Lysophospholipase D from *Thermocrispum* limits psoriatic inflammation by hydrolyzing epidermal lysoplasmalogen produced by group IIF secreted phospholipase A_2

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Abstract

Epidermal lipids play important roles in skin homeostasis and diseases. Psoriasis is an inflammatory disease characterized by keratinocyte hyperproliferation and Th17 immune responses. We previously reported that ethanolamine-type lysoplasmalogen (P-LPE), preferentially produced by group IIF secreted PLA_2 ($sPLA_2$-IIF/PLA2G2F) that is expressed in the suprabasal epidermis, promotes epidermal hyperplasia in psoriatic inflammation. Herein, we show that forcible degradation of epidermal P-LPE by topical application of recombinant lysophospholipase D (LyPls-PLD) from *Thermocrispum*, a lysoplasmalogen-specific hydrolase, attenuated epidermal hyperplasia and inflammation in imiquimod-induced and K5.Stat3C-transgenic mouse psoriasis models. In humans, P-LPE levels were elevated in the tape-stripped stratum corneum of patients with psoriasis. Moreover, in primary cultured human epidermal keratinocytes, aberrant cell proliferation and activation by psoriatic cytokines were $sPLA_2$-IIF/P-LPE-dependent and were suppressed by the addition of LyPls-PLD with a decrease in P-LPE. These findings confirm that the $sPLA_2$-IIF/P-LPE axis in the epidermis indeed regulates psoriasis, that P-LPE is a lipid biomarker that predicts the severity of psoriasis, and that pharmacological removal of this bioactive lipid is useful to prevent the disease. Thus, our study may lead to the development of drug discovery and diagnostic techniques based on this pathway.

Keywords: group IIF phospholipase A_2, lysoplasmalogen, *Thermocrispum* lysophospholipase D, psoriasis, lipidomics.

List of abbreviations:

DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ESI	electrospray ionization
IMQ	imiquimod
IL-17A	interleukin-17A
IL-23	interleukin-23
IL-36A	interleukin-36A
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LT	leukotriene
LyPls-PLD	lysoplasmalogen-specific phospholipase D
MRM	multiple reaction monitoring
MS	mass spectrometry
P-LPC	plasmalogen LPC (choline-type lysoplasmalogen)
P-LPE	plasmalogen LPE (ethanolamine-type lysoplasmalogen)
PG	prostaglandin
PLA_2	phospholipase A_2
PUFA	polyunsaturated fatty acid
qPCR	quantitative RT-PCR
$sPLA_2$-IIF/PLA2G2F	group IIF secreted PLA_2
Tg	transgenic
TNFα	tumor necrosis factor-α
TPA	12-O-tetradecanoylphorbol 13-acetate

1. Introduction

Psoriasis is a systemic disease that affects around 1% of the population worldwide causing chronic inflammation of the skin [1]. Epidermal hyperplasia, infiltration of inflammatory cells such as neutrophils and lymphocytes into the dermis and epidermis, and vascular dilation are characteristic histologic features of psoriasis. Although the fundamental etiology of psoriasis is still unclear, it is now widely accepted that cytokines such as interleukin (IL)-17A, IL-23 and tumor necrosis factor α (TNFα) play pivotal roles in its development [2, 3]. In fact, biological drugs that target these cytokines or their receptors have shown significant therapeutic efficacy against psoriasis [4].

Recently, lipids have been reported to display diverse and significant effects on skin homeostasis and disease [5, 6]. Various epidemiologic studies have noted a correlation between systemic metabolic diseases and the severity and incidence of psoriasis, suggesting a link of this skin disease with the quantity and quality of lipids consumed from the diet [7-9]. In particular, sustained elevation of saturated fatty acids in the blood due to excessive intake of fatty diets results in aberrant activation of keratinocytes with increased Th17-type immune responses [10]. In addition, polyunsaturated fatty acids (PUFAs) are converted to a wide range of lipid mediators that play exacerbating or ameliorating roles in psoriasis [11]. For example, several eicosanoids (such as prostaglandins (PGs) and leukotrienes (LTs)) derived from ω6 arachidonic acid contribute to exacerbation of psoriasis [10, 12], while specialized pro-resolving lipid mediators (such as resolvins, protectins, and maresins) derived from ω3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) improve skin inflammation and promote tissue repair [13, 14]. Lysophospholipid-derived mediators (e.g., lysophosphatidic acid (LPA) and platelet-activating factor) are also involved in skin pathophysiology by regulating psoriatic inflammation, skin barrier function, and hair growth [15-17]. Biosynthesis of these PUFA- and lysophospholipid-derived lipid mediators is in general initiated by *sn*-2 hydrolysis of membrane phospholipids by phospholipase A_2 (PLA_2).

The PLA_2 superfamily consists of about 50 enzymes in mammals and is classified into several subfamilies based on their structural relationship [18]. Recent studies using transgenic or knockout mice for PLA_2 enzymes revealed that several intracellular and extracellular PLA_2s are involved in the promotion or prevention of skin diseases by mobilizing distinct lipid metabolites [18]. Of these, group IIF secreted PLA_2 ($sPLA_2$-IIF) is induced in epidermal keratinocytes by Th17 cytokines derived from γδT and Th17 cells and promotes epidermal hyperplasia and inflammation such as psoriasis, contact hypersensitivity, and skin cancer [19].

The most common forms of membrane glycerophospholipids contain two acyl chains attached to the *sn*-1 and *sn*-2 positions of the glycerol backbone via ester bonds. However, there are glycerophospholipids that possess an *sn*-1 ether bond instead of an ester bond. Additionally,

some of these ether-containing glycerophospholipids also possesses a *cis* double bond that is conjugated with the ether oxygen, forming a vinyl ether bond (1-*O*-alkenyl type) [20]. 1-Alkenyl glycerophospholipids are called plasmalogens and annotated with a "P" before *sn*-1 hydrocarbon chain abbreviation. Importantly, our previous study supported the idea that $sPLA_2$-IIF promotes psoriasis by producing plasmalogen-type lysophosphatidylethanolamine (lysoplasmalogen; P-LPE) [19], although the contribution of P-LPE to psoriasis is still incompletely understood. Recently, Matsumoto *et al* isolated lysoplasmalogen-specific phospholipase D (LyPls-PLD) from thermophilic actinomycete, *Thermocrispum sp* [21]. By taking advantage of LyPls-PLD, we herein provide an additional line of evidence that $sPLA_2$-IIF/P-LPE axis indeed contributes to the exacerbation of psoriasis and demonstrate that forcible degradation of P-LPE has prophylactic and therapeutic effects on this disease.

2. Materials and Methods

2.1. Animals

Balb/c and FVB/NJcl mice were purchased from Japan SLC. *Pla2g2f*$^{-/-}$ mice and K5.Stat3C-transgenic (Tg) mice were described previously [19, 22]. The mice were housed in climate-controlled facilities at 23°C with a 12 h light-dark cycle, with free access to standard laboratory food (CE2, CLEA) and water. All animal experiments were approved by Tokushima University (approval numbers T27-95, T30-118, T2021-99) and conformed to the Japanese Guide for the Use of Laboratory Animals.

2.2. Imiquimod (IMQ)-induced psoriatic inflammation

Mice (Balb/c WT and *Pla2g2f*$^{-/-}$) received a daily topical application of 12.5 mg of 5% (w/v) IMQ (Mochida Pharma) on the ventral surfaces of the ears for 5 days. As required for the experiments, dorsal and ventral surfaces of the ears were daily topically treated with LyPls-PLD in 50 mM Tris-HCl (pH 7.4) and/or dexamethasone (Wako) in ethanol. Ear thickness was monitored with a micrometer. At the end of experiments, the mice were sacrificed and subjected to subsequent analyses.

2.3. Collection of stratum corneum samples by tape stripping

Mouse stratum corneum samples were collected by tape stripping from the both sides of the ear. A 10 × 10 mm piece of film masking tape (Teraoka) was stuck to both sides of the ear and removed. The procedure was repeated five times using a new piece of tape. The collected tape samples were put into a microtube and stored at -80°C.

2.4. Lipidomics

Mass spectrometry (MS)-based lipidomic analysis was performed according to our published protocol [23]. Briefly, for the detection of phospholipids, after extraction of lipids from the samples by the method of Bligh and Dyer [24], electrospray ionization (ESI)-MS analysis was performed using a QTRAP4000 (Sciex) or an LCMS-8040 (Shimadzu) triple quadrupole-linear ion trap hybrid mass spectrometer with a reverse-phase LC (NexeraX2 system, Shimadzu). The samples were injected by an autosampler, applied to a Kinetex C18 column (2.1 x 3 x 150 mm, 1.7 μm particle, Phenomenex) coupled to ESI-MS, and separated by a step gradient with mobile phase A (acetonitrile/methanol/water = 1:1:1 [v/v/v] containing 5 μM phosphoric acid and 1 mM ammonium formate) and mobile phase B (2-propanol containing 5 μM phosphoric acid and 1 mM ammonium formate) at a flow rate of 0.2 mL/min at 50°C. Lipids were identified using multiple reaction monitoring (MRM) transition and retention times, and quantification was performed based on the peak area of the MRM transition and the calibration curve obtained with an authentic standard for each compound. As internal standards, 100 pmol *d5*-EPA (Cayman) and 18:1-*d7* LPE (Avanti) were added to each sample.

