

Pathogen-Associated Molecular Pattern-induced TLR2 and TLR4 Activation Increases Keratinocyte Production of Inflammatory Mediators and is Inhibited by Phosphatidylglycerol

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Running title: Phosphatidylglycerol reduces TLR2/4 activation *in vitro*

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The abbreviations used are: AQP3, aquaporin-3; DAMP, danger- or damage-associated molecular pattern; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; PAMP, pathogen-associated molecular pattern; PG, phosphatidylglycerol; PLD, phospholipase D; PLD2, phospholipase D2; TPA, 12-O-tetradecanoylphorbol 13-acetate.

Keywords: epidermis, inflammation, keratinocytes, 12-O-tetradecanoylphorbol 13-acetate (TPA), phosphatidylglycerol, psoriasis, skin

ABSTRACT

Skin serves not only as a protective barrier to microbial entry into the body but also as an immune organ. The outer layer, the epidermis is composed predominantly of keratinocytes, which can be stimulated to produce pro-inflammatory mediators. Although some inflammation is useful to defend against infection, excessive or persistent inflammation can lead to the development of inflammatory skin diseases, such as psoriasis, a common skin disorder affecting approximately 2% of the US population. We have previously found that phosphatidylglycerol (PG) derived from soy can inhibit inflammation in a contact irritant ear edema mouse model. Here we investigated the ability of soy PG to inhibit inflammatory mediator expression in response to activators of the pattern recognition receptors, toll-like receptor-2 (TLR2) and -4 (TLR4). We found that in epidermal keratinocytes soy PG inhibited TLR2 and TLR4 activation and inflammatory mediator expression in response to a synthetic triacylated lipopeptide and lipopolysaccharide, respectively, as well as an endogenous danger-associated molecular pattern. However, at higher concentrations soy PG alone enhanced the expression of some pro-inflammatory cytokines, suggesting a narrow therapeutic window for this lipid. Dioleoylphosphatidylglycerol (DOPG), but not dioleoylphosphatidylcholine, exerted a similar inhibitory effect, completely blocking keratinocyte inflammatory mediator expression induced by TLR2 and TLR4 activators, as well as NF κ B activation in a macrophage cell line (RAW264.7); however, DOPG was not itself pro-inflammatory even at high concentrations. Further, DOPG had no effect on NF κ B activation in response to a TLR7/8 agonist. Our results suggest that DOPG could be used to inhibit excessive skin inflammation.

SIGNIFICANCE STATEMENT

Although inflammation is beneficial for clearing an infection, in some cases the infection can be excessive and/or become chronic thereby resulting in considerable tissue damage and pathological conditions. We show here that the phospholipid, phosphatidylglycerol can inhibit the activation of toll-like receptors-2 and -4 of the innate immune system, as well as the downstream inflammatory mediator expression, in response to microbial component-mimicking agents in epidermal keratinocytes that form the physical barrier of the skin.

INTRODUCTION

Skin serves as a protective barrier to microorganisms and as such plays an important role as a component of the innate immune system (Pivarcsi, et al. 2003). The outer layer, the epidermis is composed predominantly of keratinocytes, the proliferation and differentiation of which is precisely regulated in order to properly form the epidermal mechanical and water-permeability barrier (Goldsmith 1991; Yuspa, et al. 1990). Dysregulation of this programmed pattern of growth and maturation can lead to an abnormal barrier which characterizes a variety of skin diseases, including psoriasis (Langley 2005). Psoriasis is an immune-mediated skin disease, exhibiting hyperproliferation and abnormal differentiation of keratinocytes in addition to inflammation. Patients with psoriasis report a reduction in their quality of life similar to that described by patients with more life-threatening illnesses (Rapp, et al. 1999; Stern, et al. 2004). Accumulating evidence points to a possible involvement of the innate immune system in inflammatory skin diseases like psoriasis [reviewed in (Schon 2019)].

We previously found that phosphatidylglycerol (PG) can be generated by the combined action of the glycerol channel, aquaporin-3 (AQP3), and the lipid-metabolizing enzyme phospholipase D2 (PLD2) (Bollag, et al. 2007; Xie, et al. 2014; Zheng and Bollag 2003). These two proteins are colocalized in epidermal keratinocytes (Zheng and Bollag 2003), with PLD able to convert the glycerol transported by AQP3 to PG. PG levels were found to increase upon keratinocyte differentiation in response to medium with elevated calcium concentrations, with maximal levels reached at a calcium concentration that is optimal for inducing early keratinocyte differentiation (Zheng et al. 2003), suggesting a potential role in this process. Furthermore, manipulations to alter the function of this novel AQP3/PLD2 signaling pathway affected keratinocyte differentiation (Bollag et al. 2007; Choudhary, et al. 2015). Importantly, egg-derived

PG normalizes keratinocyte function, inhibiting the proliferation of keratinocytes that are rapidly dividing and stimulating growth in cells that are dividing slowly. The effect seems to be specific to PG since a similar phospholipid phosphatidylpropanol did not significantly affect keratinocyte proliferation (Bollag et al. 2007). We subsequently showed that the fatty acids composing PG also played an important role in the effect of different PG species on keratinocyte proliferation. Thus, PG species containing polyunsaturated fatty acids are more effective at inhibiting, and those with saturated or monounsaturated fatty acids are better able to stimulate, keratinocyte growth (Xie et al. 2014). Soy PG possesses a large percentage of polyunsaturated fatty acids, with particular efficacy to inhibit keratinocyte proliferation (Xie et al. 2014), suggesting that it might be used to suppress the keratinocyte hyperproliferation characteristic of psoriasis. Indeed, we showed that soy PG inhibits inflammation and mouse ear swelling induced by the contact irritant, 12-O-tetradecanoylphorbol 13-acetate (TPA) *in vivo* (Xie, et al. 2018). However, the mechanism by which soy PG functions to suppress inflammation is unclear.

Toll-like receptors (TLRs) are pattern recognition receptors, which upon activation can stimulate the innate immune system. In response to microbial components (so-called pathogen-associated molecular patterns or PAMPs) or endogenous molecules released by threatened or injured cells (danger- or damage-associated molecular patterns, i.e., DAMPs), TLRs are known to initiate a signaling cascade that results in downstream activation of nuclear factor-kappaB (NF κ B). NF κ B activation occurs through adaptor proteins like myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter inducing interferon- β (TRIF), E3 ligases like TNF receptor-associated factor-6 (TRAF6), and kinases such as IL1 receptor-associated kinases (IRAK)-1, -2 and -4, receptor-interacting protein-1 (RIP1) and TGF β -activating kinase-1 (TAK1) (reviewed in (Adelaja and Hoffmann 2019)). The cascade results in TAK1-mediated

activation of inhibitor of kappaB ($I\kappa B$) kinase (IKK) to phosphorylate and induce the degradation of $I\kappa B$ resulting in NF κB nuclear translocation and activation (Adelaja and Hoffmann 2019). The transcription factor NF κB is the principal regulator of inflammatory gene expression (Adelaja and Hoffmann 2019).

In a previous study we found that in keratinocytes and a macrophage cell line, dioleoylphosphatidylglycerol (DOPG) inhibits inflammatory mediator production in response to TLR2 and TLR4 activation by the anti-microbial peptide S100A9 (Choudhary, et al. 2019). In this study we were also able to show an inhibition of skin inflammation *in vivo* in an imiquimod-induced mouse model of psoriasis (Choudhary et al. 2019). Whether or not soy PG and DOPG inhibit TLR activation in keratinocytes stimulated by PAMPs is also unknown. Also unclear is whether PG can inhibit TLR-mediated NF κB activation in response to PAMPs to exert its anti-inflammatory activity.

To test the idea that soy PG might affect sterile and infectious inflammation through effects on TLR2 and TLR4 activation, keratinocytes and the macrophage cell line RAW264.7 were treated with TLR2 and TLR4 agonists in the presence and absence of soy PG, and inflammatory mediator expression was monitored. Our results provide evidence for the anti-inflammatory effects of PG, suggesting the potential use of this phospholipid to treat inflammatory skin disorders such as psoriasis.

MATERIALS AND METHODS

Materials: Recombinant S100A9 was purchased from R&D Systems (Minneapolis, MN), primary antibodies recognizing p65-NF κ B phosphorylated on serine 536 (pNF κ B; catalog #3033) or total p65-NF κ B (catalog #6956) from Cell Signaling Technology (Danvers, MA), HEK-Blue hTLR2 (catalog #hkb-htlr2), HEK-Blue hTLR4 (catalog #hkb-htlr4) and Resiquimod from InvivoGen (San Diego, CA) and Graphpad Prism from Graphpad (La Jolla, CA). Pam₃CSK₄ and LPS were purchased from Sigma-Aldrich (St. Louis, MO).

Keratinocyte Preparation and Cell Culture: Primary cultures of mouse epidermal keratinocytes were prepared from 1- to 3-day-old ICR strain CD-1 outbred neonatal mice of both sexes as described in detail in (Bollag, et al. 1993; Griner, et al. 1999). All procedures were approved by the Institutional Animal Care and Use Committees of Augusta University or the Charlie Norwood VA Medical Center. RAW 264.7 cells, purchased from American Type Culture Collection (Manassas, VA), were kindly provided by Drs. Qing Zhong and Carlos Isales (Augusta University) and were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin.

Quantitative RT-PCR (qRT-PCR): Keratinocytes were treated with or without a synthetic triacylated lipoprotein (Pam₃CSK₄), a TLR1/TLR2 agonist, lipopolysaccharide, a TLR4 agonist, or recombinant human S100A9 (as in (Choudhary et al. 2019)) for 2 hours in the presence and absence of different concentrations of soy PG or dioleoylphosphatidylglycerol (DOPG) as indicated. Cells were harvested and RNA isolated using PerfectPure RNA tissue kits (5 PRIME, Inc, Gaithersburg, MD, USA) as per the manufacturer's protocol. Following verification of RNA integrity using a Nanodrop instrument (NanoDrop Technologies, Wilmington, DE), RNA was reverse-transcribed to cDNA with iScript cDNA synthesis kits (Bio-Rad Laboratories, Hercules,

CA, USA) and analyzed by real-time PCR using Taqman primer/probe sets from Applied Biosystems (Life Technologies, Grand Island, NY). Reactions were performed using Fast Reagent PCR Master Mix (Applied Biosystems) and a StepOnePlus Real-Time PCR System (Applied Biosystems) as instructed by the manufacturer. Expression of genes of interest was determined using the delta-delta Ct method normalized to the expression of GAPDH as the endogenous housekeeping gene and expressed as the fold increase relative to the appropriate control. For each individual experiment the maximum fold increase was then identified and set to 100% with all values expressed relative to this maximal value. The results were analyzed in this way to account for differences in the magnitude of the response (i.e., the fold change) in experiments performed over time by normalizing the responses between 0 and 100%.

