then stimulated with LPS (50 ng/ml) and EMSA were performed as indicated in *Material and Methods*. (B) AMs were stimulated with LPS (50 ng/ml) during the indicated times and I κ B α levels analysed by Western blot. (C) AMs were pretreated with LT (PA 1 μ g/mL + increasing concentrations of LF) or MG-132 (1 μ M) 1 h before 30 min stimulation with LPS (50 ng/mL). (D) AMs were pretreated with SB 203580 (10 μ M) for 1 h and then stimulated with LPS (50 ng/mL) for 30 min. Cell extracts were subjected to I κ B α analysis by western blot.

Figure 4: LT alters NF- κ B activation in CHO cells *via* MAPK-dependent process

(A) CHO were transfected with an NF- κ B luciferase construct. Transfected cells were pretreated with LT (PA 1 μ g/mL + indicated concentrations of LF) 1 h before IL-1 β (10 ng/mL) stimulation. (B) Cells co-transfected with the NF- κ B luciferase plus DN-p38 (0.1-10 pg/mL) constructs were stimulated with IL-1 β (10 ng/mL). (C) Cells co-transfected with the NF- κ B luciferase plus AC-p38 (0.1-10 pg/mL) constructs were pretreated with LT (500 ng/mL) 1 h before IL-1 β (10 ng/mL) stimulation. Luciferase activity was measured 24 h after the addition of IL-1 β . (D) Non transfected CHO cells were pretreated with LT (1 μ g/mL) for 1 h and then stimulated with IL-1 β (10 ng/mL) at the indicated time intervals. p-38 phosphorylation was analyzed by western blot.

Figure 5: LT reduces sPLA₂-IIA activity in BALF and guinea pig ears

Guinea pigs received subcutaneous administration of LT (PA 10 μ g + LF 5 μ g) 1 h before a second instillation of both LT (PA 5 μ g + LF 2.5 μ g) and LPS (100 μ g). 14 h later, sPLA₂-IIA activity was measured in BALF (A) and ears (B) as detailed in the Methods.

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Paris, September 21, 2009

Dear Editor,

We are pleased to submit for publication in “Biochemical Pharmacology” a manuscript entitled “Anthrax lethal toxin down-regulates type-IIA secreted phospholipase A₂ expression through MAPK/NF- κ B inactivation”.

This article identifies a mechanism by which Lethal Toxin, a major virulence factor of *B. anthracis*, the agent of Anthrax, inhibits the expression of type-IIA phospholipase A₂ and shows the importance of MAPK and NF- κ B in this process. As type-IIA phospholipase A₂ is endowed with bactericidal activity its inhibition by Lethal Toxin may predispose the host to infection by this bacterium.

We hope that this work will be of interest for your Journal.

Best regards,

Dr. L. Touqui

Anthrax lethal toxin down-regulates type-IIA secreted phospholipase A₂ expression through MAPK/NF- κ B inactivation

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Abstract

Bacillus anthracis, the etiological agent of anthrax, produces lethal toxin (LT) that displays a metallo-proteolytic activity toward the *N*-terminus of the MAPK-kinases. We have previously shown that secreted type-IIA phospholipase A₂ (sPLA₂-IIA) exhibits potent anthracidal activity. *In vitro* expression of sPLA₂-IIA in guinea pig alveolar macrophages (AMs), the major source of this enzyme in lung tissues, is inhibited by LT. Here, we examined the mechanisms involved in sPLA₂-IIA inhibition by LT. We first showed that chemical inhibitors of p-38 and ERK MAPKs reduced PLA₂-IIA expression in AMs indicating that these kinases play a role in PLA₂-IIA expression. Transfection studies using CHO cells showed that LT inhibited IL-1 β -induced PLA₂-IIA promoter activation. This effect was mimicked by co-transfection with dominant negative construct of p38 (DN-p38) and reversed by the active form of p38 MAPK (AC-p38). Both LT and DN-p38 decreased IL-1 β -induced NF- κ B luciferase activity. This contrasted with the effect of AC-p38, which enhanced this activity. However, LT failed to interfere with LPS induced I κ B α degradation or NF- κ B nuclear translocation in AMs. Finally, we showed that administration of LT to guinea pig reduced sPLA₂-IIA levels in broncho-alveolar lavages, suggesting that LT is able to interfere with the *in vivo* sPLA₂-IIA production. We conclude that sPLA₂-IIA expression is induced via a sequential MAPK-NF- κ B activation and that LT inhibits this expression likely by interfering with the transactivation of NF- κ B in the nucleus. This inhibition, which is operating both *in vitro* and *in vivo*, may represent a mechanism by which *B. anthracis* subvert host defense.