2.5. Enzymatic reaction

The enzymatic reaction of recombinant actinomycete LyPls-PLD [21] was performed using a natural membrane assay, as described previously [23]. Briefly, total lipids were extracted from IMQ-induced ear skin by the method of Bligh and Dyer [24]. The samples were applied to Sep-Pak Silica Cartridges (Waters), and then washed sequentially with acetone and chloroform/methanol (9/1; v/v), eluted with chloroform/methanol (3/1; v/v), and dried up under N_2 gas. The membrane mimic composed of tissue-extracted lipids (typically 1–10 μM) was sonicated for 5 min in 50 mM Tris–HCl (pH 7.4) containing 2 mM $CaCl_2$, and then incubated for 60 min with various concentrations of recombinant LyPls-PLD (1.6-100 ng/mL) at 37°C. After incubation, the lipids were mixed with internal standards (100 pmol *d5*-EPA and 18:1-*d7* LPE), extracted, and subjected to LC-MS for detection of lysophospholipids and phospholipids.

2.6. Psoriatic inflammation in K5.Stat3C-Tg mice

The generation of psoriasiform lesions in psoriasis-prone K5.Stat3C-Tg mice were conducted as described previously [22]. Briefly, K5.Stat3C-Tg mice and control FVB mice were topically applied with 0.68 nmol of 12-O-tetradecanoylphorbol 13-acetate (TPA) in 10 μL of acetone on days 1 and 3. On day 4, the mice were sacrificed and subjected to subsequent analyses. As required for the experiments, dorsal and ventral surfaces of the ears were daily topically treated with LyPls-PLD in 50 mM Tris-HCl (pH 7.4) and/or STA-21 (Ochromycinone; Cayman) in dimethylsulfoxide.

2.7. Quantitative RT-PCR (qPCR)

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using a High Capacity cDNA Reverse transcription kit (Applied Biosystems). PCR reactions were performed using a Power SYBR Green PCR system (Applied Biosystems) or a TaqMan Gene Expression System (Applied Biosystems) on the Step One Plus Real-Time PCR System (Applied Biosystems). The probe/primer sets used are listed in Table 1.

2.8. Histology and immunohistochemistry

Tissues from mice were fixed with 10% Formalin Neutral Buffer Solution (Wako) and embedded in paraffin, and 5 μm sections were cut and stained with hematoxylin and eosin. Mouse tissue sections were incubated with 1x Blockace (DS Pharma Bio Medical) in PBS-T for 30 min, washed three times with PBS-T for 5 min each, and incubated with rabbit anti–mouse or –human $sPLA_2$-IIF antibody [25] at 1:1,000 dilution in a 10-fold-diluted Blockace for overnight at 4°C. The sections were then washed 3 times with PBS-T for 5 min each time and incubated with Alexa Fluor 488–labeled goat anti–rabbit IgG antibody (Molecular Probes; 1:1,000) at 4°C for 5 h. Counterstaining was performed using Vectashield Plus Antifade Mounting Medium with 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories). Stained sections were analyzed with All-in-one fluorescence microscope BZ-X800 (Keyence).

2.9. Human samples

Human stratum corneum samples prepared from volunteers via tape stripping were obtained from Nagoya University (approval number 2018-0310) and Tokushima University (approval number 18011 (2019.3.29)) following approval by the Institutional Research Ethics Committee with informed consent. Stratum corneum samples were collected from healthy and psoriatic volunteers. A 25 × 60 mm piece of film masking tape (Teraoka) was stuck to the inner forearm and then pressed and removed. The procedure was repeated five times using a new piece of tape. The collected tape samples were applied to a plastic sheet (A4 size; 210 mm x 297 mm) and stored at -80°C.

2.10. Cell culture

Immortalized newborn human epidermal keratinocytes (NHEK/SVTERT3-5, Evercyte) were cultured with HuMedia KG2 Medium (KURABO) at 37°C under a humidified atmosphere of 7% (v/v) CO_2 in air. NHEK/SVTERT3-5 cells were transfected with 10 nM human *PLA2G2F* siRNA (Flexi Tube siRNA, Hs_PLA2G2F_2; 5-TACCAGGAACTCTTTGACCAA-3;

QIAGEN) or control siRNA (All Stars Negative Control siRNA; QIAGEN) using Lipofectamine RNAiMAX (Invitrogen), in accordance with the manufacturer's instructions. Then the medium was replaced with 3D cell culture medium (CnT-PR-D; CELLnTEC) with or without a cocktail of psoriatic cytokines (IL-1β, IL-6, IL-17A, IL-17F, IL-22, and TNFα; 10 ng/mL for each) (Peprotech) exposed at the air–liquid interface for 7 days. Immortalized human adult fibroblasts (HDF/TERT164, Evercyte) were cultured with DMEM/Ham's F-12 Medium (Wako) containing 10% (v/v) fetal calf serum (FCS; HyClone) at 37°C under a humidified atmosphere of 7% CO_2 in air. The cells were treated with or without a cocktail of psoriatic cytokines for 2 days. As required for experiments, LyPls-PLD was added to the cultures. At the end of the experiments, the samples were subjected to qPCR, histochemistry, or immunoblotting. *In vitro* skin irritation studies were performed using a LabCyte EPI-MODEL24 kit (J-TEC).

2.11. SDS-PAGE/Immunoblotting

Cell lysates were subjected to SDS-PAGE using 10% (w/v) gels under a reducing condition. The separated proteins were electroblotted onto PVDF membranes (Biorad) using a semi-dry blotter (Biorad). After blocking with Blockace (DS Pharma Bio Medical) in PBS-T, the membranes were probed with the respective antibodies against STAT3 (with a Stat3 monoclonal antibody #124H6; Cell Signaling) and P-STAT3 (with a phospho-Stat3 (Ser727) polyclonal antibody #9134; Cell Signaling) for 2 h, followed by incubation with appropriate horseradish peroxidase-conjugated anti-IgG (Amersham Biosciences) for 1 h, and were visualized using the ECL Western blot system (Amersham Biosciences).

2.12. Statistical analysis

The experiments were performed and analyzed in non-randamized and non-blinded manner. The data were expressed as mean ± SEM and analyzed using Prism9 by GraphPad. Comparisons of two groups were performed by Student's *t*-test. For multiple-group comparisons, one-way ANOVA followed by Turkey's multiple comparisons test was used. The number of replicate samples per group (*n*) is specified in the figure legends.

3. Results

3.1. LyPlsPLD specifically hydrolyzes lysoplasmalogens

Matsumoto *et al* previously reported that LyPls-PLD preferentially hydrolyzed choline-type lysoplasmalogen (plasmalogen lysophosphatidylcholine; P-LPC) and to a lesser extent ethanolamine-type lysoplasmalogen (P-LPE) to produce P-LPA (Figure 1A), whereas it hardly hydrolyzed ester-linked LPC and LPE [21]. To confirm the substrate specificity of LyPls-PLD for a wide range of phospholipids in natural membranes, LyPls-PLD at various concentrations was incubated with phospholipids prepared from psoriasis-induced (IMQ-treated) mouse ears as substrates, and the changes in the lipids were analyzed comprehensively by lipidomics. The results showed that LyPls-PLD hydrolyzed both P-LPC and P-LPE, with greater hydrolysis of P-LPC at lower concentrations of the enzyme, to produce P-LPA and did not react with ester-linked lysophospholipids (LPC and LPE) and phospholipids (Figure 1B). These results confirmed that LyPls-PLD has high substrate specificity for lysoplasmalogens in skin-extracted natural membranes.

3.2. Topical application of LyPls-PLD to the skin improves psoriasis in mouse models

We hypothesized that forcible degradation of P-LPE, which is markedly increased in psoriatic epidermis by the action of $sPLA_2$-IIF [19], by LyPls-PLD could alleviate psoriasis. To evaluate the potential therapeutic effect of LyPls-PLD on psoriasis, we applied LyPls-PLD to mouse ears that had been treated with IMQ for 5 days (Figure 2A). Topical application of LyPls-PLD for 2 days accelerated recovery from IMQ-induced ear thickening (Figure 2B). Under this condition, the amount of P-LPE in the skin was reduced by application of LyPls-PLD, while other lysophospholipids were unaffected (Figure 2C). The skin level of P-LPA, a hydrolytic metabolite of P-LPE by LyPls-PLD, was very low and, unlike in the *in vitro* enzyme assay (Figure 2B), it was not significantly elevated even after LyPls-PLD treatment (Figure 2C), suggesting that P-LPA might be unstable and converted rapidly to some other metabolite(s) in the skin microenvironment. Furthermore, along with the improvement of ear swelling, expression levels of the psoriasis markers *S100a9* and *Tnf* were decreased in LyPls-PLD-treated skin compared to vehicle-treated skin (Figure 2D). These results indicate that topical application of LyPls-PLD improves pre-existing psoriasis.

We then examined the prophylactic effect of LyPls-PLD on psoriasis. When IMQ and LyPls-PLD were applied simultaneously to mouse skin, the IMQ-induced ear thickening was attenuated by LyPls-PLD in a dose-dependent manner, even though the effect was partial (Figure 3A and 3B). The reduced ear swelling by the highest dose of LyPls-PLD in WT mice was nearly comparable to the swelling observed in IMQ-treated *Pla2g2f*$^{-/-}$ mice. As a positive control, topical application of dexamethasone completely suppressed skin thickening in both

WT and *Pla2g2f*$^{-/-}$ mice (Figure 3B). The skin level of P-LPE was markedly reduced by the application of LyPls-PLD, while those of LPC, LPE and LPA were not significantly affected (Figure 3C). Concomitantly, *S100a9* and *Tnf* expression was decreased in LyPls-PLD-treated skin (Figure 3D). These results suggest that the sPLA$_2$-IIF/P-LPE axis contributes significantly to ear swelling and inflammation in IMQ-induced psoriasis.

