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Soy Phosphatidylglycerol Reduces Inflammation in a Contact Irritant Ear Edema Mouse Model *in vivo*

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The abbreviations used are: AQP3, aquaporin-3; PG, phosphatidylglycerol; PLD, phospholipase D; PLD2, phospholipase D2; TNFα, tumor necrosis factor-alpha; TPA, 12-O-tetradecanoyl-phorbol 13-acetate; Vitamin D, 1,25-dihydroxyvitamin D_3.

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ABSTRACT

We have previously shown that phosphatidylglycerol (PG) regulates the function of keratinocytes, the predominant cells that comprise the epidermis, inhibiting the proliferation of rapidly dividing keratinocytes. In particular, soy PG, a PG mixture with a high proportion of polyunsaturated fatty acids, is efficacious at inhibiting these proliferating keratinocytes. Psoriasis is a skin disorder characterized by hyperproliferation of keratinocytes and inflammation. Data in the lung suggest that PG in pulmonary surfactant inhibits inflammation. To investigate the possibility of using PG containing polyunsaturated fatty acids for the treatment of psoriasis, in the current study we examined the effect of soy PG on inflammation induced by the application of 12-O-tetradecanoylphorbol 13-acetate (TPA), a contact irritant, to mouse ears *in vivo*. We monitored ear thickness and weight as a measure of ear edema, as well as CD45-positive immune cell infiltration. Our results indicate that soy PG when applied together with 1,25-dihydroxyvitamin D_3 (Vitamin D), an agent known to acutely disrupt the skin barrier, suppressed ear edema and inhibited the infiltration of CD45-positive immune cells. On the other hand, neither PG nor Vitamin D alone was effective. The combination also decreased tumor necrosis factor-alpha (TNFα) levels. This result suggested the possibility that PG was not permeating the skin barrier efficiently. Therefore, in a further study we applied PG in a penetration-enhancing vehicle, and found that it inhibited inflammation induced by the phorbol ester and decreased CD45-positive immune cell infiltration. Our results suggest the possibility of using soy PG as a topical treatment option for psoriasis.

INTRODUCTION

Keratinocytes undergo a precisely regulated pattern of proliferation and differentiation that is essential for proper formation of the epidermis as a physical and water-permeability barrier (Goldsmith 1991; Yuspa, et al. 1990). Defects in the regulation of this growth program result in an abnormal barrier and a variety of skin diseases, such as psoriasis (Langley 2005). Psoriasis is characterized by hyperproliferation and abnormal differentiation of epidermal keratinocytes, as well as inflammation, and results in a reduced quality of life similar to that observed in patients with life-threatening illness (Rapp, et al. 1999; Stern, et al. 2004).

Our previous studies have suggested that the lipid second messenger phosphatidylglycerol (PG) can be formed by a signaling module composed of the glycerol channel, aquaporin-3 (AQP3), and phospholipase D2 (PLD2) (Bollag, et al. 2007; Xie, et al. 2014; Zheng and Bollag 2003), which are colocalized in epidermal keratinocytes (Zheng and Bollag 2003). PLD is a lipid-metabolizing enzyme that can catalyze both phospholipid hydrolysis to produce phosphatidate and a transphosphatidylation reaction using primary alcohols to generate phosphatidylalcohols. We have previously found that both *in vitro* and in intact keratinocytes, PLD can convert glycerol, a physiological alcohol, to PG and that PG levels increase upon stimulation of keratinocytes with a differentiating agent, elevated extracellular calcium levels (Zheng, et al. 2003). PG production is maximal (Zheng et al. 2003) at a calcium concentration that is optimal for triggering early differentiation (Yuspa, et al. 1989), suggesting a potential role in this process. Furthermore, manipulating this novel AQP3/PLD2 signaling module alters keratinocyte differentiation (Bollag et al. 2007; Choudhary, et al. 2015). Importantly, egg PG inhibits proliferation of rapidly dividing keratinocytes whereas in slowly dividing cells egg PG

stimulates proliferation; however, the related phospholipid phosphatidylpropanol has no effect (Bollag et al. 2007). A subsequent study from our laboratory demonstrated that PG species possessing polyunsaturated fatty acids are effective at inhibiting keratinocyte proliferation; in contrast, PG species containing saturated or monounsaturated fatty acids stimulate the growth of slowly dividing keratinocytes (Xie et al. 2014). Soy PG, which contains a large percentage of polyunsaturated fatty acids, is particularly effective at inhibiting keratinocyte proliferation (Xie et al. 2014), suggesting its possible use as a treatment to suppress the keratinocyte hyperproliferation observed in psoriasis.