TLR2 or TLR4 activation in the HEK-Blue-hTLR2 reporter cell line: TLR2 or TLR4 activation was determined in the HEK-Blue-hTLR2 or HEK-Blue-hTLR4 reporter cell lines according to the supplier's instructions. These HEK293 cells are engineered to express human TLR2 or TLR4 together with the MD2 and CD14 co-receptors. They also express secreted embryonic alkaline phosphatase (SEAP) under the control of a promoter with binding sites for the transcription factors nuclear factor- κ B (NF κ B) and activator protein-1 (AP-1). Pam₃CSK₄ or LPS with or without DOPG or DOPC (in 20 μ L) was added to a 96-well cell culture plate. HEK-Blue hTLR2 or hTLR4 cells at 70-80% confluence were detached in phosphate-buffered saline (PBS). Cells resuspended in HEK-Blue Detection medium (at a density of approximately 280,000 cells/mL in 180 μ L) were added to each well with or without phospholipid and incubated at 37°C and 5% CO₂ for 18 or 24 hours as indicated. SEAP activity was measured as absorbance at 620 nm using a BIO-TEK Synergy HT microplate reader with Gen5 analysis software.

Western Analysis and Immunocytochemistry: Immunocytochemical analysis of NF κ B

nuclear translocation and Western analysis of NF κ B phosphorylation was performed as described previously (Choudhary et al. 2019).

Statistical Analyses: Statistical analyses were performed using ANOVA followed by Tukey's post-hoc tests, as performed by GraphPad Prism (La Jolla, CA), with significance established at $p \leq 0.05$.

RESULTS

Effects of TLR2 activation with a synthetic triacylated lipoprotein, Pam₃CSK₄, a TLR1/2 agonist, on keratinocyte expression of inflammatory mediators: We have previously shown that S100A9 and β -defensin-2 can activate TLR2 (Choudhary et al. 2019), presumably by acting as danger-associated molecular patterns. To determine if pathogen-associated molecular patterns can also induce keratinocyte expression of inflammatory mediators, we next examined the effect of TLR2 stimulation with the synthetic triacylated lipoprotein TLR1/2 agonist Pam₃CSK₄, which mimics the acylated amino terminus of bacterial lipoproteins, on the mRNA levels of keratinocyte inflammatory mediators. Pam₃CSK₄ dose-dependently stimulated the expression of IL1 α and TNF α . On the other hand, IL6 was induced to a maximal plateau by the lowest Pam₃CSK₄ concentration and IL1 β was induced to the greatest extent by the lowest concentration with higher doses actually producing a lesser response (Fig. 1). Further, we examined the time course of the effects of Pam₃CSK₄ on mRNA levels of these inflammatory mediators and found a time-dependent ability of Pam₃CSK₄ to increase the expression of these cytokines, with maximal expression of IL1 α and TNF α at 2 hours and of IL1 β and IL6 at 6 hours (Fig. 2). Interestingly, Pam₃CSK₄ also induced the expression of S100A9 (Fig. 2), indicating that stimulation of TLR2 promotes the expression of a TLR2 agonist (S100A9) and suggesting the possibility that a feed-forward loop might be established to promote chronic skin inflammation.

Effects of soy PG on the keratinocyte expression of inflammatory mediators upon stimulation with Pam₃CSK₄: We previously showed that soy PG inhibits inflammation in a contact irritant ear edema mouse model *in vivo* (Xie et al. 2018). To determine whether soy PG's effect could result from an inhibition of TLR2 activation to block keratinocyte expression of inflammatory mediators, we treated primary mouse epidermal keratinocytes with 0, 10 and 50

$\mu\text{g/mL}$ soy PG in the presence or absence of Pam₃CSK₄. As shown in Figure 3, we found that Pam₃CSK₄ increased the mRNA expression of the inflammatory mediators TNF α , IL6, IL1 α and IL1 β at 2.5 $\mu\text{g/mL}$ Pam₃CSK₄. Importantly, pretreatment with 10 and 50 $\mu\text{g/mL}$ concentrations of soy PG blocked the increase in TNF α and IL6; 10 $\mu\text{g/mL}$ soy PG also returned the Pam₃CSK₄-induced increase in IL1 α to a level that was not statistically significantly different from the control value. However, soy PG had no effect on Pam₃CSK₄-elicited IL1 β expression. This result suggests that soy PG can inhibit some of the inflammatory mediators that are expressed upon the activation of TLR1/2, which may be activated in psoriasis as a result of the up-regulation of agonists like S100A8 and S100A9 (Choudhary et al. 2019). In addition, it is possible that the expression of other (unexamined) cytokines may be similarly affected, as we have observed previously (Choudhary et al. 2019).

Effects of soy PG on the keratinocyte expression of inflammatory mediators upon stimulation with LPS: To determine whether soy PG can also inhibit TLR4 activation in keratinocytes thereby blocking keratinocyte expression of inflammatory mediators, we treated primary mouse epidermal keratinocytes with 0, 10 and 50 $\mu\text{g/mL}$ soy PG in the presence or absence of LPS. As shown in Figure 4, we found that LPS increased the mRNA expression of the inflammatory mediators IL1 α , IL1 β and TNF α , as well as the receptor for Pam₃CSK₄, TLR1 and TLR2. Pretreatment with 10 and 50 $\mu\text{g/mL}$ concentrations of soy PG blocked the increase in IL1 β and TLR1 and TLR2. However, soy PG at the higher dose actually increased LPS-induced IL1 α and TNF α inflammatory mediator expression. This result suggests that soy PG can inhibit some of the inflammatory mediators that are expressed upon the activation of TLR4, but that soy PG itself might be slightly pro-inflammatory/irritating, suggesting that this PG mixture could cause skin inflammation and therefore would not be ideal for treatment of dermatological disorders.

Effects of soy PG on the keratinocyte expression of inflammatory mediators upon stimulation with recombinant S100A9:

We have previously shown that S100A9 acts as a DAMP to activate TLR2 and TLR4 and induce keratinocyte inflammatory mediator production and that DOPG blocks the activation of both pattern recognition receptors (Choudhary et al. 2019). In addition, in prior findings we determined that soy PG inhibits inflammation induced by a contact irritant (Xie et al. 2018), and we postulated that this inhibition might relate to an ability of soy PG to block inflammatory mediator production in response to contact irritant-induced protein expression of DAMPs such as S100A9. To examine whether the inhibitory effect on DAMP-stimulated inflammatory mediator induction was specific to DOPG and/or whether the inhibition of inflammation by soy PG could involve its blocking of DAMP-induced inflammatory mediator production, we determined the effect of soy PG on S100A9-induced inflammatory mediator expression in keratinocytes. We observed that, as with DOPG (Choudhary et al. 2019), soy PG inhibited S100A9-induced IL1 β and IL6 as well as TLR2 and TLR4 expression (Fig. 5). On the other hand, soy PG at a concentration of 10 μ g/mL slightly but statistically significantly increased TNF α mRNA levels and 50 μ g/mL soy PG markedly elevated expression, with no additional stimulatory or inhibitory effect with S100A9. The fact that soy PG alone exhibits some inflammatory effects again suggests that another PG species may be a better choice for use as a treatment for inflammation associated with infection or skin diseases such as psoriasis.

Effects of DOPG on the Pam₃CSK₄-induced keratinocyte expression of inflammatory mediators: Soy PG has a fatty acid composition that includes palmitic acid (16:0; 17%), stearic acid (18:0; 6%), oleic acid (18:1; 13%), linoleic acid (18:2; 59%) and linolenic acid (18:3; 5%). Soy PG was selected in our previous experiment (Xie et al. 2018) because PG species comprised

of polyunsaturated fatty acids were most efficacious in inhibiting keratinocyte proliferation (Xie et al. 2014). To determine if the ability of PG to inhibit TLR2-induced inflammatory mediators was unique to its high proportion of polyunsaturated fatty acids, we also examined the effect of DOPG on Pam₃CSK₄-elicited inflammatory mediator expression. As shown in Figure 6, both concentrations of DOPG (50 and 100 μg/mL) statistically significantly inhibited the Pam₃CSK₄-induced increase in the expression of all cytokines tested. Thus, DOPG produced similar, but not identical, effects on the TLR1/2-induced cytokine expression as did soy PG.

Effects of DOPG on the Pam₃CSK₄-induced expression of inflammatory mediators in a RAW264.7 macrophage cell line: Keratinocytes and inflammatory mediators released by these cells are likely involved in inflammatory skin diseases like psoriasis; nevertheless, inflammation is thought to be predominantly mediated by the immune system. Therefore, we investigated the ability of PG to inhibit inflammatory mediator expression in the cell line, RAW264.7 (RAW), which serves as a model for macrophages, an immune cell. Similarly to the keratinocytes, upon stimulation with Pam₃CSK₄, RAW cells also up-regulated their mRNA levels of IL1α, IL1β, IL6 and TNFα (Fig. 7). Co-treatment with DOPG (100 μg/mL) resulted in an inhibition of Pam₃CSK₄'s induction of IL1α, IL1β and IL6; the lower (50 μg/mL) DOPG concentration also inhibited IL1α and β expression. Consistent with this result, we also found, as illustrated in Figure 8A and B, that DOPG inhibited the phosphorylation (activation) of NFκB. In addition, DOPG, but not the control lipid dioleoylphosphatidylcholine (DOPC), inhibited Pam₃CSK₄-induced nuclear translocation (i.e., activation) of NFκB (Fig. 8C), a transcription factor downstream of pattern recognition receptors such as TLR2 and TLR4.

The effect of DOPG on NFκB activation in response to the TLR7/8 agonist R848 (Resiquimod) was also examined. As illustrated in Figure 9, Resiquimod, like Pam₃CSK₄,

increased the levels of phosphorylated (activated) NF κ B. However, in contrast to the TLR2 agonist and consistent with its minimal effects on Resiquimod-induced inflammatory mediator expression (Choudhary et al. 2019), DOPG did not affect Resiquimod-induced NF κ B activation, suggesting that DOPG was not a general inhibitor of TLR and/or NF κ B activation.