To assess whether the sPLA$_2$-IIF/P-LPE axis is also operative in different psoriasis models, we next employed K5.Stat3C-Tg mice, in which constitutive Stat3 overexpression in keratinocytes under the *K5* promoter produces a psoriasis-like phenotype [22]. In these mice, epidermal Stat3 is activated by topical application of TPA, leading to the development of psoriasis-like lesions. This phenotype meets many of the pharmacological criteria of psoriasis and, in addition to the IMQ-induced psoriasis model as mentioned above [26, 27], is one of the most physiologically relevant animal models of psoriasis [28]. In this model, TPA challenge onto the ear skins of K5.Stat3C-Tg mice elicited greater induction of *S100a9* and *Tnf* as well as *Pla2g2f* than those of control FVB mice (Figure 4A), as in the case of the IMQ-induced psoriasis model [19]. Consistently, sPLA$_2$-IIF protein was increased in the thickened epidermis of TPA-treated K5.Stat3C-Tg mice relative to control mice (Figure 4B). We then analyzed lipid profiles in the tape-stripped stratum corneum from the epidermis of K5.Stat3C-Tg mice in comparison with those of WT mice by lipidomics analysis, and the changes in lysophospholipids are summarized as a heatmap in Figure 4C. A class of lysoplasmalogens, but not ester-linked lysophospholipids, was markedly increased in the stratum corneum of TPA-treated K5.Stat3C-Tg mice relative to control mice (Figure 4C), which was consistent with the lipidomics of the stratum corneum from IMQ-treated mice, where lysoplasmalogens, particularly P-LPE species, were highly elevated in WT mice following IMQ treatment and sPLA$_2$-IIF deficiency partially attenuated this response (Figure 4D). These results confirmed that, regardless of the psoriasis models, P-LPE is the major lysophospholipid that is markedly increased in the stratum corneum of psoriatic skins and that sPLA$_2$-IIF contributes profoundly if not solely to P-LPE generation.

Having ascertained that the sPLA$_2$-IIF/P-LPE axis is also involved in the K5.Stat3C-Tg psoriasis model, we next examined the therapeutic effect of LyPls-PLD on this model. Topical treatment of TPA-challenged K5.Stat3C-Tg mice with LyPls-PLD (a schematic procedure in Figure 5A) reduced ear swelling (Figure 5B) and epidermal thickening (Figure 5C, D), decreased the amount of P-LPE but not other lysophospholipids in the stratum corneum (Figure 5E), and markedly attenuated the expression of *S100a9* and *Tnf* in the skin (Figure 5F). The suppressing effects of LyPls-PLD on P-LPE generation (Figure 5E) and *S100a9* and *Tnf* expression (Figure 5F) were comparable to those of STA-21, a Stat3 inhibitor that alleviates psoriasis [29]. Taken together, these results suggest that the enzymatic removal of P-LPE by LyPls-PLD improves multiple models of psoriasis in mice. Furthermore, the decrease of P-LPE

in STA-21-treated skin (Figure 5E) implies that the improvement of psoriasis is accompanied by reduced production of P-LPE, giving an additional insight into this lysophospholipid as a biomarker that reflects the severity of psoriasis.

3.3. The sPLA$_2$-IIF/P-LPE axis contributes to psoriasis in human

Immunohistochemical staining of human skin showed that sPLA$_2$-IIF is localized mainly in the suprabasal epidermis [19]. As in mice, P-LPE levels in the tape-stripped stratum corneum of human psoriasis patients were found to be higher than those in healthy controls or in non-lesion area of the patients (Figure 6A). The expression of sPLA$_2$-IIF in primary newborn human epidermal keratinocytes (NHEK/SVTERT3-5) was much higher than that in primary human dermal fibroblasts (HDF/TERT164), confirming that sPLA$_2$-IIF is expressed in the epidermis (Figure 6B). In the epidermal 3D culture model, expression levels of *PLA2G2F* and *S100A9*, a keratinocyte activation marker, were significantly increased in response to a cocktail of psoriatic cytokines (IL-1β, IL-6, IL-17A, IL-17F, IL-22, and TNFα), whereas treatment with *PLA2G2F* siRNA decreased the expression of *S100A9* without affecting the constant expression of *KRT14* (Figure 6C). The increased phosphorylation of STAT3 and thickening of the keratinocyte layer by psoriatic cytokines in these cells were markedly abrogated by treatment with *PLA2G2F* siRNA (Figures 6D and 6E). Furthermore, even without cytokine stimulation, supplementation of *PLA2G2F*-knockdown cells with P-LPE significantly restored keratinocyte layer thickness and *S100A9* expression (Figures 6E and 6F), suggesting that P-LPE alone can stimulate keratinocyte hyperproliferation and activation.

Differentiated keratinocytes secrete lipids (e.g., ceramides, cholesterol, and phospholipids) from lamellar granules and this extracellular phospholipid pool may serve as a substrate for sPLA$_2$ [30]. In fact, we had demonstrated sPLA$_2$-IIF-dependent extracellular release of P-LPE in mouse primary keratinocytes [19]. As in the case of mouse keratinocytes, we confirmed that P-LPE was released into the culture supernatant of cytokine-stimulated NHEK/SVTERT3-5 cells and that this release was reduced by the addition of LyPls-PLD to the culture (Figure 7A). In addition, cytokine-induced expression of *S100A9*, *TNF*, and *IL36A* was suppressed by treatment with LyPls-PLD (Figure 7B). Although the addition of psoriatic cytokines to HDF/TERT164 cells also increased *IL1B* expression, it was unaffected by LyPls-PLD (Figure 7C). An *in vitro* skin irritation study using 3D-cultured NHEK/SVTERT3-5 cells showed no apparent cell death after application of LyPls-PLD (Figure 7D), ensuring the safety of LyPls-PLD treatment. These results collectively suggest that treatment of the skin with LyPls-PLD improves psoriasis in both mouse and human models. Therefore, we conclude that P-LPE produced from sPLA$_2$-IIF activates keratinocytes to promote psoriasis and that this pathway could be a novel drug target for this disease.

4. Discussion

Several lines of evidence obtained from experiments using LyPls-PLD as a tool, we conclude that P-LPE produced by sPLA$_2$-IIF promotes psoriatic inflammation. First, topical application of LyPls-PLD reduced cutaneous P-LPE levels and alleviated IMQ-induced psoriasis, which was accompanied by reduced expression of psoriasis-related inflammatory genes such as *S100a9* and *Tnf*. Second, in the K5.Stat3C-Tg psoriasis model, TPA challenge to the ear skin elicited epidermal hyperplasia [31], with marked increases in sPLA$_2$-IIF and its product P-LPE in the thickened epidermis. Topical application of LyPls-PLD to the K5.Stat3C-Tg psoriasis model relieved psoriatic inflammation with a decrease in P-LPE, as in the case of the IMQ-induced model. Third, P-LPE was increased in the tape-stripped stratum corneum of psoriatic patients. Finally, in primary cultured human epidermal keratinocytes, aberrant cell proliferation and activation by psoriatic cytokines were sPLA$_2$-IIF/P-LPE-dependent, an event that was suppressed by the addition of LyPls-PLD with a decrease in P-LPE. Overall, we confirm that the sPLA$_2$-IIF/P-LPE axis in the epidermis indeed regulates psoriasis, that P-LPE is a lipid biomarker that predicts psoriasis severity, and that pharmacological removal of this bioactive lipid is useful for prevention of the disease. Thus, this lipid-driven pathway may provide a novel drug target for treatment and/or diagnosis of psoriasis.

Although little is known about the biological activity and mechanistic action of P-LPE, we show here that it can be a regulator and biomarker of psoriasis. Since sPLA$_2$-IIF is expressed primarily in the suprabasal epidermis, P-LPE is expected to be generated at the same location, likely within the extracellular milieu. Currently, epidermal lipid analysis has focused on ceramides, which were detected primarily by classical thin-layer chromatography and gas chromatography [32, 33]. Entering the 21st century, MS-based lipidomics has been developed for the analysis of all lipid classes, including ceramides and phospholipids, and this method is superior in specificity, quantitation, and sensitivity, making them an indispensable technology for lipid research. As a result, it has become clear that more than 1,500 different types of diverse ceramide molecules exist in the human stratum corneum, constituting the barrier function of the skin [34]. Based on the lipidomics technology, it is now possible to quantitatively measure the levels of P-LPE in the stratum corneum, which can be easily collected by tape stripping from human subjects, allowing this unique lysophospholipid as a novel diagnosis marker for psoriasis.

LyPls-PLD is a 334 amino acid, 33-kDa molecular mass enzyme isolated from the thermophilic actinomycete, *Thermocrispum sp* [21]. The amino acid sequence of LyPls-PLD shows low similarity to those of already known PLD enzymes and is more similar to those of glycerophosphodiesterases. Its enzyme activity requires mM order of Ca^{2+} and is active in wide temperature (4−50°C) and pH (pH 4.1−9.7) ranges. The enzyme preferentially hydrolyzes P-

LPC followed by P-LPE, indicating that LyPls-PLD acts specifically on lysoplasmalogens. Furthermore, the fact that this enzyme does not hydrolyze phospholipids means that its topical application to the skin is unlikely to disrupt cell membranes. Given that the skin pH can vary from acidic to neutral depending on disease states [5], LyPls-PLD, when topically applied to the skin, is capable of hydrolyzing P-LPE without losing its enzymatic activity and therefore appears to be useful in treating psoriasis and possibly other skin diseases involving hyperproliferation and activation of epidermal keratinocytes. We do not rule out the possibility that P-LPA, a metabolite of P-LPE by LyPls-PLD, might have the ability to inhibit psoriasis through LPA receptors, although the potential contribution of P-LPA appears to be less likely due to its low abundance and instability in the skin. Nonetheless, the possibility that LyPls-PLD might have a double-edged benefit, decreasing P-LPE and increasing P-LPA, should be taken into consideration.