Psoriasis is also characterized by immune cell infiltration into the skin and inflammation, and it has been proposed that psoriasis is an immune-mediated skin disease (reviewed in (Helwa, et al. 2013)). However, recent data have suggested that there is a complex interplay between keratinocytes and immune cells, with keratinocytes producing cytokines that recruit and activate immune cells, which secrete cytokines that further stimulate keratinocytes, establishing a vicious cycle of inflammation (reviewed in (Brotas, et al. 2012; Lowes, et al. 2013; Sabat and Wolk 2011)). The importance of keratinocytes to skin lesion development can be observed in a transgenic mouse model in which c-Jun and JunB are deleted only in epidermal keratinocytes (under the control of the keratin 14 promoter). These mice exhibit psoriasiform lesions that persist in conditional double knockout mice lacking a fully functional immune system (also deficient in Rag2 or TNFR1), indicating that T cells, although important, are not the sole mediators of the observed skin phenotype (Zenz, et al. 2005).

PG is produced by alveolar cells as a significant component of pulmonary surfactant and has been shown to suppress inflammation induced by microorganisms and microbial products in the lung (Kuronuma, et al. 2009). Furthermore, PG inhibits infection of airway epithelial cells by

respiratory syncytial virus and influenza A *in vitro* and protects the lungs from the deleterious effects of these viruses *in vivo* (Numata, et al. 2010; Numata, et al. 2012). These results suggest the possibility that PG may possess not only anti-proliferative but also anti-inflammatory actions. To test whether PG is anti-inflammatory in skin, we used soy PG in a contact irritant mouse ear edema model, to determine whether PG can inhibit skin inflammation *in vivo*. Our results indicate that PG can inhibit inflammation *in vivo* and suggest the possibility that it might do so by suppressing TNFα levels in the skin.

MATERIALS AND METHODS

In Vivo Contact Irritant Ear Edema Mouse Model

Experiments were performed as described in (Clark, et al. 2013). Briefly, the ears of three to five male ICR CD-1 outbred mice [25-30 g; 5-6 weeks of age from Harlan Laboratories (Indianapolis, IN)] were treated with acetone or 12-O-tetradecanoylphorbol 13-acetate (TPA; 0.03% in acetone), and at 1h and 4h after TPA, the appropriate vehicle or the treatment in vehicle was applied to each ear. Ear thickness was measured using a caliper both before and at approximately 18-20 h after TPA treatment prior to sacrifice. After sacrifice, a circular ear punch biopsy (4 mm^2) was taken, weighed, and fixed in formalin. Histological evaluation included immunohistochemical staining for CD45 to quantify the number of infiltrating immune cells as well as for TNFα immunoreactivity (see below). All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Immunohistochemical and Immunofluorescent Staining

Sections (10 μm) were cut from formalin-fixed paraffin-embedded ear biopsies and deparaffinized and rehydrated as described in (Voss, et al. 2011). After antigen retrieval, inhibition of endogenous peroxidase with hydrogen peroxide, and blocking of non-specific antibody binding, sections were incubated with anti-CD45 antibody (BD Pharmingen, Franklin Lakes, NJ) or anti-TNFα antibody (Novus Biologicals, Littleton, CO) and visualized with diaminobenzidine (brown staining) with counterstaining using hematoxylin (blue staining in

figures). Alternatively, after staining with an anti-TNFα antibody (Abcam, Cambridge, MA) staining was visualized using a Cy3-conjugated secondary antibody. All staining, except for a portion (right ear immunoreactivity) of the analysis shown in Figure 6 for CD45-positive cells, was performed by Georgia Pathology Research Services (Augusta, GA) using standard protocols. Multiple random sections (4 to 8 per mouse separately from the left and right ear) were counted by two independent observers in a blinded fashion, counts averaged to determine a value for each mouse ear and values statistically analyzed as described below. TNFα immunofluorescence was determined using ImageJ analysis of random fields in 4 sections per mouse (from the left ear) and was quantified in terms of fluorescent intensity per area (in arbitrary units) in the demarcated epidermis.