1. Introduction

1 Anthrax is primarily a disease of herbivores due to *Bacillus anthracis* infection, but humans
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5 are also susceptible (1). *B. anthracis*, the ethiological agent of anthrax disease, is a gram-
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9 positive, spore-forming bacterium. Although infection by this bacterium frequently occurs *via*
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12 the cutaneous or gastrointestinal route, pulmonary infection causes the highest mortality in
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15 human. Macrophages and/or dendritic cells take up inhaled spores leading to their
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18 germination. This is followed by the migration of these cells to the draining lymph nodes (2).
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21 Encapsulated bacilli then enter the blood compartment and disseminate in the whole
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24 organism. Despite appropriate therapy, all these forms of infection may progress to fatal
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27 systemic anthrax, leading to shock-like symptoms and respiratory failure (3).

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Studies have suggested that type-IIA secreted phospholipase A₂ (sPLA₂-IIA) may represent
an effector involved in host defense against invading bacteria. Indeed, sPLA₂-IIA is a highly
bactericidal enzyme, especially toward Gram positive bacteria (4-6) and is found at high
levels in airways of humans (7,8) and various animal species (9-11). sPLA₂-IIA belongs to a
family of enzymes that catalyzes the hydrolysis of phospholipids leading to the generation of
lysophospholipids and free fatty acids (12). We have recently shown that sPLA₂-IIA is highly
bactericidal towards *B. anthracis* (13) and protects infected mice against *B. anthracis*
infection (14). These findings suggest that sPLA₂-IIA may represent an efficient weapon
participating in host defense against anthrax.

However, a number of studies have reported the ability of *B. anthracis* to subvert immune
response (15-18). This inhibition mainly results from the action of specific *B. anthracis*
toxins. Indeed, *B. anthracis* carry two plasmids, pXO1 (19) and pXO2 (20), which encode the
primary virulence factors: lethal and edema toxins, and the proteins required for capsule
synthesis, respectively. The toxins are composed of three secreted proteins: protective antigen

(PA), lethal factor (LF) and edema factor (EF). These proteins act in pairs (21) leading to the lethal toxin (LT) and the edema toxin (ET), compose of the association of LF/PA and EF/PA respectively. Uptake of LT by the target cells is followed by the release of LF within the cytoplasm of these cells. There, LT displays its metallo-proteolytic activity toward the *N*-terminus of the MAPK-kinase (MAPKK), thereby interfering with the MAPK pathway (22). We have recently reported that LT was able to inhibit sPLA₂-IIA expression by guinea pig AM (13), the main source of pulmonary sPLA₂-IIA in guinea pig (10). However, the mechanisms involved in this inhibition have not been examined. The present study has been addressed to identify the signaling pathways involved in this modulation and whether LT is able to interfere with sPLA₂-IIA production *in vivo*.

2. Materials and Methods

2.1. Animals and reagents

Male Hartley guinea pigs were purchased from Charles River Laboratories. RPMI 1640 cell culture medium was obtained from Invitrogen (Cergy-Pontoise, France) and Fetal calf serum (FCS) from Hyclone (Logan, USA). SB 203580 and PD 98059 were purchased from Biomol (Le Perray-en-Yvelines, France). MG-132 and *Pseudomonas aeruginosa* LPS were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies directed against, IκBα (sc-371) and β-actin (sc-81178) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant LF and PA from *B. anthracis* were purchased from List Biological Laboratory (USA).

2.2. Preparation of AMs and incubation procedures

Guinea pig broncho alveolar lavages (BALs) were performed with PBS and AMs were isolated as previously described (23). AMs, adjusted at 2×10^6 cells/mL in RPMI 1640 with 3% FCS, were pretreated either with LT, MAPK inhibitors or NF-κB inhibitor 1 h before LPS

stimulation. After stimulation, media and cell lysis were collected for sPLA₂-IIA activity, EMSA and western blot analyses were performed as described below.