Several PLA_2s and lipid metabolites have currently been implicated in the exacerbation or amelioration of psoriasis [18]. Various eicosanoids derived from arachidonic acid, which is released in many if not all cases by cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$/PLA2G4A), are abundantly present in human and mouse psoriatic skins and crucially affect immune responses [35]. For instance, thromboxane A_2-TP signaling promotes psoriasis by facilitating IL-17A production from γδ T cells [36], PGE_2 produced by dendritic cells promotes IL-23 receptor expression on Th17 cells through EP2 and EP4 to augment Th17-driven skin inflammation [37], and LTB_4-BLT1 signaling acts on neutrophils, dendritic cells, and γδT cells to promote their recruitment into psoriatic lesions [12, 13]. In addition to $cPLA_2\alpha$, two other $cPLA_2$ isoforms, $cPLA_2\delta$ (PLA2G4D) and $cPLA_2\varepsilon$ (PLA2G4E), are also expressed in psoriasis lesions [38]. $cPLA_2\delta$, reportedly secreted from mast cells via extracellular vesicles, is captured by Langerhans cells to produce lipid neoantigens, which are then presented on CD1a leading to activation of T cells that produce IL-17A and IL-22 [39, 40], while $cPLA_2\varepsilon$ induced in keratinocytes promotes the biosynthesis of *N*-acylethanolamine-related lipids that limit psoriatic inflammation [41]. Besides $sPLA_2$-IIF, other $sPLA_2$s have also been shown to contribute indirectly to the regulation of psoriasis [42]. Group IID $sPLA_2$ ($sPLA_2$-IID/PLA2G2D), which is expressed in dendritic cells in the draining lymph nodes, counteracts Th17 immunity through production of ω3 PUFA metabolites, thereby putting a break on psoriasis [43, 44], while group IIA $sPLA_2$ ($sPLA_2$-IIA/PLA2G2A), which is secreted from intestinal Paneth cells, acts as an antimicrobial protein to shape the gut microbiota, thereby secondarily affecting psoriasis in distal skin [45]. Thus, multiple PLA_2-driven lipid pathways are involved in the pathology of psoriasis, and $sPLA_2$-IIF is unique in that it mobilizes a unique lysophospholipid (P-LPE) extracellularly in keratinocytes compared to the other lipid mediator pathways as mentioned above.

In conclusion, our present study using LyPls-PLD has provided evidence that the $sPLA_2$-IIF/P-

LPE axis could be a novel drug target for psoriasis. It remains to be clarified whether LyPls-PLD also reduces P-LPE levels and thereby attenuates other forms of skin diseases such as atopic dermatitis, contact hypersensitivity, and skin cancer. Since LyPls-PLD is heat-stable and retains its activity under a wide range of pH, it may be effective in improving psoriasis also in humans. However, the antigenic property of LyPls-PLD may limit its clinical application, since its repeated use could increase the risk of unfavorable immune responses as an adverse effect. In this viewpoint, development of an $sPLA_2$-IIF-specific inhibitor could be a novel strategy for treatment of psoriasis. Alternatively, although the receptor for P-LPE has not yet been identified, application of an antagonist for the putative P-LPE receptor could be another potential therapeutic option for psoriasis. Overall, future development of agents that could block the $sPLA_2$-IIF/P-LPE pathway may be useful in the treatment of psoriatic patients, even those who have failed current psoriasis treatments targeting Th17-related cytokines or their receptors.

Author contributions

H. Hakoi, Y. Miki, S. Nomura, M. Murakami and K. Yamamoto designed the study and wrote the manuscript; K. Nakajima and S. Sano contributed to the generation of K5.Stat3C-Tg mice; C. Terashima–Murase,T. Takeichi, and M. Akiyama contributed to collect human samples; S. Sakasegawa contributed to generate recombinant LyPls-PLD. All authors have read and agreed to published version of the manuscript.

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Institutional review board statement

This study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics committee at Nagoya University (approval number 2018-0310) and Tokushima University (approval number 18011 (2019.3.29)) following approval by the Institutional Research Ethics Committee with informed consents. This study was also approved by the ethics committee on animal care at the Tokushima University (approval numbers T27-95,

T30-118, T2021-99) and conformed to the Japanese Guide for the Use of Laboratory Animals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Evaluation of the enzymatic activity of LyPls-PLD *in vitro*.
(A) LyPls-PLD enzyme reaction. Hydrolysis of LPE(P-18:0) and LPC(P-18:0) by LyPls-PLD produces P-LPA(18:0). These lipids were identified by ESI-MS using the MRM transition (Q1/Q3) shown in the figure. (B) Substrate specificity of LyPls-PLD for a wide range of phospholipids. Various concentrations of LyPls-PLD were incubated for 60 min with 10 μM phospholipid mixture prepared from IMQ-treated mouse ears as substrates and the changes in individual phospholipid and lysophospholipid species were analyzed by ESI-MS (n = 6). The values represent the relative percentage of each lipid without addition of the enzyme. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; Student t test.

Figure 2. Therapeutic effect of LyPls-PLD on the IMQ-induced psoriasis model.
(A) Experimental procedure for the topical application of LyPls-PLD to the IMQ-induced psoriasis model. IMQ was applied to the ear skin once daily for 5 consecutive days (elicitation) and then treated with 10 μg of LyPls-PLD. (B) Changes in ear swelling in IMQ-challenged mice after treatment for the indicated periods with or without LyPls-PLD (n = 8). (C) Lysophospholipid levels in the ear skin of IMQ-challenged mice after treatment for 2 days with or without LyPls-PLD (n = 6). (D) qPCR of *S100a9* and *Tnf* in the ear skin of IMQ-challenged mice after treatment for 2 days with or without LyPls-PLD (n = 5). The expression levels were normalized to housekeeping *Gapdh*. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; (B) two-tailed Mann-Whitney test; (C, D) one-way ANOVA.

Figure 3. Prophylactic effect of LyPls-PLD on the IMQ-induced psoriasis model.
(A) Experimental procedure for the analyses for the prophylactic effect of LyPls-PLD on the IMQ-induced psoriasis model. IMQ and LyPls-PLD (10 μg) were applied simultaneously to the ear skin. (B) Various doses of LyPls-PLD and/or 0.005% dexamethasone (DEX) were applied simultaneously with IMQ to the ear skin of wild-type (WT) and *Pla2g2f*$^{-/-}$ mice. Changes in ear swelling after each treatment were evaluated. “n” at the X axis means the number of mice used for each treatment. (C) Lysophospholipid levels in the ears of mice with or without treatment for 2 days with IMQ or LyPls-PLD (10 μg) (n = 6). (D) qPCR of *S100a9* and *Tnf* in the ears of mice with or without treatment for 2 days with IMQ or LyPls-PLD (n = 5). The expression levels were normalized to *Gapdh*. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; (B) two-tailed Mann-Whitney test; (C, D) one-way ANOVA.

Figure 4. sPLA$_2$-IIF expression and P-LPE levels in the K5.Stat3C-Tg psoriasis model.

K5.Stat3C-Tg and control FVB mice were topically applied with 0.68 nmol of TPA in 10 μL of ethanol on days 1 and 3. On day 4, the skin of these mice was subjected to qPCR (A), immunohistochemistry (B), and lipidomics (C). (A) qPCR of *S100a9, Tnf,* and *Pla2g2f* in the ears of K5.Stat3C-Tg and control mice treated for 4 days with TPA or vehicle (ethanol) (n = 5). The expression levels were normalized to *Gapdh*. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; one-way ANOVA. (B) Immunofluorescence microscopy of $sPLA_2$-IIF (green) in K5.Stat3C-Tg and control skins treated for 4 days with TPA or vehicle, with DAPI counterstaining (blue). Bar, 20 μm. (C, D) Heatmap representations of the levels of lysophospholipids in the tape-stripped stratum corneum from K5.Stat3C-Tg and control skins treated for 4 days with or without TPA (C) and those from WT and *Pla2g2f*$^{-/-}$ skins treated for 5 days with or without IMQ (D).

Figure 5. Therapeutic effect of LyPls-PLD on the K5.Stat3C-Tg psoriasis model.
(A) Experimental procedure for the topical application of LyPls-PLD to the TPA-induced K5.Stat3C-Tg psoriasis model. (B) Ear swelling in TPA-challenged K5.Stat3C-Tg mice treated with or without LyPls-PLD (10 μg) (n = 16) or STA-21 (20 μM) as a positive control (n = 10). Histology (C) and epidermal thickness (D) in the ear skin of K5.Stat3C-Tg and control mice treated with or without LyPls-PLD for 2 days (n = 18). Bar, 100 μm. (E) Lysophospholipid levels in the ear skins of K5.Stat3C Tg and control mice treated for 2 days with or without LyPls-PLD or STA-21 (n = 6). (F) qPCR of *S100a9* and *Tnf* in the ear skins of K5.Stat3C Tg and control mice treated for 2 days with or without LyPls-PLD or STA-21. The expression levels were normalized to *Gapdh*. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; (B) two-tailed Mann-Whitney test; (D) two-tailed Mann-Whitney test; (E, F) one-way ANOVA.