Statistical Analyses

For all experiments illustrating cumulative data, values for each animal are shown as individual symbols, with a line indicating the mean value for each group. Values from the left and right ears of each mouse were averaged. Rank transformations of the data were used in order to stabilize variances and account for outlying observations. For the experiment testing the effect of PG and Vitamin D alone and in combination, a 2x2 ANOVA was performed and the interaction was tested. For the experiment with the penetration-enhancing vehicle, one-way ANOVA was used. Tukey's test was used to adjust for the post-hoc multiple comparisons of the mean ranks for significant effects from all analyses. SAS© 9.4 (SAS Institute Inc., Cary, NC) was used for all analyses and significance was determined using a Type I error of 5%.

RESULTS

Effects of soy PG, alone and in combination with 1,25-dihydroxyvitamin D_3, on TPA-induced ear edema

Our previous results (Bollag et al. 2007; Xie et al. 2014) suggested the possibility that PG, in particular soy PG, might be useful to inhibit keratinocyte proliferation in skin diseases like psoriasis characterized by hyperproliferation. However, there is no widely accepted animal model of psoriasis that reproduces all of the hallmarks of this disease (reviewed in (Danilenko 2008)). On the other hand, inflammation is a key aspect of psoriasis and can be induced by the application of the contact irritant, TPA, to mouse ears (e.g., (Fowler, et al. 2003; Sheu, et al. 2002; Sur, et al. 2008)). Therefore, we used the TPA-induced ear edema model to examine the ability of PG and 1,25-dihydroxyvitamin D_3 (Vitamin D) to decrease phorbol ester-elicited ear edema. Vitamin D was included because of concern about the potential ability of PG, which has a molecular weight of approximately 750 daltons, to penetrate the permeability barrier of the skin, which tends to exclude agents with molecular weights greater than 600 daltons (Pathan and Setty 2009): acute exposure to Vitamin D is known to disrupt the epidermal barrier (von Brenken, et al. 1997). In addition, Vitamin D has been used successfully to treat psoriasis in humans (reviewed in (Samarasekera, et al. 2013)). After measuring the initial thickness, mouse ears were treated with TPA in acetone (0.03% weight:volume) or acetone alone at time zero; 1h and 4h later ice-cold 95% ethanol/5% water as vehicle or this vehicle containing soy PG, vitamin D or the combination was applied to each ear. Approximately 20h later, the mice were sacrificed, ear thickness measured and a 4 mm punch biopsy taken, weighed and fixed in formalin.

Shown in Figure 1A is the change in ear thickness (from time zero) in ears treated with the indicated agents. TPA clearly increased ear thickness relative to the untreated control by

more than 0.3 mm. (Please note that there was no change in ear thickness in the untreated control, demonstrating the reproducibility of the measurement.) Neither soy PG alone nor Vitamin D alone had any statistically significant effect on the increase in ear thickness induced by TPA. However, the combination of soy PG and vitamin D significantly reduced (by approximately 40 percent) the change in ear thickness observed upon TPA exposure. Similar effects were observed in terms of the weights of the 4 mm punch biopsies, with TPA inducing a significant increase of almost 5 mg and the combination of PG and vitamin D suppressing the TPA-induced change in ear weight by about 50% (Figure 1B).