2.3. Assay of sPLA₂-IIA activity

sPLA₂-IIA activity was assayed using [³H]-oleic acid labeled membranes of *Escherichia coli*, following a modification of the method of (24) as modified by (25). Briefly, the *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.6% sodium dihydrogen orthophosphate, pH 5.0 and grown for 6-8 h at 37 °C in the presence of 5 µCi/mL [³H]-oleic acid (specific activity 10 Ci/mmol) until growth approached the end of the logarithmic phase. After centrifugation at 1,800 x g for 10 min at 4 °C, the membranes were washed in buffer (0.7 M Tris-HCl, 10 mM CaCl₂, 0.1% bovine serum albumin, pH 8.0), re-suspended in saline and autoclaved for 30-45 min. The membranes were then washed, centrifuged again and frozen at -70 °C. At least, 95% of the radioactivity was incorporated into the phospholipid fraction.

Aliquots (5-20 µL) of AM supernatants, broncho alveolar lavage fluids (BALF) or ear homogenates were incubated with 250 µL of buffer A (100 mM Tris-HCl, 1 mM CaCl₂, pH 7.5), for 15 min 37 °C in the presence of 10 µL autoclaved [³H]-oleic acid-labeled membranes. The reactions were stopped by addition of 100 µL ice-cold stop solution (0.25% BSA in 100 mM Tris-HCl, 1 mM CaCl₂, pH 7.5). After centrifugation at 1,800 x g for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting. BALF and ear homogenates were treated with the sPLA₂-IIA inhibitor, LY311727 to check that hydrolysis is due to sPLA₂-IIA activity. The latter was expressed as pmoles hydrolyzed substrate per min per mg ear homogenates or per ml BALF or AM supernatants.

2.4. Nuclear protein extraction and Electrophoretic Mobility Shift Assays

Nuclear proteins were extracted from 2.10^6 AMs as previously described (26). The NF- κ B double-stranded oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, CA) consensus sequence 5'-AGT TGA GGG GAC TTTT CCC AGG C-3' was γ - 32 P-labeled with T4 polynucleotide kinase (Biolabs) on the overhanging ends. Protein concentrations were determined by using Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Binding reactions were performed as follow: briefly, incubations were performed by adding 5 μ g of nuclear extract, 10 μ L of 2 X binding buffer (40 mM HEPES, pH 7, 140 mM KCl, 4 mM DTT, 0.02 % Nonidet P-40, 8% Ficoll, 200 μ g/ml BSA, 1 μ g of poly(dI:dC)), and 1 μ L of γ - 32 P-labeled probe, in a total volume of 20 μ L for 20 min at room temperature. The reaction mixtures were separated on a 5% polyacrylamide gel in 0.5% Tris / borate / EDTA buffer at 150 V for 2 h. Gels were dried and exposed for 2 to 12 h. We have previously shown, using supershift analysis, that antibodies directed against NF- κ B's p50 and p65 subunits shift the NF- κ B band in LPS-stimulated AMs, thus confirming that the observed complexes belong to the NF- κ B family (27).

2.5. Protein extraction and Western blot analyses

Proteins from AMs were extracted in lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 3 mM EDTA, 100 μ M leupeptin, 100 μ M aprotinin, 1 μ M soybean trypsin inhibitor, 5 mM NEM, 1 mM PMSF, 5 mM benzamidine and 1% Triton X-100, pH 7.4) and electrophoresed under reducing conditions. Semidry transferred proteins were applied onto polyvinylidene difluoride membranes. Nonpecific binding sites were blocked overnight with 5% BSA in 20 mM Tris-HCl, pH 7.6, 140 mM NaCl and 0.1% Tween 20. Blots were probed for 1 h with anti-MEK3. After washing, the immunoreactive bands were visualized using a peroxidase-conjugated,

goat anti-rabbit IgG (1/10,000 dilution) and an ECL Plus Western Blotting Detecting System (Amersham Biosciences, Orsay, France).

2.6. Plasmid constructions and transfection

Chinese hamster ovary (CHO), adjusted at 2×10^4 cells/ml (24-well plates) in HAM F12 with 10% FCS, 4 mM glutamine, 100 units/mL penicillin and 100 mg/ml streptomycin were transfected or cotransfected with the Ig-(κ B) luciferase plasmid (28), [-1153; +46]-sPLA₂-IIA luciferase plasmid (28), dominant negative construct of p-38 (DN-p38) or active form of p38 MAPK (AC-p38) using LIPOFECTAMINETM Plus (Invitrogen). After 24 h of transfection the cells were pretreated with LT (PA + LF) 1 h before LPS (1 μ g/mL) or IL-1 β (10 ng/mL) stimulation. 24 h after stimulation luciferase activity was determined in duplicate using a luciferase reporter assay kit (Promega), with signal detection for 12 s by a luminometer (Berthold, Pforzheim, Germany), and normalized by dividing the relative light units by β -galactosidase activity.