DOPG-mediated inhibition of TLR2 and TLR4 activation occurs in human cells expressing these pattern recognition receptors: We next sought to extend our findings in mouse keratinocytes and macrophages to human cells expressing these receptors. To do so we made use of two reporter cell lines, one that overexpresses and reports on human TLR2 and the other that overexpresses and reports on human TLR4. We first validated these reporter cell lines, demonstrating a dose-dependent increase in reporter activity with Pam₃CSK₄ in the hTLR2 and LPS in the hTLR4 cell lines (Fig. 10A and B). We then showed that DOPG markedly inhibited reporter activity in both cell lines, whereas DOPC, as a negative control, exerted a slight (but statistically significant) inhibition of activity (Fig. 10C and D).

DISCUSSION

We have previously shown in primary mouse keratinocytes that AQP3 and PLD2 associate (physically and functionally) and that this association allows the generation of PG, a lipid second messenger involved in regulating keratinocyte proliferation and differentiation (reviewed in (Qin, et al. 2011)). “PG” comprises different species depending on its fatty acid composition, and our prior study has indicated that these different species can have differential effects on keratinocytes (Xie et al. 2014). This idea is consistent with findings in the lung demonstrating that unsaturated PG species inhibit surfactant protein A’s anti-inflammatory effects on macrophages exposed to lipopolysaccharide but saturated PG do not (Chiba, et al. 2006). This differential PG molecular species effect might also underlie the unexpected finding that Pam₃CSK₄-induced IL1 β expression was not always inhibited by PG; thus, in RAW264.7 cells DOPG was unable to repress the induction of IL1 β expression, although DOPG, but not soy PG, reduced IL1 β expression in keratinocytes. This result suggests the possibility that the TLR2 agonist’s ability to interact or not with its receptor is influenced by the fatty acid composition of the phospholipid, or perhaps it suggests more than one mode of induction of IL1 β , particularly in immune cells. In addition, it is known that multiple signal transduction pathways can crosstalk with and regulate TLR signaling and downstream NF κ B, including mitogen-activated protein kinase (MAPK) and mammalian target of rapamycin (mTOR) and those kinases activated by TNF and interferons, as well as the endoplasmic reticulum (ER) stress-activated kinase, pancreatic eIF2 α ER kinase (PERK) and the nutrient deprivation-activated protein kinase, general control non-derepressible-2 (GCN2) (reviewed in (Adelaja and Hoffmann 2019)). Thus, differences in the activity of one or more of these other pathways in the two cell types might result in PG’s differential repression of IL1 β expression in response to the TLR2 agonist.

We previously found that synthetic polyunsaturated fatty acid-containing PGs showed the greatest efficacy to inhibit keratinocyte proliferation *in vitro* (Xie et al. 2014); however, synthetic PGs may be costly to produce, possibly impeding their development as a psoriasis therapy. On the other hand, soy PG is relatively inexpensive to produce and is also a natural product, suggesting possible advantages to its use as a treatment for psoriasis. Thus, in a previous study we selected soy PG, which is a mixture of PG species with a high percentage of polyunsaturated fatty acids, to act as a potential anti-inflammatory agent and found that soy PG did in fact inhibit inflammation in a contact irritant mouse model *in vivo* (Xie et al. 2018). In the present study we investigated the potential mechanism of soy PG's anti-inflammatory properties. Our results indicated that keratinocytes responded to TLR2 and TLR4 agonists with inflammatory mediator expression and that soy PG inhibited TLR2 and TLR4 activation in both keratinocytes and the macrophage cell line. However, we observed that soy PG had some pro-inflammatory effects itself, particularly at higher concentrations.

The contact irritant, 12-O-tetradecanoylphorbol 13-acetate (TPA), used in our previous study is known to induce the expression of S100A8 and S100A9 as well as various inflammatory cytokines in keratinocytes (for example, (Carlsson, et al. 2005); Helwa, et al. 2015) and skin (e.g., (Gebhardt, et al. 2002); reviewed in (Mueller 2006)). Based on the results presented here, it seems possible that soy PG may inhibit inflammation in this ear edema model at least in part by blocking TLR activation by DAMPs like S100A9 (Choudhary et al. 2019), as well as PAMPs expressed by the skin microbiome. Our results showing that in keratinocytes soy PG inhibits DAMP-elicited expression of TLR1/2 (Figure 5), which in addition to di- and triacylated microbial peptides responds to DAMPs such as S100A9 and β -defensin that are also up-regulated in psoriasis (Choudhary et al. 2019; Nakajima, et al. 2011; Racz, et al. 2011; Waite and Skokos 2012), suggests

the potential utility of soy PG for the treatment of inflammation in psoriasis. Similarly, its repression of IL6, for which serum levels are markedly elevated in psoriasis patients in comparison with healthy controls (Arican, et al. 2005), provides additional support for the use of soy PG to suppress skin inflammation. However, as noted above our results demonstrating that soy PG induces TNF α expression are problematic, since TNF α levels are elevated in psoriasis, with drugs targeting the TNF α pathway showing efficacy for treatment of the disease (reviewed in (Brotas, et al. 2012)). Thus, our findings suggest that soy PG exhibits a narrow therapeutic window, and despite its ability to inhibit the proliferation of rapidly dividing keratinocytes, soy PG would likely not be practicable as a therapy to treat of inflammatory skin diseases such as psoriasis.

Both soy PG and DOPG reduced the TLR1/2-elicited mRNA levels of IL1 α , IL6 and TNF α in keratinocytes in response to Pam₃CSK₄. Soy PG appeared to be quite efficacious, since a concentration as low as 10 μ g/mL produced a statistically significant inhibition of IL6 and TNF α mRNA levels. It should be noted that the effect of soy PG and DOPG on the inflammatory mediator profile was similar but not identical, with soy PG exerting little effect on IL1 β , whereas DOPG inhibited the Pam₃CSK₄-induced keratinocyte expression of all of the tested inflammatory mediators. In contrast to keratinocytes, in the RAW264.7 macrophage cell line, DOPG did not reduce the effect of Pam₃CSK₄ on IL1 β or TNF α expression, although it inhibited the Pam₃CSK₄-induced increase in mRNA levels of the other two inflammatory mediators. Together, these results argue against a non-specific effect of PG to inhibit Pam₃CSK₄'s action, an idea that is further confirmed by the inability of another lipid (DOPC) to block TLR2 or TLR4 activation. The lack of effect of PG alone on the expression of several of the inflammatory mediators also suggests that the inhibitory effects of PG are not the result of cytotoxicity of this phospholipid. Nor did DOPG exhibit a general inhibition of all TLRs since DOPG inhibited neither NF κ B activation (Figure 9)

nor inflammatory mediator expression (Choudhary et al. 2019) induced by the TLR7/8 agonist Resiquimod. Furthermore, our results indicated that DOPG had a broad therapeutic range for the inhibition of TLR2 and TLR4 activation by both microbial components (these studies) and endogenous molecules that engage these pattern recognition receptors (Choudhary et al. 2019), with limited or no ability to induce inflammatory mediator production on its own. Thus, our results provide evidence for the potential use of DOPG to treat inflammatory skin disorders such as psoriasis.

A comparison of the results observed in keratinocytes with those in the lung and bronchial epithelial cells is also in order. In general, Voelker and colleagues have demonstrated an effect of palmitoyl-oleoylphosphatidylglycerol (POPG) to reduce TLR2-mediated cellular responses. Thus, POPG inhibits TLR2-mediated arachidonic acid release from mouse and human macrophages exposed to *Mycoplasma pneumonia* membrane despite having no effect on *Mycoplasma* binding to the cell surface (Kandasamy, et al. 2011). In these experiments DOPG induces a small but not statistically significant decrease in arachidonic acid release. Nevertheless, these authors have also shown that DOPG is most potent in terms of inhibition of IL8 production in BEAS2B human bronchial epithelial cells (Numata, et al. 2013). Consistent with this result, DOPG has been found in macrophages to inhibit endotoxin-stimulated NF κ B activation, thereby reducing Type IIA secretory phospholipase A₂ levels and activity (Wu, et al. 2003), although these authors did not investigate other PG species.

Finally, the molecular mechanism by which PG inhibits TLR2/4 activation is reported to be through the protein CD14. Thus, Kuronuma et al. (Kuronuma, et al. 2009) have shown that PG binds to MD2 and CD14, an accessory protein and a co-receptor, respectively, for TLR2 and TLR4 (Chun and Seong 2010; He, et al. 2016; van Bergenhenegouwen, et al. 2013). Subsequent studies

showed that the ability of PG to inhibit TLR2 and TLR4 activation corresponded with its interaction with CD14 rather than MD2 (Kandasamy, et al. 2016). PG inhibits the binding of di[3-deoxy-D-manno-octulosonyl]-lipid A (a recombinant LPS analog) to CD14, likely interfering with its ability to present such microbial components to the appropriate TLR (Kandasamy et al. 2016), but further investigation is required.