We also performed immunohistochemistry on the ear biopsies, staining for CD45 (or leukocyte common antigen), which is specifically expressed by hematopaeotic cells other than erythrocytes and plasma cells and is thus a marker of immune cells. This procedure allows the determination of the effect of treatment on immune cell infiltration into the ear. Random fields from multiple sections were then photographed and the number of CD45-positive cells counted in a blinded fashion. As shown in Figure 2, TPA treatment significantly increased the number of immune cells infiltrating into the treated ear approximately 5-fold, and PG alone slightly but significantly reduced this increase (by about 25%). Vitamin D alone had no significant effect on the number of CD45-positive infiltrating immune cells, but the combination produced a statistically significant inhibition of about 90% in the number of CD45-positive immune cells infiltrating into the ear in response to TPA, returning this parameter to a value that was not statistically different from the control.

Finally, TNFα immunoreactivity in the skin was examined by immunohistochemistry. We elected to focus on this particular cytokine based on the ability of anti-TNFα reagents to successfully treat psoriasis (Brotas et al. 2012). As shown in Figure 3, TPA induced a striking

increase in TNFα staining that was not dramatically affected by either PG or Vitamin D alone. However, treatment with both PG and Vitamin D greatly reduced TNFα staining, returning it to a level not substantially different from the control.

Immunohistochemistry is difficult to quantify; therefore, we performed further quantitative immunofluorescence analysis of TNFα levels in additional sections from each mouse. Fluorescent staining of the epidermis was quantified in 4 sections per mouse and the results presented in Figure 4, with each symbol representing an individual mouse and the lines showing the mean values. TPA caused an approximate doubling of TNFα in the epidermis, with no significant effect of PG observed. On the other hand, Vitamin D induced a significant decrease in TNFα immunofluorescence that was not further affected by the concomitant addition of PG, returning both groups to a value that was not significantly different from the control. The disparity observed between Figures 3 and 4 in terms of effects of Vitamin D alone likely reflects the different techniques used for visualization; thus, enzyme (horseradish peroxidase)-based immunohistochemistry amplifies the signal and may accentuate low-intensity staining relative to immunofluorescence.

Effects of different concentrations of soy PG in a penetration-enhancing vehicle on TPA-induced ear edema

Although Vitamin D has been used successfully as a therapy for psoriasis, as noted above, the acute effect of Vitamin D treatment on mouse skin is a disruption of the epidermal permeability barrier (von Brenken et al. 1997). The ability of vitamin D, a barrier disruptor, to manifest an anti-inflammatory effect of PG, in the absence of its own effects on inflammation, suggested that permeation of the lipid through the skin could be an issue. We, therefore,

hypothesized that application of PG in a vehicle that enhances permeability might improve the skin response to PG alone. In consultation with Avanti Polar Lipids, we selected a vehicle for topical application composed of triacylglycerol, in particular, trioctanoin (8:0, 8:0, 8:0) and magnesium-stearate (18:0) at a ratio that yields a cream with a consistency suitable for easy application. Again, we first measured the thickness of both ears of each mouse with a digital caliper (at time zero) followed by application of 0.03% TPA (in acetone) to both ears. We then applied vehicle or vehicle containing 0.02% or 0.2% soy PG at 1h and 4h after TPA exposure.

At 18h after the TPA application, each mouse ear was again measured with the digital caliper and the mice were sacrificed. Two punch biopsies were taken from each ear to measure weight and for immunohistochemical analysis. Because many vehicles themselves are known to exert effects in skin (reviewed in (Surber and Smith 2005)), an additional set of mice received TPA and a sham treatment (their ears were rubbed without applying vehicle or PG), providing a sham control to determine potential effects of the vehicle. A final set of mice received neither TPA stimulation nor treatment of any kind, providing a null control for TPA exposure and ear weight. Values were then calculated as the change in ear thickness (relative to time zero) or weight (relative to an average weight obtained from the ears of the null controls).

Similar to its effect in the previous experiment, TPA with the sham treatment resulted in an increase in ear thickness of approximately 0.3 mm; however, in comparison vehicle treatment significantly reduced (by about 70%) the TPA-induced increase in ear thickness (Figure 5). Nevertheless, as illustrated in Figure 5A, soy PG at both 0.02% and 0.2% applied topically in the trioctanoin vehicle was able to further reduce ear thickness by approximately 65% relative to the vehicle alone (n = 4-5 animals), with the low-dose PG appearing to be as effective as the higher dose in inhibiting inflammation. Again, there was essentially no change in ear thickness in the

sham control. Similar results were obtained in terms of a reduction in ear weight (Figure 5B), with the two PG doses returning the weight measure to a value that was not statistically different from the control. Although the change in weight values for the two PG doses were not a statistically different from the vehicle alone, this could be the result of the fact that each mouse could not be biopsied at time zero to serve as its own control for this measurement.