2.7. In vivo experiments

Guinea pigs weighing 500-600 g were anesthetized by i.m. injection of ketamine hydrochloride (50 mg/kg body weight; Parke Davis, Courbevoie, France) and xylazine (2 mg/kg body weight; Bayer, Leverkusen, Germany). LT (PA + LF) and LPS were injected subcutaneously in each ear. A first injection (PA 10 μ g + LF 5 μ g in 20 μ l of sterile PBS) was administrated 1 h before a second injection (PA 5 μ g + LF 2.5 μ g + 100 μ g of LPS in 20 μ L of sterile PBS). Control experiments were performed using PBS instead of LT. After overnight treatment with LPS the animals were euthanized with pentobarbital sodium (40 μ g/kg; Sanofi, Libourne, France) and broncho-alveolar lavages (BALs) and ear homogenates were performed as described below.

2.8 Preparation of ear homogenates

12 mm sections of ears were punched after local anesthesia and homogenized. Briefly, frozen tissues were suspended in 0.25 mM sucrose solution containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM PMSF, 2 μ g/mL leupeptin, and 2 μ g/mL aprotinin to yield a 10% (w/v) homogenate. The tissues were disrupted with a mixer (Ultra-Turrax T-25, Janke and Kunkel, Staufen, Germany) at 4 °C and then centrifuged for 5 min at 1,000 x g. The resulting supernatant was centrifuged at 20,000 x g for 20 min and supernatants were used for sPLA₂-IIA activity analyses as described above.

2.9. Control of cell viability

Cell viability was checked by the trypan blue dye exclusion test. Cell lysis was controlled by measuring the release of lactate dehydrogenase activity using a commercial kit from Boehringer (Mannheim, Germany). No changes of cell viability or lysis were observed in AMs or CHO cells after addition of LT or LPS.

2.10. Statistical analysis

The results are presented as mean $\pm$ SEM; and are represented of at least 3 independent experiments. The level of statistical significance was determined by two-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

3. Results

3.1. A role for MAPK in the inhibition of sPLA₂-IIA expression by LT

Our previous report showed that LT inhibited LPS-induced sPLA₂-IIA synthesis by guinea pig AMs (13). LT was able to cleave MEK-3 in AMs (data not shown), which is in agreement with previous reports in various cell types (22). Thus, it is likely that inhibition of MAPK activation by LT may play a key role in the inhibition of sPLA₂-IIA expression in AMs. We first examined the effect of known inhibitors of MAPK cascade on sPLA₂-IIA production by AMs. Pre-incubation of AM with PD 98059 and SB 203580, respectively inhibitors of ERK and p38 MAPK pathways, reduced LPS-induced sPLA₂-IIA secretion (Fig. 1A and B).

We then investigated the impact of LT on the activation of sPLA₂-IIA promoter construct transfected in CHO cells. Pretreatment of CHO cells with LT inhibited IL-1 β -induced sPLA₂-IIA promoter activity (Fig. 2A). Similar results were observed when the cells were stimulated with LPS instead of IL-1 β (data not shown). Co-transfection of CHO cells with DN-p38 construct inhibited IL-1 β -induced sPLA₂-IIA promoter activity (Fig. 2B). In contrast, co-transfection with AC-p38 construct significantly increased sPLA₂-IIA transcriptional activity and reversed the inhibitory effect of LT on this activity (Fig. 2C).

3.2. Inhibition of NF- κ B transactivation but not on its translocation by LT: implication of MAPK

Because NF- κ B is known to play a pivotal role in the induction of sPLA₂-IIA expression (29) we examined the effect of LT on NF- κ B activation in AMs. EMSA analysis showed that LT failed to interfere with LPS-induced NF- κ B nuclear translocation in AMs (Fig. 3A). The use of the p38 MAPK inhibitor SB 203580 also failed to interfere with LPS-induced NF- κ B translocation in AMs. Pretreatment of AMs with LPS induced a transient degradation of I κ B α

(Fig. 3B). LPS-induced I κ B α degradation was not altered by LT, whereas MG-132, a proteasome inhibitor, abolished LPS-induced I κ B α degradation, as suspected (Fig. 3C).