Figure 6. Evaluation of the $sPLA_2$-IIF/P-LPE axis in human skin and 3D-cultured human keratinocytes.
(A) P-LPE levels in the stratus corneum prepared from human skin. Lipid extraction was performed on the stratum corneum collected by tape stripping from the skin of healthy (n = 16) and psoriatic (n = 12) subjects. In patients with psoriasis, the epidermis samples were collected from psoriasis and non-psoriasis lesions. Data are normalized with protein concentrations. (B) qPCR of *PLA2G2F* in human epidermal keratinocytes (NHEK/SVTERT3-5) (n = 5) and human adult fibroblasts (HDF/TERT164) (n = 10). (C) Activation of keratinocytes by psoriatic cytokines in a 3D culture. qPCR of *PLA2G2F, S100A9* and *KRT14* in NHEK-SVTERT3-5 cells with scrambled or *PLA2G2F* siRNA treatment after 3D culture for 7 days with or without a cocktail of psoriatic cytokines (IL-1β, IL-6, IL-17A, IL-17F, IL-22, and TNFα; 10 ng/mL for each) exposed at the air–liquid interface (n = 4). Expression levels of individual genes in a

monolayer culture were regarded as 1. (D) STAT3 phosphorylation in NHEK-SVTERT3-5 cells treated with scrambled or *PLA2G2F* siRNA after 3D culture with or without psoriatic cytokines for 7 days. Homogenates from keratinocytes in (C) were subject to immunoblotting for STAT3 and P-STAT3. Representative immunoblots are shown. (E) Hematoxylin and eosin staining of 3D-cultured keratinocytes. NHEK-SVTERT3-5 cells with scrambled or *PLA2G2F* siRNA treatment after 3D culture for 7 days with or without a cocktail of psoriatic cytokines or 10 nM P-LPE. Scale bar, 100 μm. (F) qPCR of *S100A9* and *KRT14* in NHEK-SVTERT3-5 cells with scrambled or *PLA2G2F* siRNA treatment after 3D culture for 7 days with or without 10 nM P-LPE (n = 4). Expression levels of individual genes in control were regarded as 1. *RN18S* (B) and *GAPDH* (C, F) were used as a reference gene for normalization. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; (A) Kruskal-Wallis test; (B) two-tailed Mann-Whitney test; (C, F) one-way ANOVA.

Figure 7. Effect of LyPls-PLD on cytokine-stimulated NHEK-SVTERT3-5 cells.
(A) P-LPE levels in the supernatants of psoriatic cytokines-stimulated NHEK-SVTERT3-5 cells after culture with or without LyPls-PLD (n = 6). (B) qPCR of *S100A9*, *TNF* and *IL36A* in NHEK-SVTERT3-5 cells after culture for 7 days with or without a cocktail of psoriatic cytokines or LyPls-PLD (10 μg). (C) qPCR of *IL1B* in HDF/TERT164 cells after culture for 2 days with or without a cocktail of psoriatic cytokines or LyPls-PLD (10 μg). Expression levels of individual genes in control were regarded as 1. (D) An *in vitro* skin irritation assay with 3D culture of human keratinocytes was performed using a LabCyte EPI-MODEL24 kit. Various concentrations of LyPls-PLD were added to 3D-cultured NHEK/SVTERT3-5 cells for 15 min or 24 h and cell viability was evaluated (n = 3). Treatment with 5% SDS is a positive control to disrupt the cells. *GAPDH* was used as a reference gene for normalization. Values are mean ± SEM; *, $p < 0.05$; **, $p < 0.01$; one-way ANOVA.