Immunohistochemical analysis using an antibody to CD45 was also conducted, as discussed above. The results are shown in Figure 6 and indicate that TPA induced a statistically significant increase in the number of CD45-positive cells relative to unexposed ears. Treatment with vehicle did not significantly reduce the number of CD45-positive immune cells infiltrating into the skin induced by TPA. However, PG treatment (analyzing the two concentrations of PG combined) resulted in a significant reduction in the number of infiltrating CD45-positive cells.

We also performed immunohistochemistry for TNFα in the ear biopsies of the treated mice. As shown in Figure 7, TNFα staining was increased in the ears exposed to TPA alone (Panel B), as compared to the control ears (Panel A), and vehicle alone reduced TNFα immunoreactivity (Panel C). There may have been a small additional effect of the vehicle containing the lower concentration of soy PG (Panel D), although the higher PG dose did not appear to decrease TNFα staining any more than the vehicle alone (Panel E).

DISCUSSION

Our previous study provided evidence for the existence in primary mouse keratinocytes of a novel lipid signaling pathway, for which PG is a key effector in the regulation of keratinocyte proliferation and differentiation. In particular, we showed that egg PG inhibits keratinocyte proliferation in rapidly dividing keratinocytes and stimulates keratinocyte proliferation in slowly dividing keratinocytes (Bollag et al. 2007). A further study suggested that PG species with different fatty acid compositions can exert different effects in keratinocytes (Xie et al. 2014). It is perhaps not surprising that PG species with different acyl groups have different signaling functions since in the lung PG species possessing saturated fatty acids cannot block the anti-inflammatory effects of surfactant protein A on lipopolysaccharide-treated macrophages while PG containing unsaturated fatty acids can (Chiba, et al. 2006). Thus, our previous studies suggested that PG might be an ideal treatment to normalize skin function, with different PG species for different skin conditions.

Although in a previous study synthetic polyunsaturated fatty acid-containing PGs, and in particular dilinoleoylphosphatidylglycerol, seemed most effective at inhibiting keratinocyte proliferation *in vitro* (Xie et al. 2014), the expense of these PGs could potentially preclude their use as a treatment for psoriasis. Therefore, in this study we investigated the ability of soy PG, a mixture of PG species containing a high proportion of polyunsaturated fatty acids, as a potential treatment for psoriasis and the inflammation that accompanies this disease using a mouse model that mimics the inflammatory aspect of the disease. Soy PG also has the advantage of being a natural product. When soy PG was applied in an ethanol/water vehicle, the lipid showed essentially no effect on ear edema (induced by TPA) by itself. However, in combination with Vitamin D, which alone also had no effect, soy PG inhibited the TPA-induced ear edema

response. This result suggests the possibility of using soy PG in conjunction with Vitamin D, analogs of which are in current clinical use to treat psoriasis (reviewed in (Samarasekera et al. 2013)), to more effectively control skin inflammation. On the other hand, because Vitamin D can disturb the epidermal barrier to allow better permeation of exogenous substances into mouse skin (von Brenken et al. 1997), this result suggested the possibility that soy PG alone was ineffective in penetrating the epidermis to exert its anti-inflammatory effects but was able to do so when allowed entry through the Vitamin D–disrupted barrier. Indeed, when applied in a penetration-enhancing cream vehicle, soy PG alone was able to inhibit TPA-induced ear edema. On the other hand, it should be noted that in this experiment the vehicle alone was able to reduce inflammation and TNFα immunoreactivity. Although the mechanism underlying the activity is unclear, an ability of vehicles to exert effects in the skin is often observed (Surber and Smith 2005). For example, petrolatum can improve psoriasis (Limaye and Weightman 1997); it is thought to do so through its ability to occlude the skin and reduce trans-epidermal water loss. Barrier disruption itself is reported to increase cytokine release (e.g., (Wood, et al. 1992)) and contribute to psoriasis in mouse models (Nakajima, et al. 2013)(Roelandt, et al. 2009) and patients (Roberson and Bowcock 2010). Alternatively, the lipid-based vehicle may sequester the hydrophobic TPA and prevent its effective penetration and action in the skin.