We then examined the effect of LT on NF- κ B activation in transfected cells. CHO cells were transfected with Ig-(κ B) luciferase plasmid followed by pre-incubation with LT 1 h before addition of IL-1 β . As shown in Figure 4A, IL-1 β induced a significant increase in luciferase activity, which was abrogated by LT. Similar results were observed when cells were stimulated with LPS (data not shown). Co-transfection of CHO cells with DN-p38 reduced IL-1 β -induced NF- κ B luciferase activity (Fig. 4B). In contrast, co-transfection with AC-p38 significantly increased this activity (Fig. 4C).

3.3. *In vivo* effects of LT on sPLA₂-IIA production

These findings prompted us to examine whether the inhibitory effect of LT on sPLA₂-IIA expression is operating *in vivo*. Guinea pigs received ear subcutaneous administration of LT before a second instillation of both LT and LPS. Our results showed that LPS enhanced the levels of sPLA₂-IIA activity, both in BALF (Fig. 5A) and ears (Fig. 5B). The levels of sPLA₂-IIA activity were lowered either in BALF and ears in animals pretreated with LT before LPS instillation.

4. Discussion

Our previous studies suggested that sPLA₂-IIA, a component of lung innate immunity present in airways of human and various animal species, plays a role in host defense against *B. anthracis* (14). However, despite the ability of sPLA₂-IIA to kill this bacterium, pulmonary anthrax has been shown to cause near 100% mortality. This suggests that *B. anthracis* probably affects sPLA₂-IIA expression and/or activity in lung tissues. Our previous studies showed that LT was indeed able to inhibit sPLA₂-IIA synthesis in AMs (13), the main pulmonary source of this enzyme in guinea pig (9), but the mechanisms involved in this inhibition have not been investigated. This inhibitory effect was not due to a cytotoxic action of LT, because guinea pig AMs were found to be resistant to the lethal effect of this toxin. Thus, guinea pig AMs belong to the group of macrophages that are resistant to the lytic action of LT, but responsive to its action, as detected by the cleavage of MEK-3 (data not shown) and inhibition of sPLA₂-IIA production (13).

LT is known to cleave MAPKK thereby interfering with the activation of p38 and ERK pathways (22). Our results clearly showed that both p38 and ERK play a role in the induction of sPLA₂-IIA expression in AMs as specific inhibitors of these kinases significantly reduced sPLA₂-IIA levels. This finding, *per se*, is of great interest, as it reveals an unrecognized role of these kinases in the modulation of sPLA₂-IIA expression. Remarkably, our studies showed that LT inhibited sPLA₂-IIA expression by interfering with p38 MAPK activation. We also demonstrated that this kinase mediates NF- κ B activation, a major transcription factor involved in sPLA₂-IIA expression in guinea pig AMs (29), and that this activation is abrogated by LT. Although the mechanisms by which this kinase stimulates NF- κ B activation in our cell system are still unclear, our findings indicated that this activation might not involve I κ B α degradation or NF- κ B nuclear translocation. It is thus likely that p38 MAPK induces

NF- κ B activation at a level located downstream nuclear translocation of this transcription factor. We hypothesize that p38 MAPK induces a nuclear transactivation of NF- κ B through either a phosphorylation of NF- κ B subunits or coactivators and that this process would lead to stimulation of sPLA₂-IIA expression. In this model, the cleavage of MAPKK by LT would result in the inhibition of NF- κ B activation ultimately leading to alteration of sPLA₂-IIA expression. Our recent report showed that LT inhibit IL-8 expression in the pulmonary epithelial cells line Beas-2B in part by altering chromatin accessibility of IL-8 promoter to activated NF- κ B without any effect on NF- κ B activation (15). Whether LT inhibits sPLA₂-IIA expression in AMs by a similar mechanism remains to be investigated. Recent reports showed that epigenetic mechanisms play a key role the modulation of sPLA₂-IIA expression (30, 31).

To confer a pathophysiological relevance to these studies we examined the effect of LT instillation on the production of sPLA₂-IIA *in vivo*. Our results showed that subcutaneous instillation of LPS in ears induced an increased production of sPLA₂-IIA either in BALF and ear homogenates. We suggest that in this model LPS may induce locally (in ear tissues) sPLA₂-IIA expression the cell sources of which remains are still to be identified. In parallel, LPS may diffuse into lung tissues where it stimulates sPLA₂-IIA by AMs, a major source of this enzyme in lungs (9). Moreover, a previous study showed that i.p. instillation of LPS induced an increased pulmonary expression of TNF α transcript, suggesting that LPS rapidly entered the blood stream and reached the competent cells within the lung tissues (32). Interestingly, our data revealed that LT instillation decreased the levels of sPLA₂-IIA both in BALF and ears of LPS-challenged guinea pigs. Although the cell targets of LT in lung tissues have not been identified it is likely that LT target AMs leading to sPLA₂-IIA inhibition.