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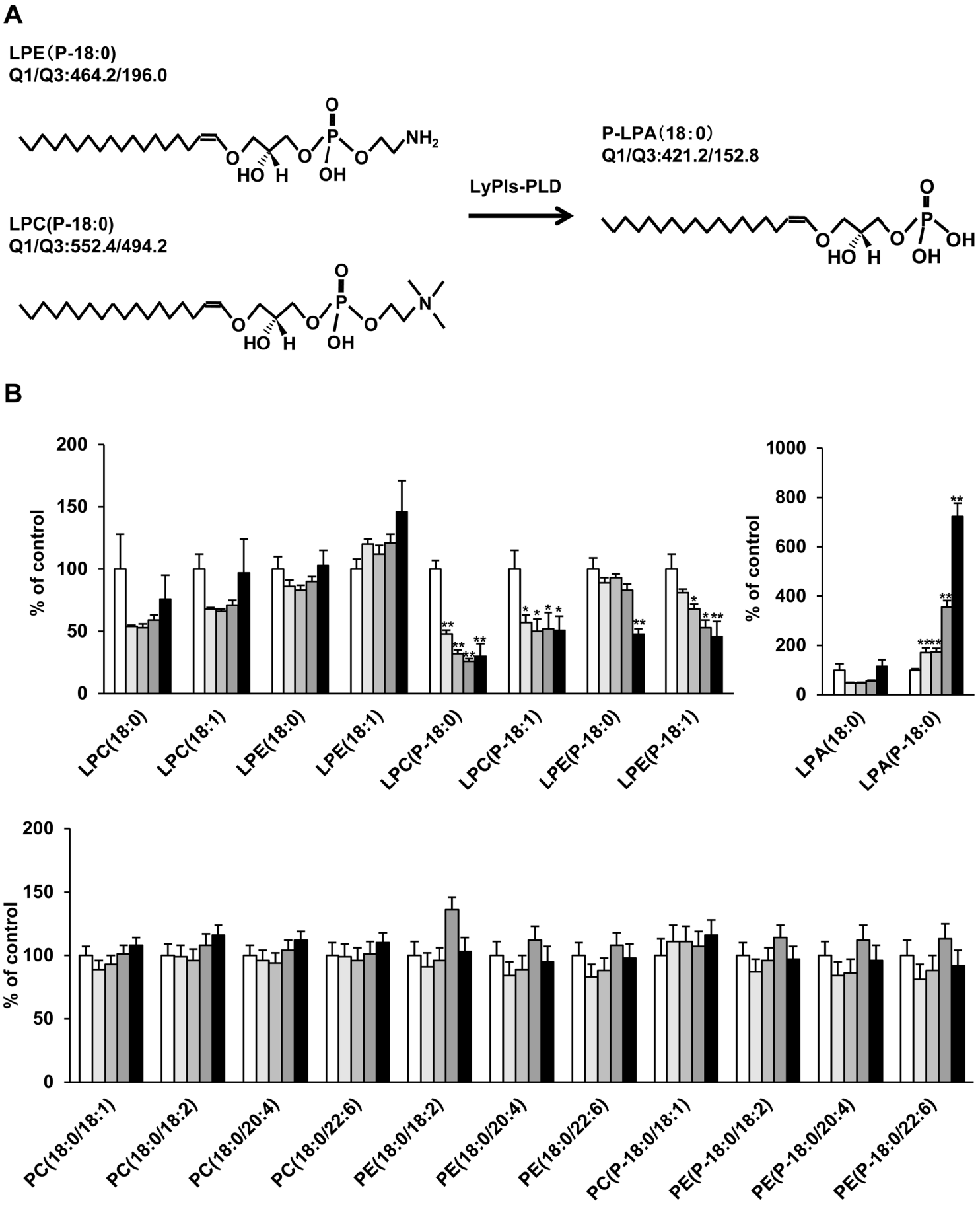
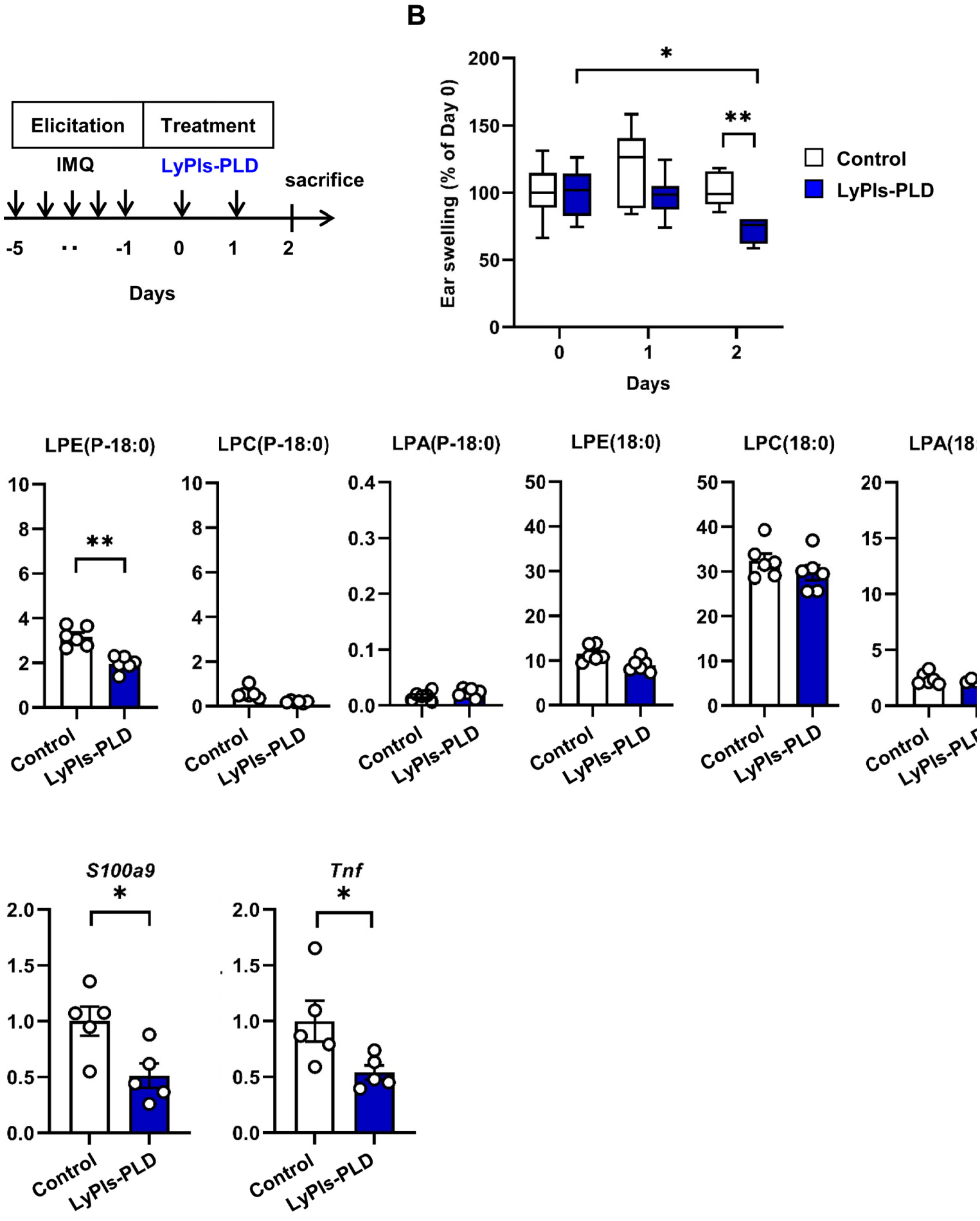
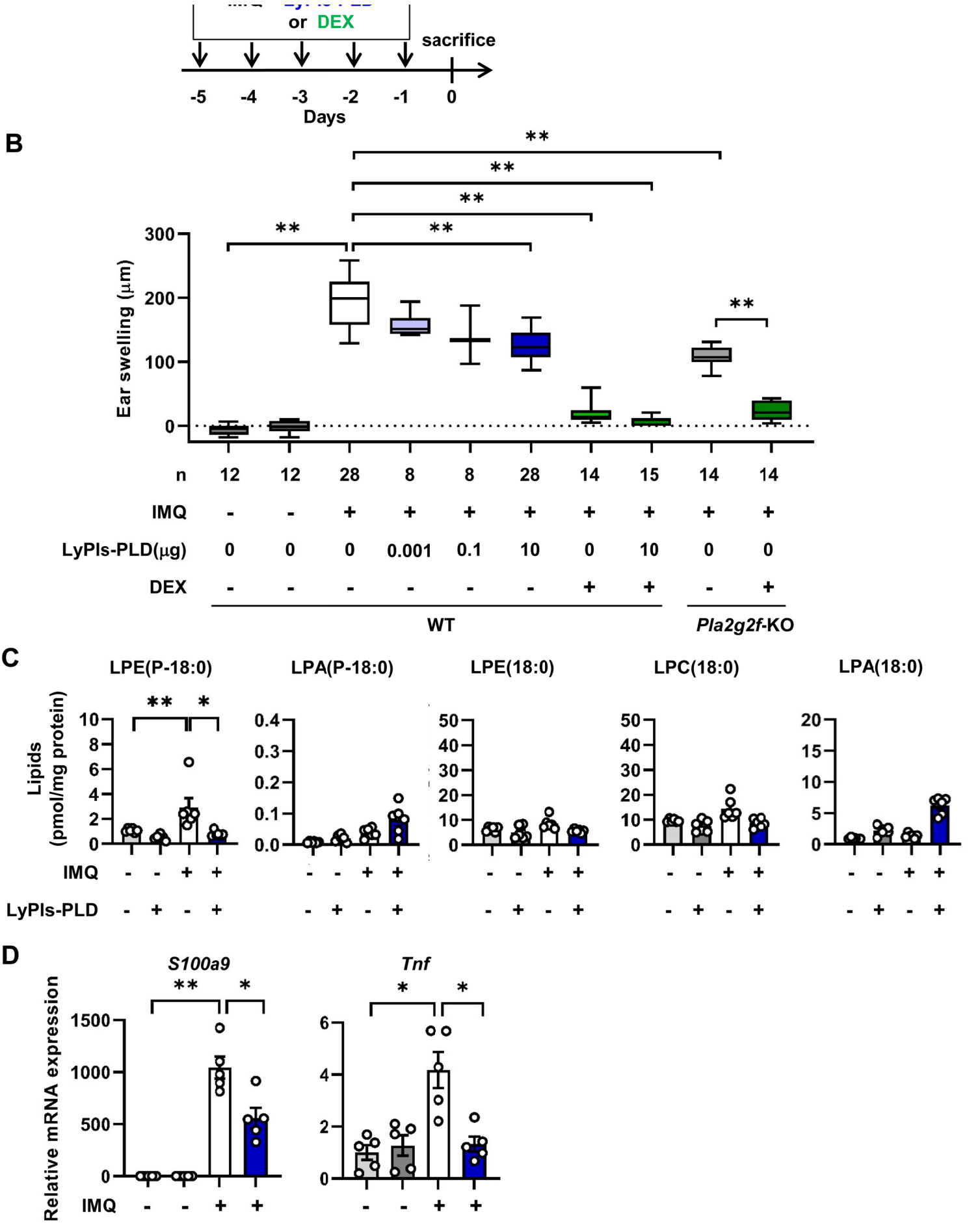
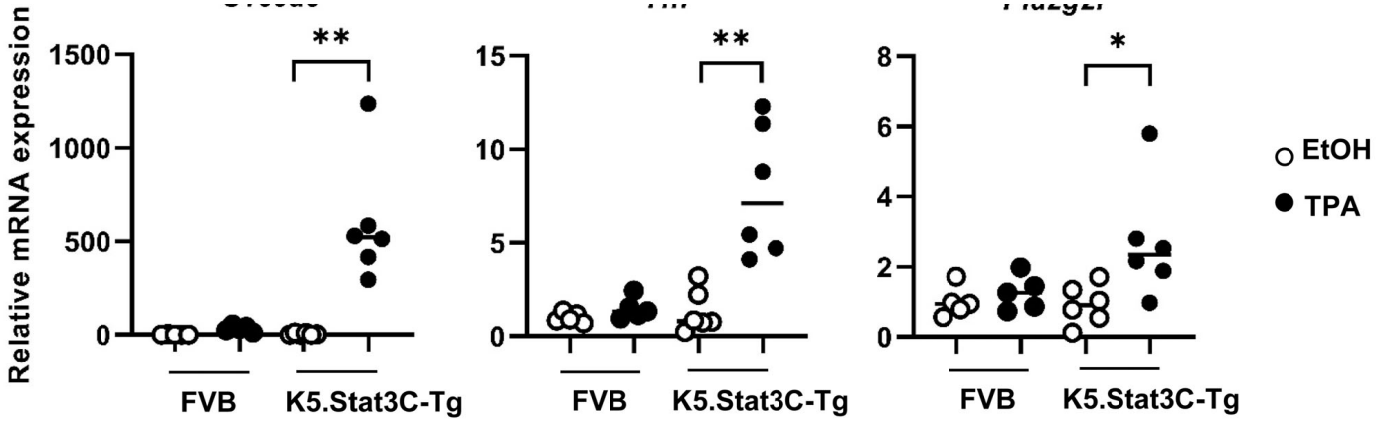
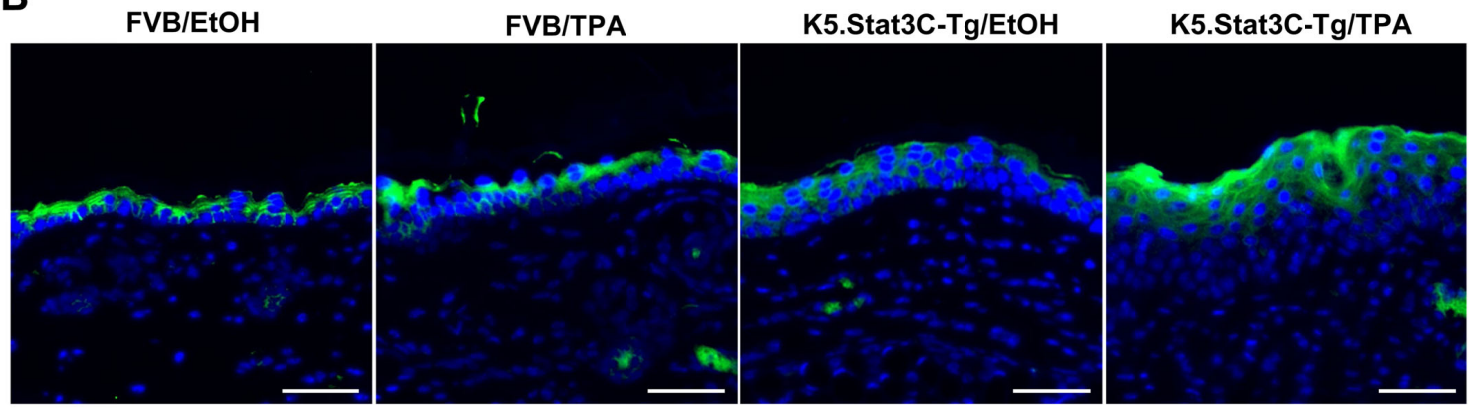

A
LPE（P-18:0)
Q1/Q3:464.2/196.0
LPC(P-18:0)
Q1/Q3:552.4/494.2
LyPls-PLD
P-LPA（18:0）
Q1/Q3:421.2/152.8
B
% of control
LPC(18:0)
LPC(18:1)
LPE(18:0)
LPE(18:1)
LPC(P-18:0)
LPC(P-18:1)
LPE(P-18:0)
LPE(P-18:1)
LPA(18:0)
LPA(P-18:0)
PC(18:0/18:1)
PC(18:0/18:2)
PC(18:0/20:4)
PC(18:0/22:6)
PE(18:0/18:2)
PE(18:0/20:4)
PE(18:0/22:6)
PC(P-18:0/18:1)
PE(P-18:0/18:2)
PE(P-18:0/20:4)
PE(P-18:0/22:6)
LyPls-PLD: □, 0 ng/mL; □, 1.6 ng/mL; ▒, 6.3 ng/mL; ▒, 25 ng/mL; ■, 100 ng/mL.