TPA induces the expression of several inflammatory cytokines in keratinocytes (e.g., (Carlsson, et al. 2005); Helwa, et al. 2015) and skin ((Gebhardt, et al. 2002) and reviewed in (Mueller 2006)). These cytokines include TNFα, which is known to be elevated in psoriasis; in fact, drugs targeting the TNFα pathway are effective in the treatment of psoriasis (reviewed in (Brotas et al. 2012)). Indeed, our results showed that combined treatment with PG and Vitamin D inhibited the TPA-induced increase in TNFα immunoreactivity in the skin. In separate

immunofluorescent TNFα staining, quantitation showed a significant inhibition by Vitamin D alone. This result suggests that the effects of the combined treatment on TNFα alone are not sufficient to explain the synergistic reduction in ear inflammation seen. Thus, in addition to inhibiting inflammation (PG) or promoting barrier disruption and reducing TNFα levels (Vitamin D), these agents may have other actions that contribute to their synergistic effects in the ear edema model. Some cytokines such as TNFα are thought to originate from immune cells in psoriasis lesions; whereas, others, such as interleukin-1α, are thought to arise mainly from the keratinocytes (Brotas et al. 2012). Thus, it is unlikely that keratinocytes are the only, or perhaps even the primary source of TNFα. On the other hand, as shown in Figure 2, TPA induces immune cell infiltration that is not completely inhibited by either PG or Vitamin D alone. Since this agent is also known to activate macrophages, among other immune cells, it seems likely that multiple cells contribute to the elevation in TNFα levels in this model. Thus, it seems possible that cytokines produced either by immune cells or by keratinocytes can recruit and activate additional immune cells, thereby helping to initiate and/or maintain a cytokine network of inflammation and promote the development of psoriatic skin lesions. In this regard, the ability of 1,25-dihydroxyvitamin D_3 to inhibit immune cell activation [reviewed in (Reichrath, et al. 2007)] may help to explain the observed inhibitory effect on epidermal TNFα levels (Figure 4).

In these experiments, we used male mice to demonstrate anti-inflammatory effects of soy PG. There is no apparent sex difference in the incidence psoriasis, although the severity of the disease is greater in men [e.g., (Hagg, et al. 2017)], which is the reason for our initial decision to focus on males. However, it is critical that these anti-inflammatory effects of PG be investigated in females as well. Nevertheless, our experiments serve as proof-of-principle for the feasibility of potentially using PG as a therapy to treat psoriasis. Thus, based on the positive effects observed

in these experiments, we have elected to examine effects of PG in both sexes in a model that more accurately mimics the psoriatic phenotype rather than this acute inflammation contact irritant model. These experiments are currently in progress.

Data from Voelker and colleagues have demonstrated an ability of palmitoyl-oleoylphosphatidylglycerol (POPG) to inhibit inflammation in alveolar cells and the lung. Indeed, POPG effectively reduces microbial product-induced arachidonic acid release from human and mouse macrophages treated with *Mycoplasma pneumonia* membrane, without inhibiting cell surface binding of *Mycoplasma* (Kandasamy, et al. 2011). These authors have also shown that dioleoylphosphatidylglycerol (DOPG) can inhibit IL8 production in BEAS2B human bronchial epithelial cells (Numata, et al. 2013). In line with this result, Wu et al. (Wu, et al. 2003) also showed that DOPG effectively inhibits endotoxin-stimulated Type IIA secretory phospholipase A_2 levels and activity via reductions in the activation of nuclear factor-κB in macrophages. Our results show that soy PG also exerts anti-inflammatory effects in the skin.