It is clearly established now that i) sPLA₂-IIA exhibits potent anthracidal properties (13), ii) this enzyme is present in airways at sufficient concentrations to kill *B. anthracis*, and iii) LT

1 is synthesized shortly after germination of *B. anthracis* in the host. Thus, the control of
2 sPLA₂-IIA expression could represent an adaptive mechanism that allows *B. anthracis* to
3 escape from the innate immune response. Our previous report showed that the decrease in
4 sPLA₂-IIA levels was paralleled by a decrease in the anthracidal activity in LT-treated AMs
5 (13). The present study identifies a mechanism by which *B. anthracis* might modulate sPLA₂-
6 IIA expression in AMs, thus opening a new therapeutic strategy for the treatment of this
7 deadly infection.
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6. Legends

Figure 1: MAPK inhibition reduces sPLA₂-IIA secretion by AMs

AMs were pretreated with (A) PD 98059 (100 μ M) or (B) SB 203580 (10 μ M) for 1 h, and then stimulated with LPS (50 ng/mL). After 24 h incubation, the levels of sPLA₂-IIA was measured in the supernatants.

Figure 2: LT inhibits sPLA₂-IIA expression in CHO cells

(A) CHO cells were transfected with the [-1153; +46]-sPLA₂-IIA luciferase construct. The cells were pretreated for 1 h with LT (PA 1 μ g/mL + increasing concentrations of LF) and then stimulated with IL-1 β (10 ng/ml). (B) Co-transfected cells with the [-1153; +46]-sPLA₂-IIA luciferase construct plus DN-p38 construct were stimulated with IL-1 β (10 ng/ml). (C) Co-transfected cells with the [-1153; +46]-sPLA₂-IIA luciferase construct plus AC-p38 were pretreated for 1 h with LT (500 ng/ml) and then stimulated with IL-1 β (10 ng/ml). Luciferase activity was measured 24 h after the addition of IL-1 β .

Figure 3: LT fails to affect NF- κ B translocation in AMs

(A) AMs were pretreated for 1 h with LT (1 μ g/mL) and then stimulated with LPS (50 ng/ml) and EMSA were performed as indicated in *Material and Methods*. (B) AMs were stimulated with LPS (50 ng/ml) during the indicated times and I κ B α levels analysed by Western blot. (C) AMs were pretreated with LT (PA 1 μ g/mL + increasing concentrations of LF) or MG-132 (1 μ M) 1 h before 30 min stimulation with LPS (50 ng/mL). Cell extracts were subjected to I κ B α analysis by western blot.

Figure 4: LT alters NF- κ B activation in CHO cells *via* MAPK-dependent process

(A) CHO were transfected with an NF- κ B luciferase construct. Transfected cells were pretreated with LT (PA 1 μ g/mL + indicated concentrations of LF) 1 h before IL-1 β (10 ng/ml) stimulation. (B) Cells co-transfected with the NF- κ B luciferase plus DN-p38 (0.1-10 pg/mL) constructs were stimulated with IL-1 β (10 ng/ml). (C) Cells co-transfected with the NF- κ B luciferase plus AC-p38 (0.1-10 pg/mL) constructs were pretreated with LT (500 ng/ml) 1 h before IL-1 β (10 ng/ml) stimulation. Luciferase activity was measured 24 h after the addition of IL-1 β .

Figure 5: LT reduces sPLA₂-IIA activity in BALF and guinea pig ears

Guinea pigs received subcutaneous administration of LT (PA 10 μ g + LF 5 μ g) 1 h before a second instillation of both LT (PA 5 μ g + LF 2.5 μ g) and LPS (100 μ g). 14 h later, sPLA₂-IIA activity was measured in BALF (A) and ears (B) as detailed in the Methods.

Figure 1:

Figure 2:

Figure 3:

Figure 4:

Figure 5:

Inflammatory signals (LPS, IL1 β) induce sPLA2-IIA expression in alveolar macrophages *via* NF- κ B and p38/ERK-dependent mechanism. *Bacillus anthracis* produces Lethal Toxin (LT) which abrogates this induction by cleaving MAPKK