B
Elicitation
Treatment
IMQ
LyPls-PLD
sacrifice
-5
-1
0
1
2
Days
Ear swelling (% of Day 0)
Control
LyPls-PLD
LPE(P-18:0)
LPC(P-18:0)
LPA(P-18:0)
LPE(18:0)
LPC(18:0)
S100a9
Tnf

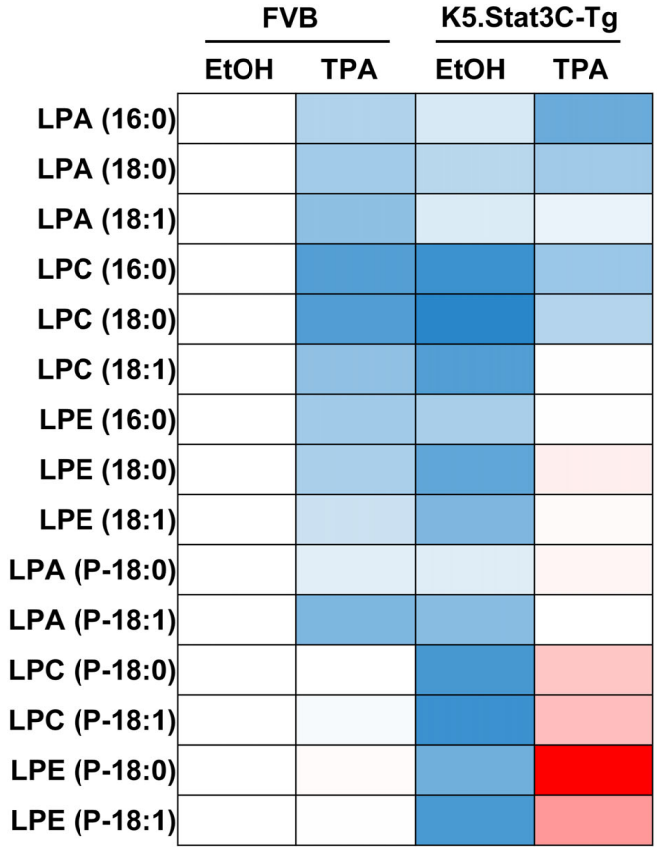

or DEX
sacrifice
-5 -4 -3 -2 -1 0
Days
B
Ear swelling (μm)
300
200
100
0
**
n 12 12 28 8 8 28 14 15 14 14
IMQ - - + + + + + + + +
LyPls-PLD(μg) 0 0 0 0.001 0.1 10 0 10 0 0
DEX - - - - - - + + - +
WT
Pla2g2f-KO
C
LPE(P-18:0)
LPA(P-18:0)
LPE(18:0)
LPC(18:0)
LPA(18:0)
Lipids (pmol/mg protein)
IMQ
LyPls-PLD
D
S100a9
Tnf
Relative mRNA expression
IMQ


Relative mRNA expression
1500
1000
500
0
**
FVB
K5.Stat3C-Tg
15
10
5
0
**
FVB
K5.Stat3C-Tg
8
6
4
2
0
*
FVB
K5.Stat3C-Tg
EtOH
TPA