In conclusion, this study shows for the first time to our knowledge that soy PG suppressed TLR2 and TLR4 activation and induction of inflammatory mediator expression by microbial components and other pathogen-associated molecular patterns in keratinocytes. In addition, the demonstrated ability of TLR2 activation to induce S100 proteins, and TLR4 activation to stimulate TLR1/2 expression, may initiate a positive feedback loop in keratinocytes that promotes and sustains skin inflammation in inflammatory skin diseases like psoriasis. Thus, our results suggest that PG, in particular DOPG, may be effective for the treatment of skin diseases characterized by inflammation.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Choudhary, Chen, and Bollag

Conducted experiments: Choudhary, Griffiths, and Chen

Performed data analysis: Choudhary, Griffiths, and Chen

Wrote or contributed to the writing of the manuscript: Choudhary, Griffiths, Chen, and Bollag

REFERENCES

- Adelaja A and Hoffmann A (2019) Signaling crosstalk mechanisms that may fine-tune pathogen-responsive NFkappaB. *Front Immunol* 10: 433.
- Arican O, Aral M, Sasmaz S and Ciragil P (2005) Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. *Mediators Inflamm* 2005: 273-279.
- Bollag WB, Ducote J and Harmon CS (1993) Effects of the selective protein kinase C inhibitor, Ro 31-7549, on the proliferation of cultured mouse epidermal keratinocytes. *J Invest Dermatol* 100: 240-246.
- Bollag WB, Xie D, Zhong X and Zheng X (2007) A potential role for the phospholipase D2-aquaporin-3 signaling module in early keratinocyte differentiation: Production of a novel phosphatidylglycerol lipid signal. *J Invest Dermatol* 127: 2823-2831.
- Brotas AM, Cunha JM, Lago EH, Machado CC and Carneiro SC (2012) Tumor necrosis factor-alpha and the cytokine network in psoriasis. *An Bras Dermatol* 87: 673-681.
- Carlsson H, Yhr M, Petersson S, Collins N, Polyak K and Enerback C (2005) Psoriasis (S100A7) and calgranulin-B (S100A9) induction is dependent on reactive oxygen species and is downregulated by Bcl-2 and antioxidants. *Cancer Biol Ther* 4: 998-1005.
- Chiba H, Piboonpocanun S, Mitsuzawa H, Kuronuma K, Murphy RC and Voelker DR (2006) Pulmonary surfactant proteins and lipids as modulators of inflammation and innate immunity. *Respirology* 11: S2-S6.
- Choudhary V, Olala LO, Qin H, Helwa I, Pan ZQ, Tsai YY, Frohman MA, Kaddour-Djebbar I and Bollag WB (2015) Aquaporin-3 re-expression induces differentiation in a phospholipase D2-dependent manner in aquaporin-3-knockout mouse keratinocytes. *J Invest Dermatol* 135: 499-507.
- Choudhary V, Uaratanawong R, Patel RR, Patel H, Bao W, Hartney B, Cohen E, Chen X, Zhong Q, Isales CM, et al. (2019) Phosphatidylglycerol Inhibits Toll-Like Receptor-Mediated Inflammation by Danger-Associated Molecular Patterns. *J Invest Dermatol* 139: 868-877.
- Chun KH and Seong SY (2010) CD14 but not MD2 transmit signals from DAMP. *Int Immunopharmacol* 10: 98-106.
- Gebhardt C, Breitenbach U, Tuckermann JP, Dittrich BT, Richter KH and Angel P (2002) Calgranulins S100A8 and S100A9 are negatively regulated by glucocorticoids in a c-Fos-dependent manner and overexpressed throughout skin carcinogenesis. *Oncogene* 21: 4266-4276.
- Goldsmith LA 1991 *Physiology, Biochemistry, and Molecular Biology of the Skin*. New York: Oxford University Press.
- Griner R, Qin F, Jung E, Sue-Ling C, Crawford K, Mann-Blakeney R, Bollag R and WB B (1999) 1,25-Dihydroxyvitamin D₃ induces phospholipase D-1 expression in primary mouse epidermal keratinocytes. *J Biol Chem* 274: 4663-4670.
- He Z, Riva M, Bjork P, Sward K, Morgelin M, Leanderson T and Ivars F (2016) CD14 is a co-receptor for TLR4 in the S100A9-induced pro-inflammatory response in monocytes. *PLoS One* 11: e0156377.
- Helwa I, Patel R, Karempelis P, Kaddour-Djebbar I, Choudhary V and Bollag WB (2015) The antipsoriatic agent monomethylfumarate has antiproliferative, prodifferentiative, and anti-inflammatory effects on keratinocytes. *J Pharmacol Exp Ther* 352: 90-97.

- Kandasamy P, Numata M, Berry KZ, Fickes R, Leslie CC, Murphy RC and Voelker DR (2016) Structural analogs of pulmonary surfactant phosphatidylglycerol inhibit toll-like receptor 2 and 4 signaling. *J Lipid Res* 57: 993-1005.
- Kandasamy P, Zarini S, Chan ED, Leslie CC, Murphy RC and Voelker DR (2011) Pulmonary surfactant phosphatidylglycerol inhibits Mycoplasma pneumoniae-stimulated eicosanoid production from human and mouse macrophages. *J Biol Chem* 286: 7841-7853.
- Kuronuma K, Mitsuzawa H, Takeda K, Nishitani C, Chan ED, Kuroki Y, Nakamura M and Voelker DR (2009) Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and U937 cells by binding the lipopolysaccharide-interacting proteins CD14 and MD-2. *J Biol Chem* 284: 25488-25500.
- Langley R 2005 *Psoriasis*. New York: Firefly Books, Inc.
- Mueller MM (2006) Inflammation in epithelial skin tumours: old stories and new ideas. *Eur J Cancer* 42: 735-744.
- Nakajima K, Kanda T, Takaishi M, Shiga T, Miyoshi K, Nakajima H, Kamijima R, Tarutani M, Benson JM, Elloso MM, et al. (2011) Distinct roles of IL-23 and IL-17 in the development of psoriasis-like lesions in a mouse model. *J Immunol* 186: 4481-4489.
- Numata M, Nagashima Y, Moore ML, Berry KZ, Chan M, Kandasamy P, Peebles RS, Jr., Murphy RC and Voelker DR (2013) Phosphatidylglycerol provides short-term prophylaxis against respiratory syncytial virus infection. *J Lipid Res* 54: 2133-2143.
- Pivarsci A, Bodai L, Rethi B, Kenderessy-Szabo A, Koreck A, Szell M, Beer Z, Bata-Csorgoo Z, Magocsi M, Rajnavolgyi E, et al. (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immunol* 15: 721-730.
- Qin H, Zheng X, Zhong X, Shetty AK, Elias PM and Bollag WB (2011) Aquaporin-3 in keratinocytes and skin: Its role and interaction with phospholipase D2. *Arch Biochem Biophys* 508: 138-143.
- Racz E, Prens EP, Kurek D, Kant M, de Ridder D, Mourits S, Baerveldt EM, Ozgur Z, van Ijcken WFJ, Laman JD, et al. (2011) Effective treatment of psoriasis with narrow-band UVB phototherapy is linked to suppression of the IFN and Th17 pathways. *J Invest Dermatol* 131: 1547-1558.
- Rapp SR, Feldman SR, Exum ML, Fleischer AB, Jr. and Reboussin DM (1999) Psoriasis causes as much disability as other major medical diseases. *J Am Acad Dermatol* 41: 401-407.
- Schon MP (2019) Adaptive and innate immunity in psoriasis and other inflammatory disorders. *Front Immunol* 10: 1764.
- Stern RS, Nijsten T, Feldman SR, Margolis DJ and Rolstad T (2004) Psoriasis is common, carries a substantial burden even when not extensive, and is associated with widespread treatment dissatisfaction. *J Investig Dermatol Symp Proc* 9: 136-139.
- van Bergenhenegouwen J, Plantinga TS, Joosten LA, Netea MG, Folkerts G, Kraneveld AD, Garssen J and Vos AP (2013) TLR2 & Co: a critical analysis of the complex interactions between TLR2 and coreceptors. *J Leukoc Biol* 94: 885-902.
- Waite JC and Skokos D (2012) Th17 response and inflammatory autoimmune diseases. *Int J Inflamm* 2012: 819467.
- Wu YZ, Medjane S, Chabot S, Kubrusly FS, Raw I, Chignard M and Touqui L (2003) Surfactant protein-A and phosphatidylglycerol suppress type IIA phospholipase A2 synthesis via nuclear factor-kappaB. *Am J Respir Crit Care Med* 168: 692-699.

- Xie D, Choudhary V, Seremwe M, Edwards JG, Wang A, Emmons AC, Bollag KA, Johnson MH and Bollag WB (2018) Soy phosphatidylglycerol reduces inflammation in a contact irritant ear edema mouse model in vivo. *J Pharmacol Exp Ther* 366: 1-8.
- Xie D, Seremwe M, Edwards JG, Podolsky R and Bollag WB (2014) Distinct effects of different phosphatidylglycerol species on mouse keratinocyte proliferation. *PLoS One* 9: e107119.
- Yuspa SH, Hennings H, Tucker RW, Kilkenny A, Lee E, Kruszewski F and Roop DR 1990 *The Regulation of Differentiation in Normal and Neoplastic Keratinocytes*. New York: Wiley-Liss, Inc.
- Zheng X and Bollag WB (2003) Aquaporin 3 colocalizes with phospholipase D2 in caveolin-rich membrane microdomains and is regulated by keratinocyte differentiation. *J Invest Dermatol* 121: 1487-1495.
- Zheng X, Ray S and Bollag WB (2003) Modulation of phospholipase D-mediated phosphatidylglycerol formation by differentiating agents in primary mouse epidermal keratinocytes. *Biochim Biophys Acta* 1643: 25-36.

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. The TLR1/2 agonist, Pam₃CSK₄, induced keratinocyte expression of inflammatory mediators in a dose-dependent manner. Keratinocytes were treated with various concentrations of the synthetic triacylated lipoprotein Pam₃CSK₄ (Pam) for 2 hours as indicated (in $\mu\text{g}/\text{mL}$). RNA was then isolated and the expression of the inflammatory mediators (A) IL1 α , (B) IL1 β , (C) IL6 and (D) TNF α was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means \pm SD of 3 separate experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus the zero concentration (Control).

FIGURE 2. Pam₃CSK₄ induced keratinocyte expression of inflammatory mediators in a time-dependent manner. Keratinocytes were treated with vehicle or 2.5 $\mu\text{g}/\text{mL}$ Pam₃CSK₄ (Pam) for 2, 4 and 6 hours. RNA was then isolated and the expression of the inflammatory mediators, (A) IL1 α , (B) IL1 β , (C) IL6 and (D) TNF α , as well as (E) S100A8 and (F) S100A9, was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means \pm SD of 3 separate experiments; Pam₃CSK₄-treated values were analyzed at each time point using one-sample t-tests versus a hypothetical mean of 1.0 (to which the vehicle-treated control value was set), as performed by GraphPad InStat, with * $p < 0.05$ and ** $p < 0.01$ versus the control at that time point.

FIGURE 3. Soy PG inhibited keratinocyte inflammatory mediator expression in response to Pam₃CSK₄. Keratinocytes were treated with 0, 1 and 2.5 $\mu\text{g}/\text{mL}$ Pam₃CSK₄ (Pam) in the presence or absence of 10 or 50 $\mu\text{g}/\text{mL}$ soy PG (s-PG) for 2 hours. RNA was then isolated and the expression

of the inflammatory mediators (A) IL1 α , (B) IL1 β , (C) IL6 and (D) TNF α was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means \pm SD of 4 separate experiments; *p<0.05, ** p<0.01 and ***p<0.001 versus the zero concentration control; ^{ff}p<0.01, ^{fff}p<0.001, ^{$\xi\xi\xi$} p<0.001 and ^{$\xi\xi$} p<0.01 versus the indicated groups.