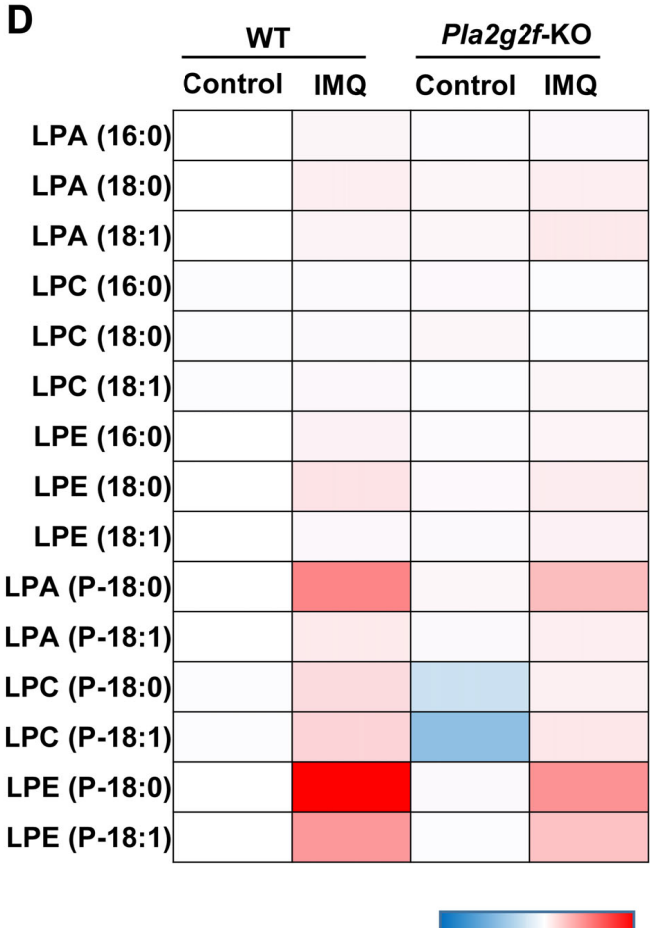
B

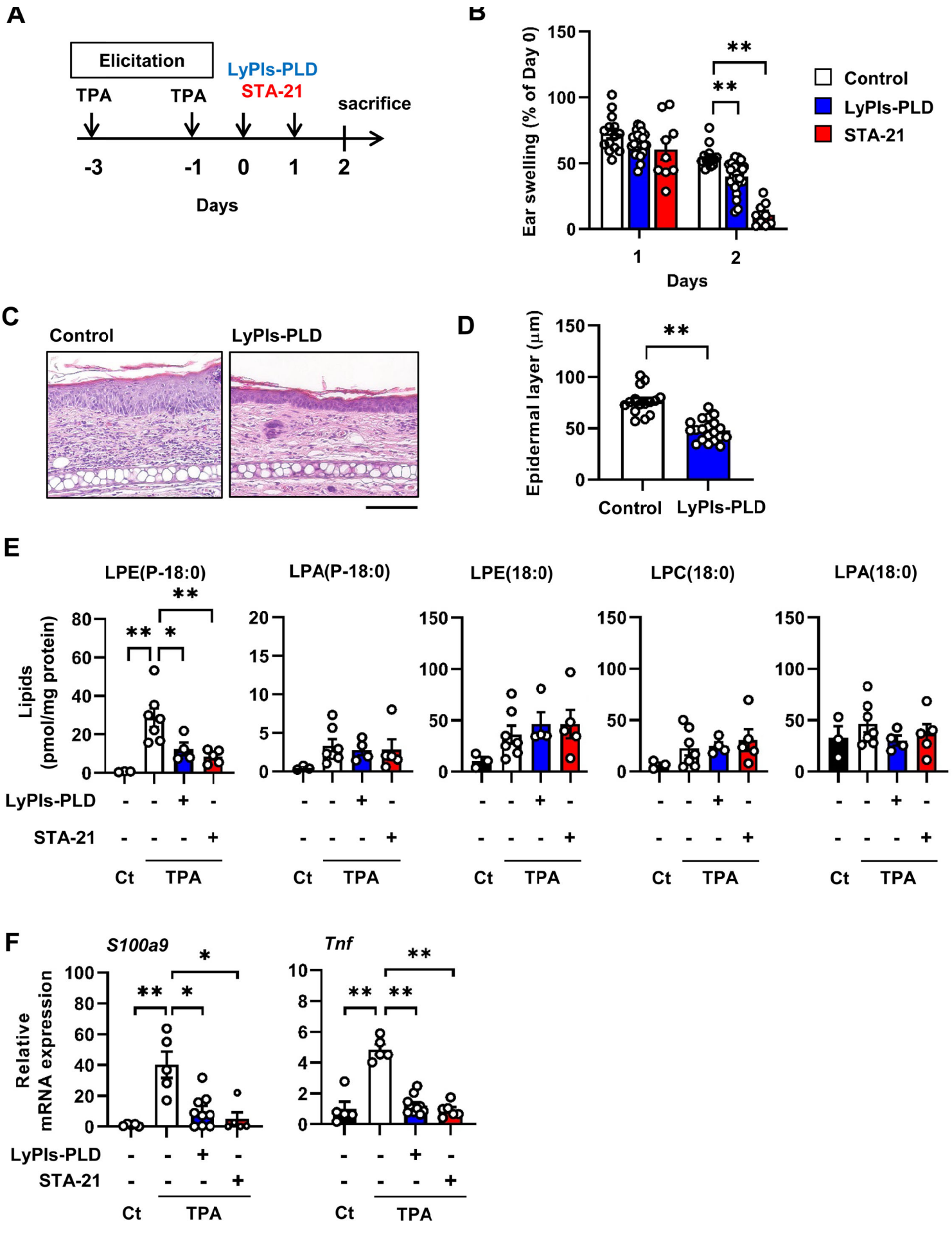

FVB/EtOH
FVB/TPA
K5.Stat3C-Tg/EtOH
K5.Stat3C-Tg/TPA

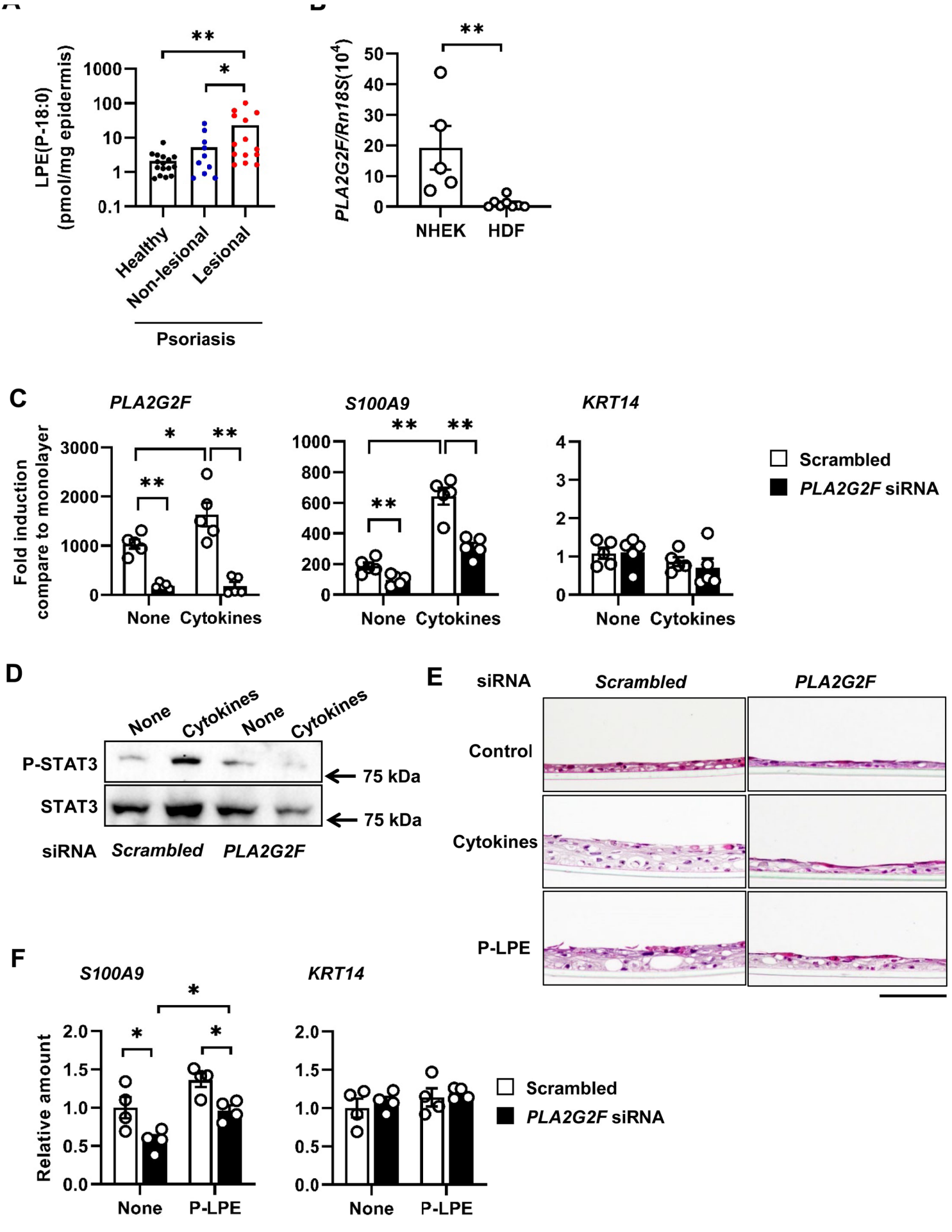
C

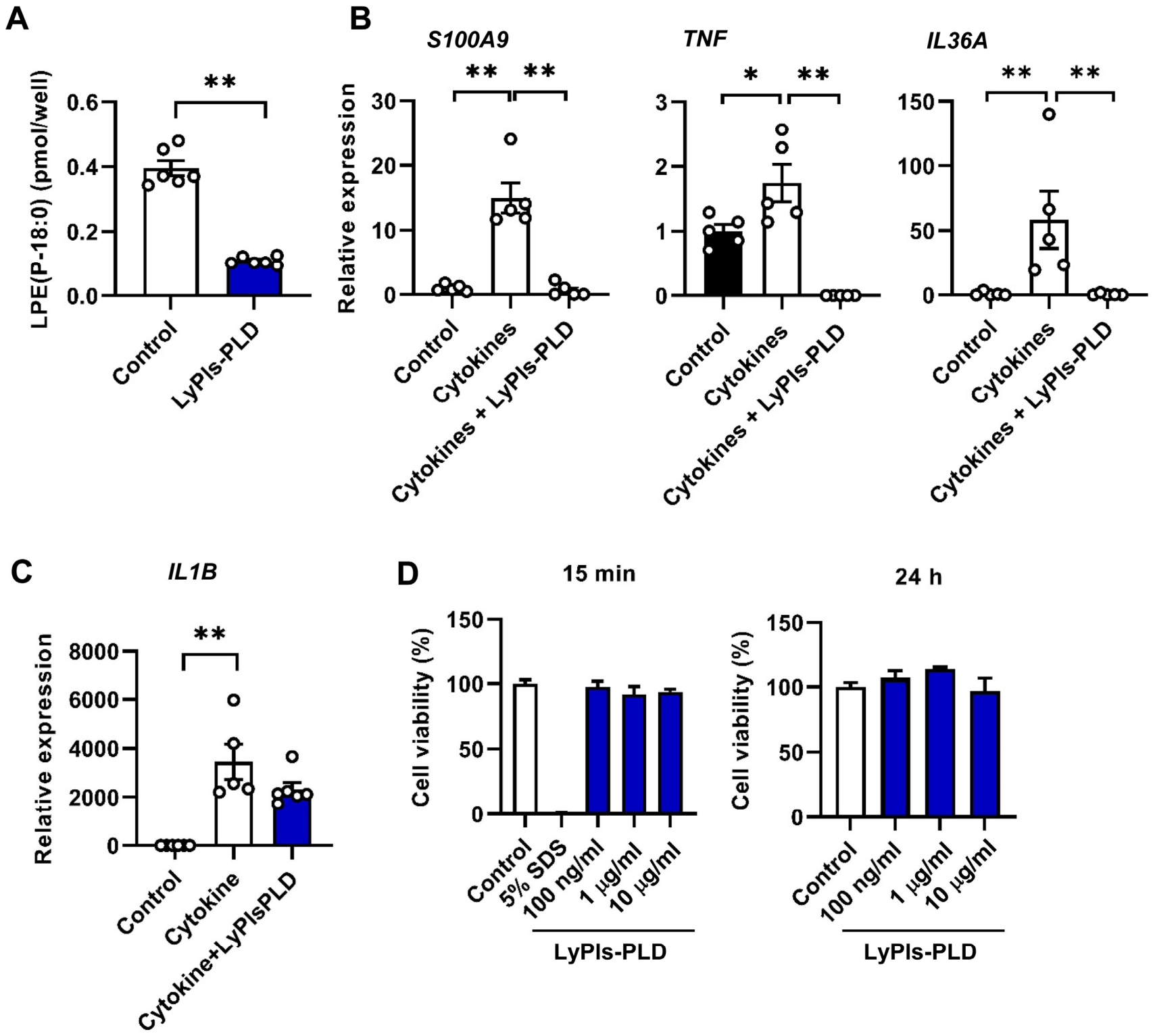

FVB
K5.Stat3C-Tg
EtOH
TPA
EtOH
TPA
LPA (16:0)
LPA (18:0)
LPA (18:1)
LPC (16:0)
LPC (18:0)
LPC (18:1)
LPE (16:0)
LPE (18:0)
LPE (18:1)
LPA (P-18:0)
LPA (P-18:1)
LPC (P-18:0)
LPC (P-18:1)
LPE (P-18:0)
LPE (P-18:1)

D


WT
Pla2g2f-KO
Control
IMQ
Control
IMQ
LPA (16:0)
LPA (18:0)
LPA (18:1)
LPC (16:0)
LPC (18:0)
LPC (18:1)
LPE (16:0)
LPE (18:0)
LPE (18:1)
LPA (P-18:0)
LPA (P-18:1)
LPC (P-18:0)
LPC (P-18:1)
LPE (P-18:0)
LPE (P-18:1)


A
Elicitation
TPA
TPA
LyPls-PLD
STA-21
sacrifice
-3
-1
0
1
2
Days
B
Ear swelling (% of Day 0)
Control
LyPls-PLD
STA-21
Days
C
Control
LyPls-PLD
D
Epidermal layer (μm)
Control
LyPls-PLD
E
LPE(P-18:0)
LPA(P-18:0)
LPE(18:0)
LPC(18:0)
LPA(18:0)
Lipids (pmol/mg protein)
LyPls-PLD
STA-21
Ct
TPA
F
S100a9
Tnf
Relative mRNA expression
LyPls-PLD
STA-21
Ct
TPA


A
LPE(P-18:0) (pmol/mg epidermis)
1000
100
10
1
0.1
**
*
Healthy
Non-lesional
Lesional
Psoriasis
B
PLA2G2F/Rn18S(10^4)
50
40
30
20
10
0
**
NHEK
HDF
C
PLA2G2F
S100A9
KRT14
Fold induction compare to monolayer
3000
2000
1000
0
1000
800
600
400
200
4
3
2
1
None
Cytokines
Scrambled
PLA2G2F siRNA
D
None
Cytokines
None
Cytokines
P-STAT3
STAT3
75 kDa
75 kDa
siRNA
Scrambled
PLA2G2F
E
siRNA
Scrambled
PLA2G2F
Control
Cytokines
P-LPE
F
S100A9
KRT14
Relative amount
2.0
1.5
1.0
0.5
0.0
None
P-LPE
Scrambled
PLA2G2F siRNA


A
LPE(P-18:0) (pmol/well)
0.6
0.4
0.2
0.0
**
Control
LyPls-PLD
B
S100A9
TNF
IL36A
Relative expression
30
20
10
0
3
2
1
0
150
100
50
0
** **
* **
** **
Control
Cytokines
Cytokines + LyPls-PLD
C
IL1B
Relative expression
8000
6000
4000
2000
0
**
Control
Cytokine
Cytokine+LyPlsPLD
D
15 min
24 h
Cell viability (%)
150
100
50
0
Control
5% SDS
100 ng/ml
1 μg/ml
10 μg/ml
LyPls-PLD