FIGURE 4. Soy PG inhibited keratinocyte inflammatory mediator expression in response to LPS. Keratinocytes were treated with 0 and 1000 units/mL LPS in the presence or absence of 10 or 50 μ g/mL soy PG (s-PG) for 2 hours. RNA was then isolated and the expression of the inflammatory mediators (A) IL1 α , (B) IL1 β , (C) IL6 and (D) TNF α , as well as (E) TLR1 and (F) TLR2, was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means \pm SD of 3 separate experiments; *p<0.05, ** p<0.01 and ***p<0.001 versus the zero concentration control; ^fp<0.05, ^{ff}p<0.01, ^{fff}p<0.001, ^{ξ} p<0.05 and ^{$\xi\xi$} p<0.01 versus the indicated groups.

FIGURE 5. Soy PG inhibited keratinocyte inflammatory mediator expression in response to S100A9. Keratinocytes were treated with 0 and 2.5 μ g/mL recombinant S100A9 in the presence or absence of 10 or 50 μ g/mL soy PG (s-PG) for 2 hours. RNA was then isolated and the expression of the inflammatory mediators (A) IL1 β , (B) IL6 and (C) TNF α , as well as (D) TLR1 and (E) TLR2, was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means \pm SD of 3 separate experiments; *p<0.05, **p<0.01 and ***p<0.001 versus the control; ^{†††}p<0.001 versus all other groups; ^fp<0.05, ^{ξ} p<0.05 and ^{$\xi\xi$} p<0.01 versus the indicated groups.

FIGURE 6. DOPG inhibited keratinocyte inflammatory mediator expression in response to Pam₃CSK₄. Keratinocytes were treated with 0, 1 and 2.5 µg/mL Pam₃CSK₄ (Pam) in the presence or absence of 50 or 100 µg/mL DOPG for 2 hours. RNA was then isolated and the expression of the inflammatory mediators (A) IL1α, (B) IL1β, (C) IL6 and (D) TNFα was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means ± SD of 4 separate experiments; *p<0.05, ** p<0.01 and ***p<0.001 versus the zero concentration control; ^{fff}p<0.001, ^τp<0.05, ^{τττ}p<0.001, ^ξp<0.05 and ^{ξξ}p<0.01 versus the indicated groups.

FIGURE 7. DOPG inhibited Pam₃CSK₄-induced inflammatory mediator expression in the macrophage cell line RAW264.7. RAW264.7 cells were treated with 0, 1 and 2.5 µM Pam₃CSK₄ (Pam) in the presence or absence of 50 or 100 µg/mL DOPG for 2 hours. RNA was then isolated and the expression of the inflammatory mediators (A) IL1α, (B) IL1β, (C) IL6 and (D) TNFα was monitored by quantitative RT-PCR with GAPDH used as the housekeeping gene. Results represent the means ± SD of 4 separate experiments; *p<0.05, **p<0.01 and ***p<0.001 versus the zero concentration control; ^{ff}p<0.01, ^τp<0.05 and ^ξp<0.05 versus the indicated groups.

FIGURE 8. DOPG inhibited Pam₃CSK₄-induced NFκB activation in the macrophage cell line RAW264.7. (A) RAW264.7 cells were treated with or without 2.5 µg/mL Pam₃CSK₄ (Pam) in the presence and absence of 100 µg/mL DOPG for 10 minutes. Cells were harvested and the phosphorylation (activation) status of NFκB determined using an antibody recognizing phosphoserine⁵³⁶ in comparison with total NFκB levels. Panel A shows a representative western blot while panel B presents the cumulative results from 3 separate experiments (means ± SD); ***p<0.001 versus the control; ^{ξξξ}p<0.001 as indicated. (C) RAW264.7 cells were treated with 0

and 2.5 μ M Pam3CSK4 (Pam) in the presence or absence of 100 μ g/mL DOPG or DOPC as indicated for 2 hours. NF κ B was then monitored by immunocytochemistry and confocal microscopy (scale bar = 5 μ m). The final row shows the same micrographs illustrating grayscale NF κ B staining as in the row above, with NF κ B staining in green and nuclear staining (with DAPI) in blue in these merged color images. Results are representative of at least 3 separate experiments.

FIGURE 9. DOPG had no effect on R848-induced NF κ B activation in the macrophage cell line RAW264.7. (A) RAW264.7 cells were treated with or without 1 μ g/mL R848 (Resiquimod) in the presence and absence of 100 μ g/mL DOPG for 10 minutes. Cells were harvested and the phosphorylation (activation) status of NF κ B determined using an antibody recognizing phosphoserine⁵³⁶ in comparison with total NF κ B levels. Panel A shows a representative western blot while panel B presents the cumulative results from 3 separate experiments (means \pm SD); *** p <0.001 versus the control.

FIGURE 10. DOPG, but not DOPC, inhibited Pam₃CSK₄- and LPS-induced TLR2 and TLR4 activation in reporter cell lines. (A) HEK-Blue-hTLR2 cells were incubated for 24 hours in the HEK-Blue detection medium with various concentrations of Pam₃CSK₄, as indicated. (B) HEK-Blue-hTLR4 cells were incubated for 24 hours in the HEK-Blue detection medium with various concentrations of LPS, as indicated. (C) HEK-Blue-hTLR2 cells were incubated in the HEK-Blue detection medium with or without 0.03 ng/mL Pam₃CSK₄ in the presence and absence of 100 μ g/mL DOPG or DOPC for 24 hours. (D) HEK-Blue-hTLR4 cells were incubated in the HEK-Blue detection medium with or without 3 ng/mL LPS in the presence

and absence of 100 μ g/mL DOPG or DOPC for 24 hours. In all cases SEAP activity was then measured as absorbance at 620 nm. Values represent the means \pm SD from at least 3 separate experiments; *** p <0.001 versus the control; ^{fff} p <0.001 and $\xi\xi\xi p$ <0.001 as indicated.

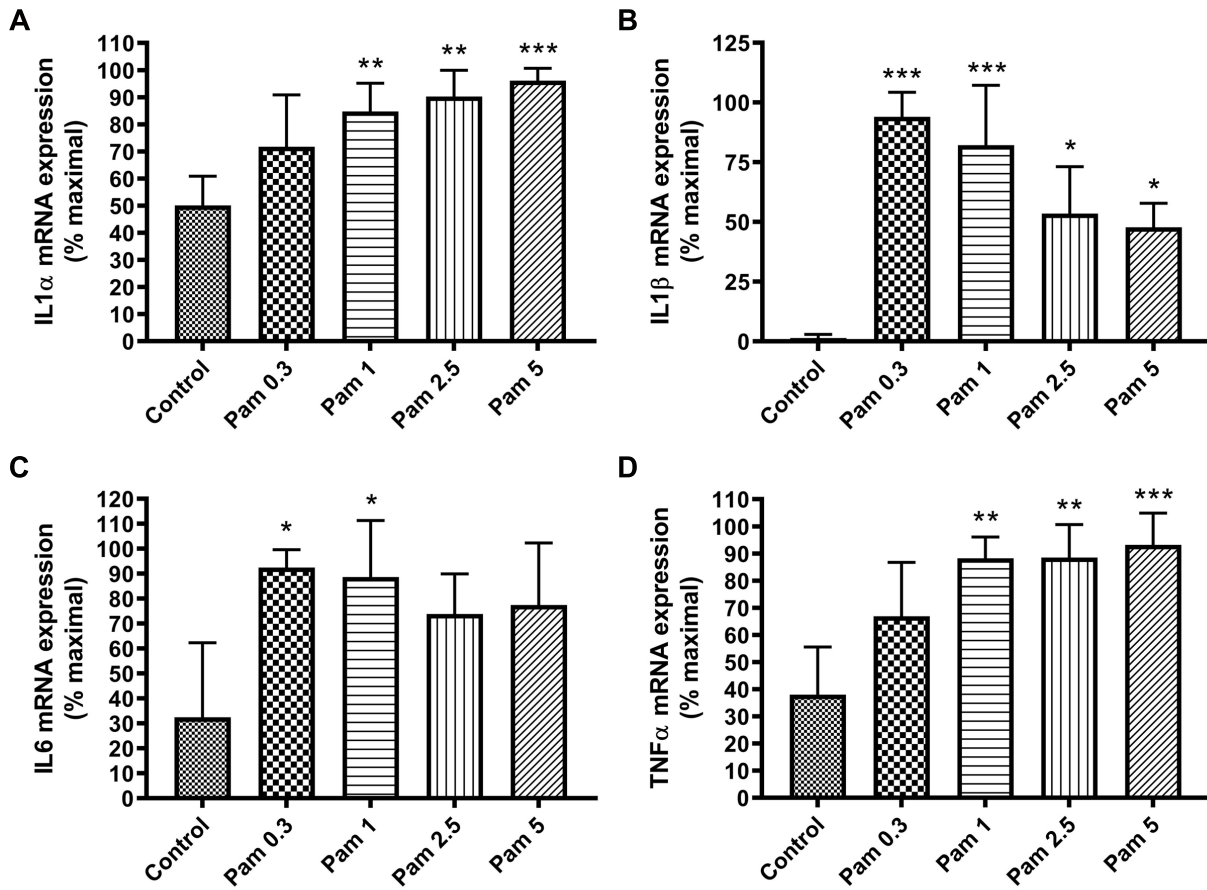


Figure 1

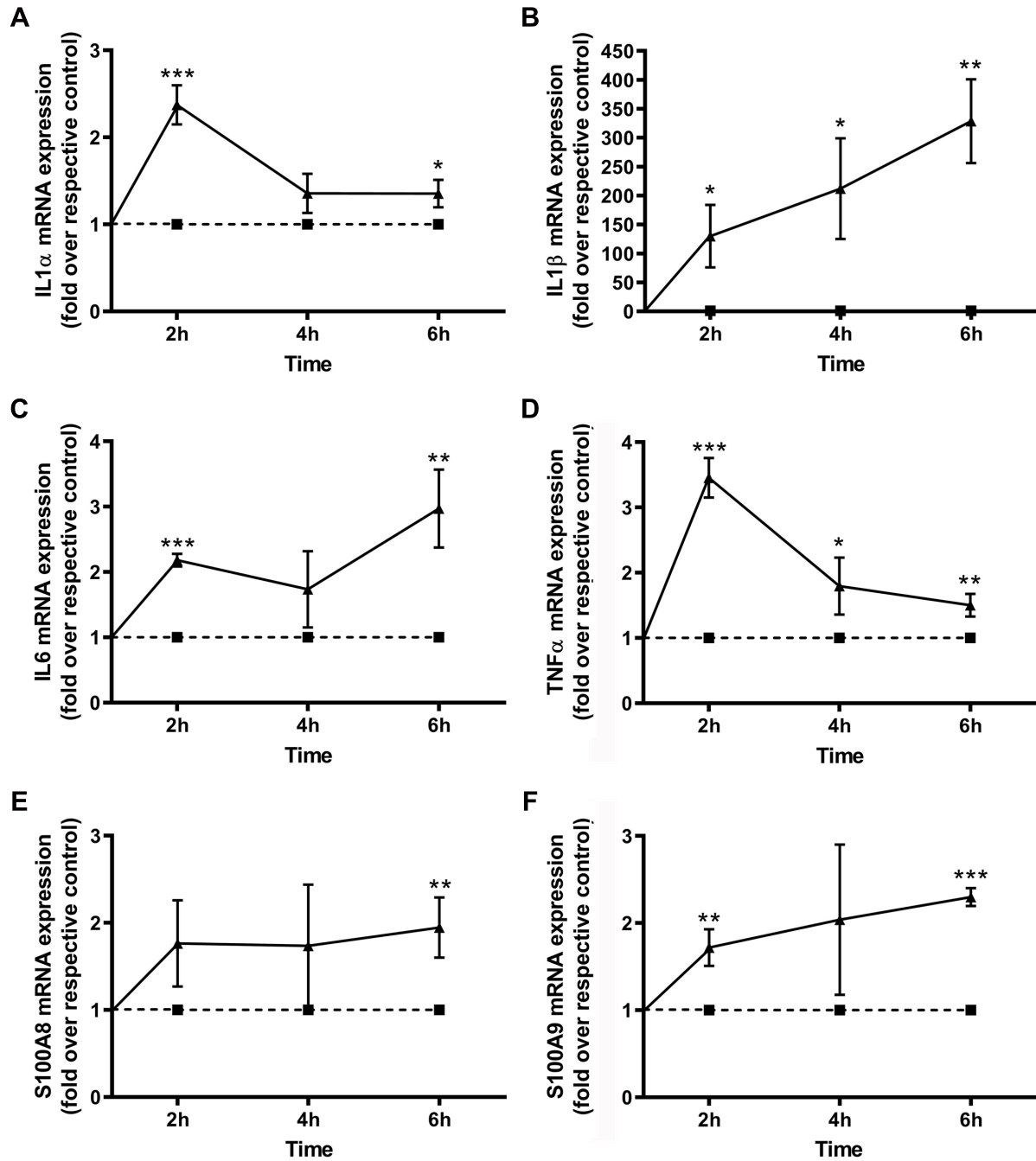


Figure 2

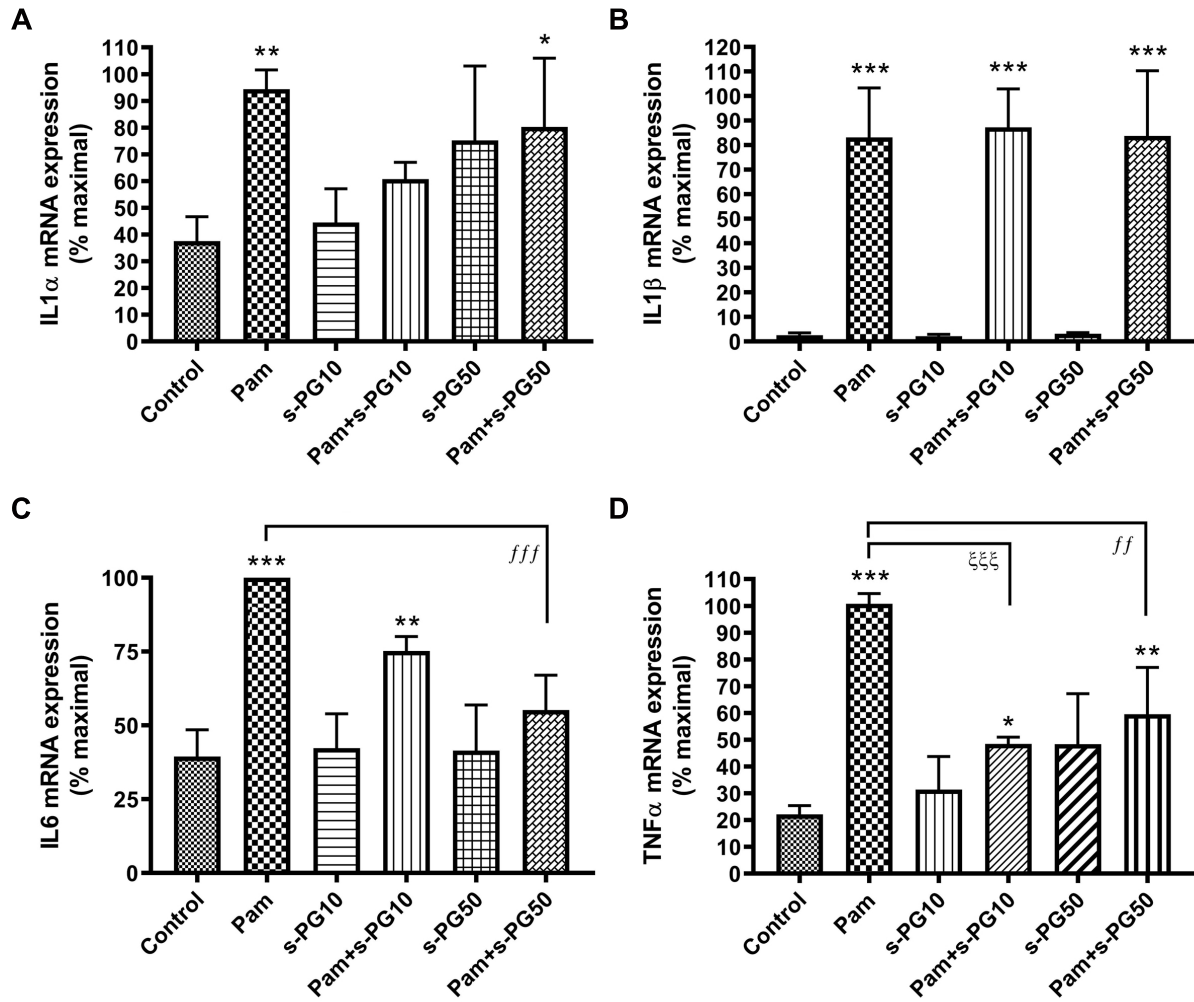


Figure 3

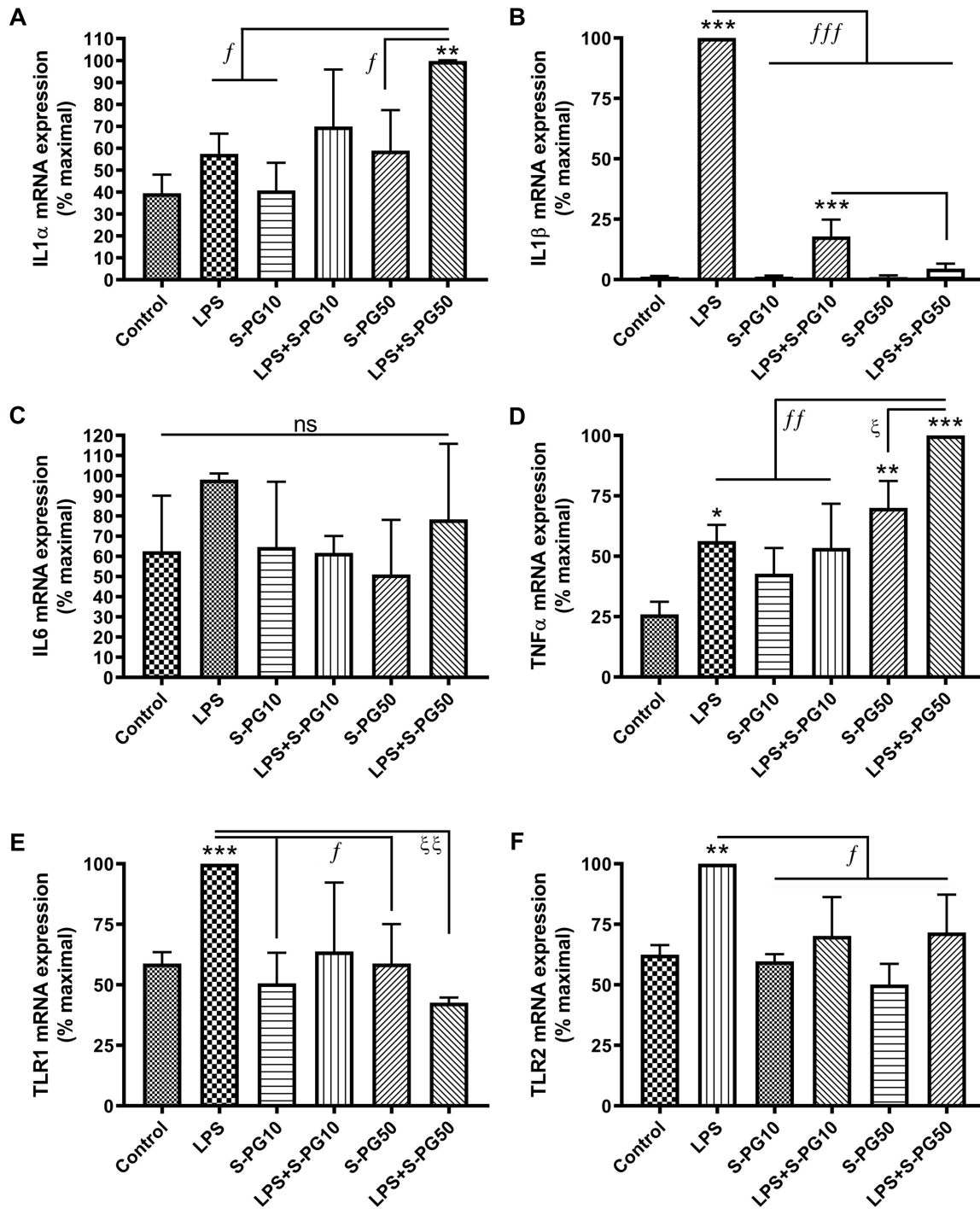


Figure 4

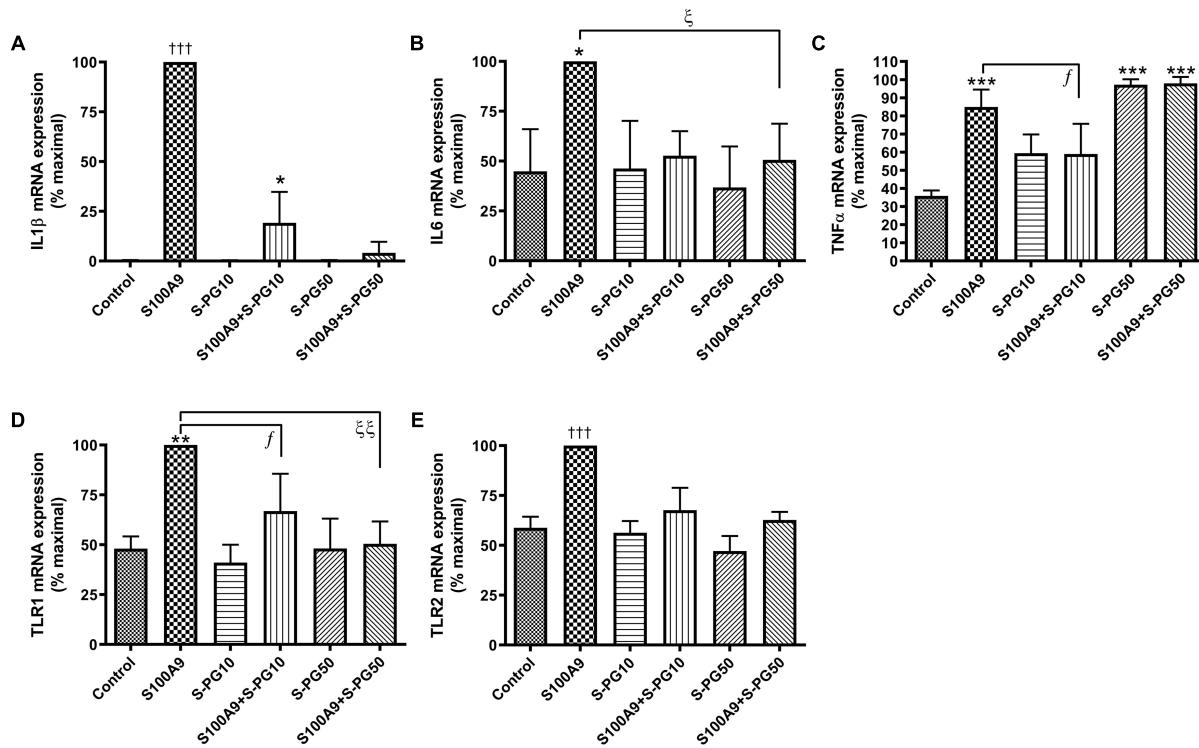


Figure 5

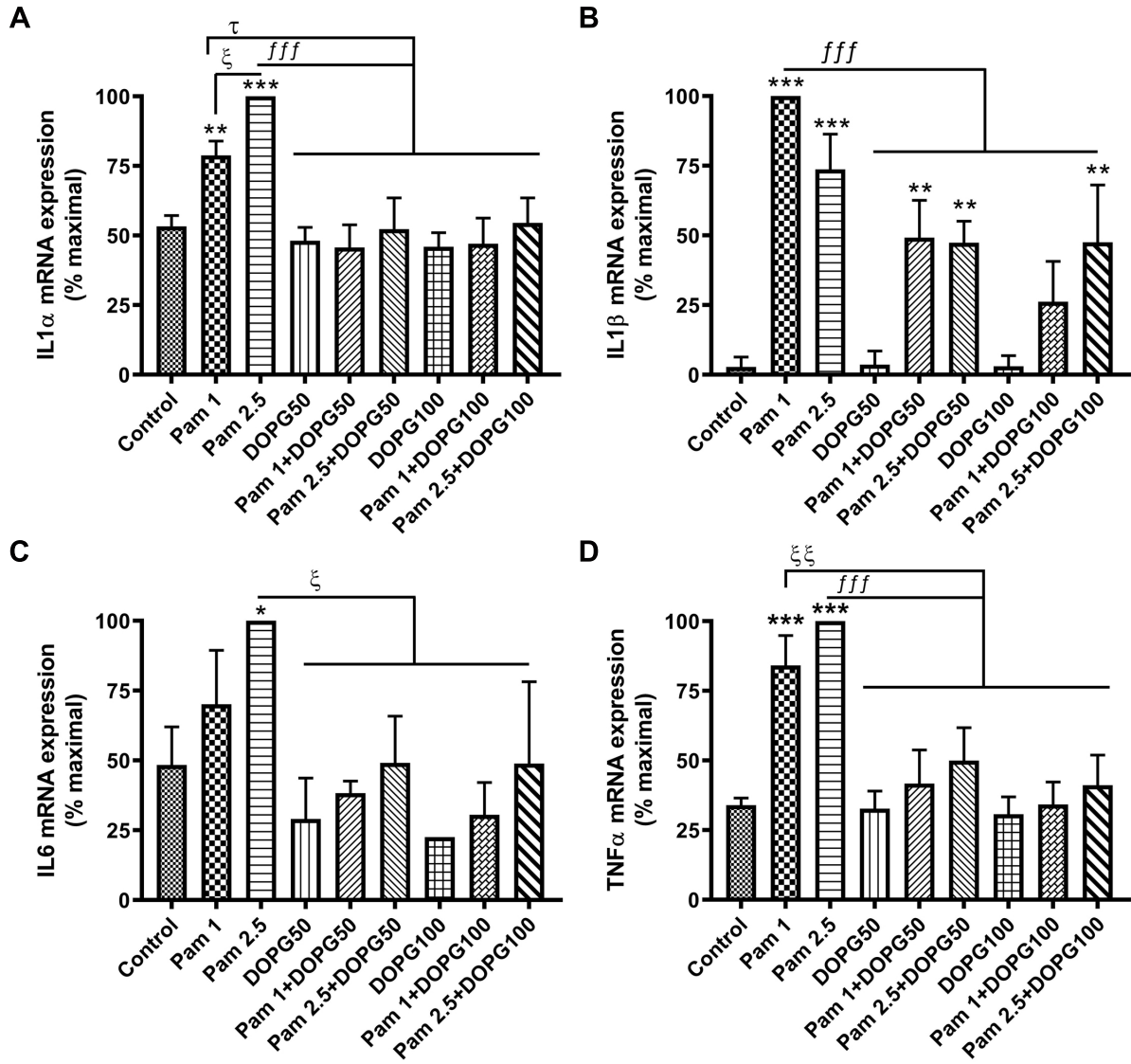


Figure 6

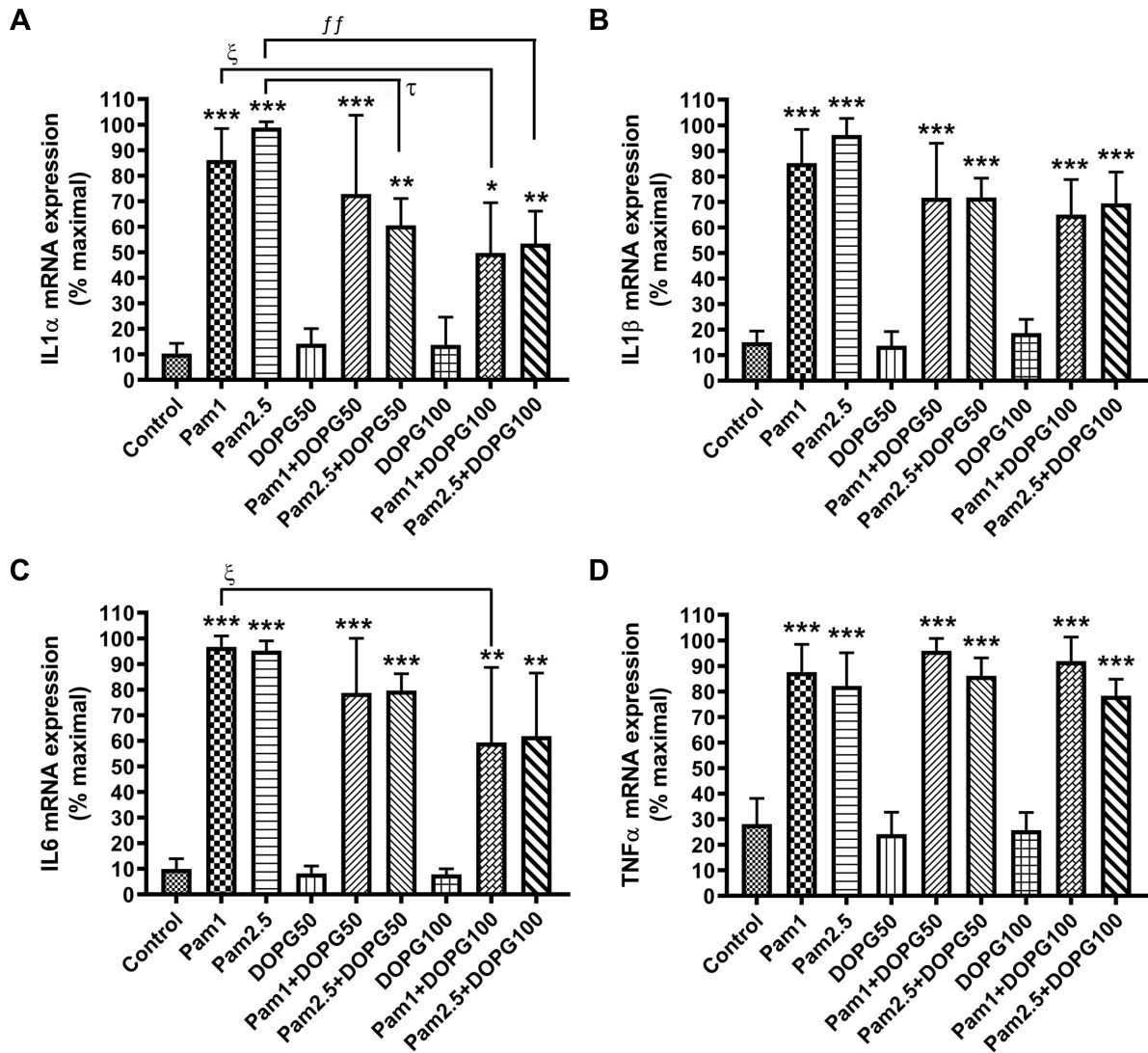


Figure 7

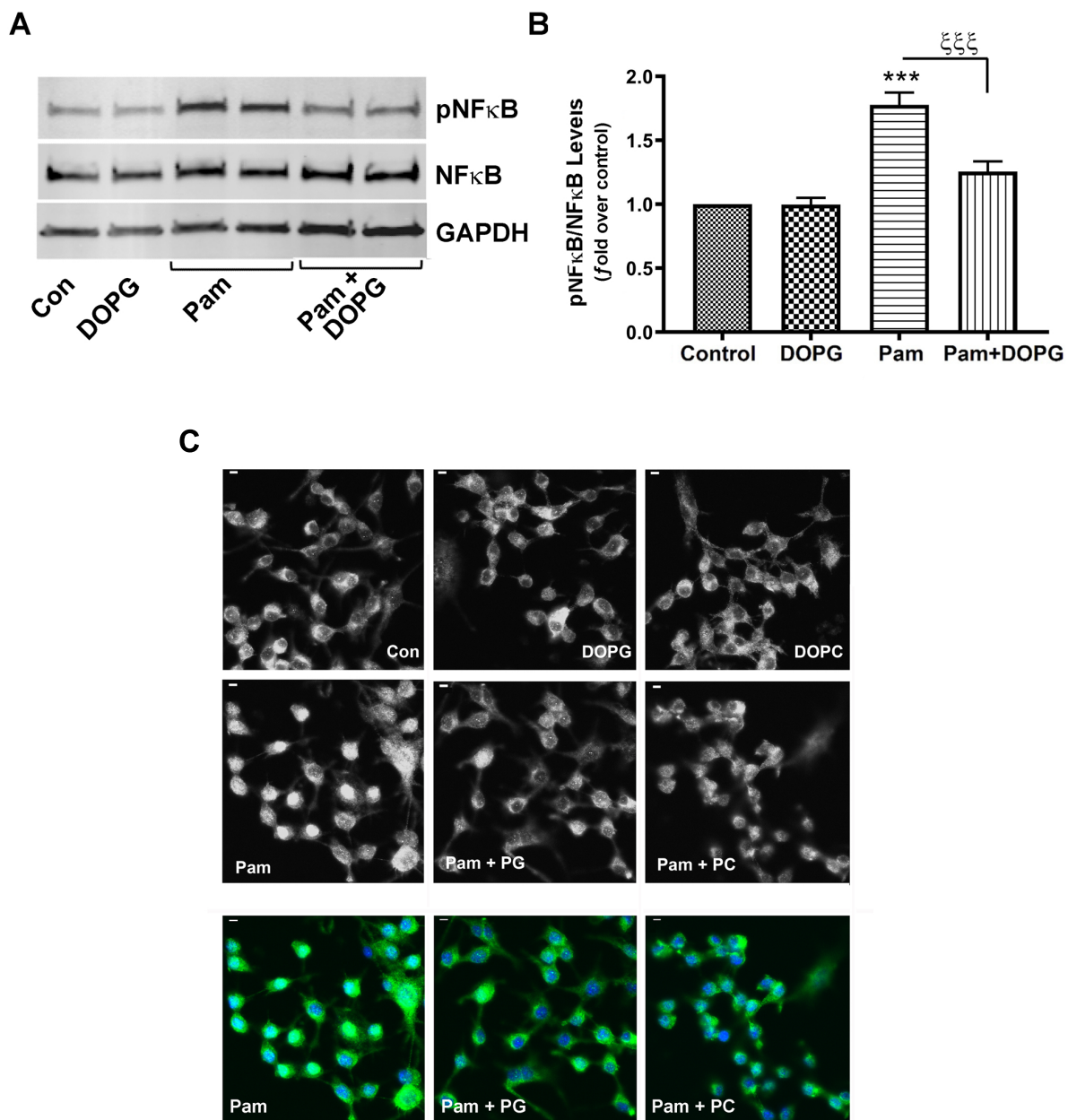


Figure 8

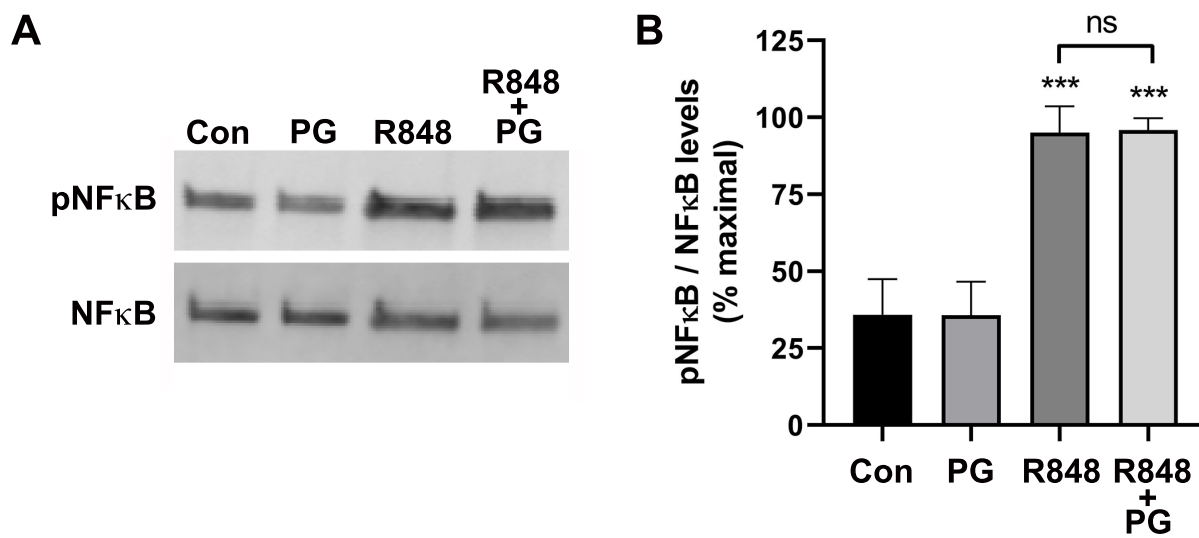


Figure 9

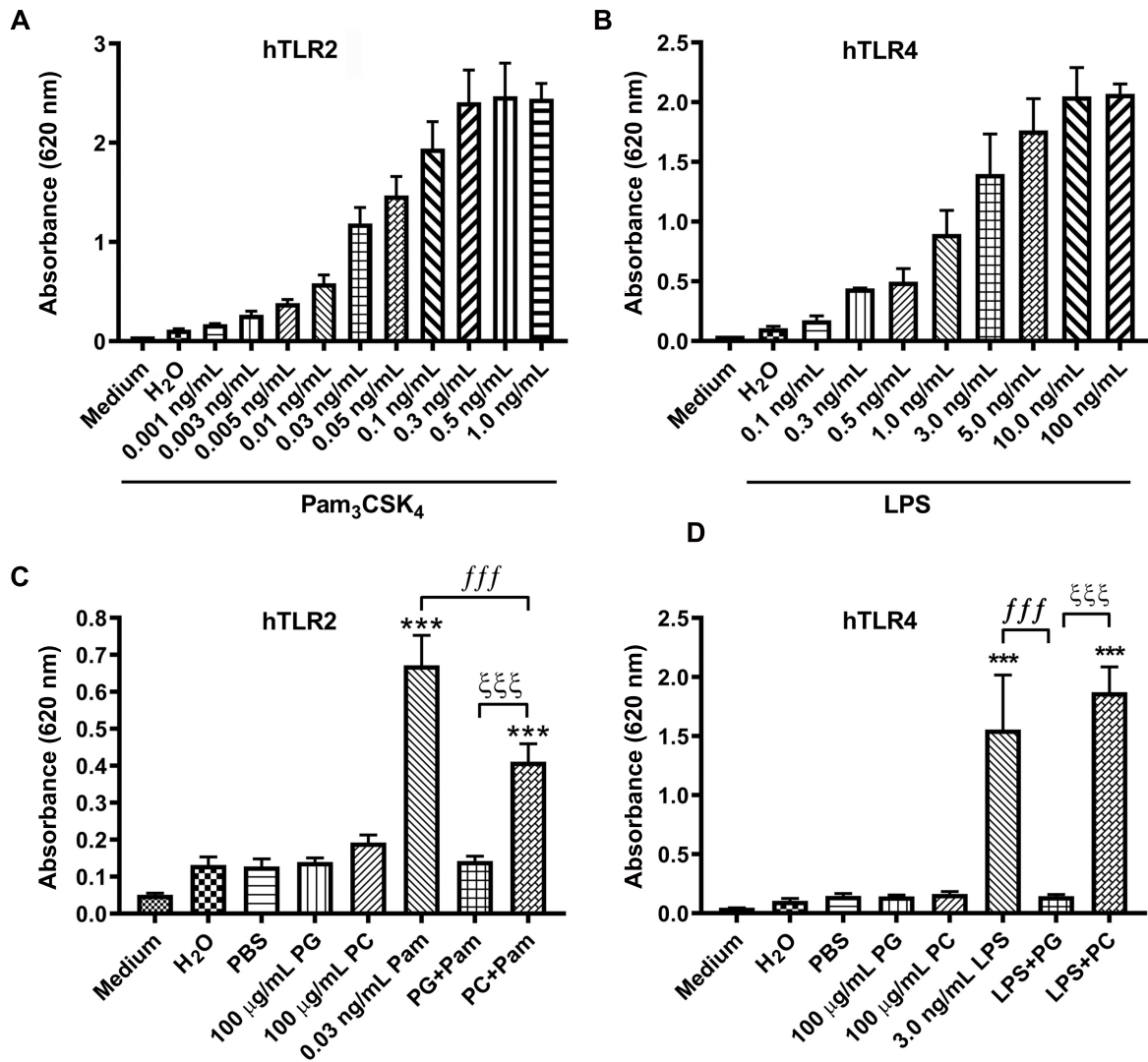


Figure 10