In conclusion, the present study shows for the first time to our knowledge that soy PG was able to suppress inflammation in response to a contact irritant (TPA) in an *in vivo* ear edema mouse model. This action may be related, at least in part, to the ability of soy PG to decrease the recruitment of immune cells, the infiltration of which is markedly increased in the inflammatory skin disease psoriasis. Thus, our results suggest that PG may be useful for treating skin diseases like psoriasis that are characterized by excessive keratinocyte proliferation and/or inflammation.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Xie, Edwards and W. Bollag.

Conducted experiments: Xie and Seremwe.

Performed data analysis: Xie, Choudhary, Wang, Emmons, K. Bollag, Johnson and W. Bollag.

Wrote or contributed to the writing of the manuscript: Xie, Choudhary and W. Bollag.

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FIGURE LEGENDS

FIGURE 1. **The combination of soy PG and Vitamin D inhibited TPA-induced ear edema.** (A) The combination of soy PG and Vitamin D suppresses the increase in ear thickness observed with TPA treatment. Ear thickness was measured with a digital caliper prior to treatment of both ears with 0.03% TPA in acetone (or acetone alone). 1h and 4h after the TPA application, vehicle (95% ethanol/5% water) or PG or Vitamin D (VitD) or the combination in vehicle was applied to the ears and 20h later ear thickness was measured again. The change in ear thickness was calculated as follows: the thickness at the end of the experiment minus the thickness of the same ear measured at time zero. (B) After TPA and treatment applications as described above, mice were sacrificed and a 4 mm punch biopsy was harvested from each ear and weighed. The change in ear weight was calculated as the weight measured at sacrifice minus the average weight of the ear biopsies from the untreated controls. Individual symbols represent individual mice with the line representing the mean value of the group of 4-5 mice; groups marked by the same letter are not, whereas those marked with different letters are, statistically significantly different ($p \leq 0.05$). A 2x2 ANOVA was performed and determined a significant interaction between PG and Vitamin D for both change in thickness ($p=0.0021$) and change in weight ($p=0.039$).

FIGURE 2. **The combination of soy PG and Vitamin D suppressed TPA-induced immune cell infiltration into the ear.** Multiple sections were cut from formalin-fixed, paraffin-embedded ear biopsies obtained from mice treated as in Figure 1 and stained for CD45 as described in Materials and Methods. Shown in panels A through E are representative sections from (A) control, (B) TPA-treated, (C) TPA- and PG-treated, (D) TPA- and Vitamin D (VitD)-treated and (E) TPA-, PG- and Vitamin D-treated mice. (F) The number of CD45-positive cells was

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determined by counting immunostained (brown) cells in at least five random fields from a minimum of two sections from the left and right ear biopsies of treated mice. Cells were counted in a blinded manner by at least two independent observers and the counts were averaged for each mouse. Mean values obtained from these counts for each mouse in a given treatment group were averaged and compared statistically as in Figure 1. Individual symbols represent individual mice with the line representing the mean value of the group of 4-5 mice; groups marked by the same letter are not, whereas those marked with different letters are, statistically significantly different ($p \leq 0.05$). The effect of each agent was found to be additive with a significant effect for both PG ($p=0.0001$) and VitD ($p=0.012$).

FIGURE 3. **The combination of soy PG and 1,25-dihydroxyvitamin D_3 suppressed TPA-induced TNFα levels.** Multiple sections were cut from formalin-fixed, paraffin-embedded ear biopsies obtained from mice treated as in Figure 1 and stained for TNFα as described in Materials and Methods, with (A) control, (B) TPA, (C) TPA + PG, (D) TPA + Vitamin D, (E) TPA + PG + Vitamin D, (F) positive control (tonsil tissue) and (G) negative control (no primary antibody included). Results are representative of sections obtained from 4-5 mice; scale bars represent 20 μm.

FIGURE 4. **TPA induced a significant increase in TNFα levels, which were significantly reduced by 1,25-dihydroxyvitamin D_3.** Additional sections were cut from formalin-fixed, paraffin-embedded ear biopsies obtained from mice treated as in Figure 3, stained for TNFα and immunofluorescence levels quantified using ImageJ as described in Materials and Methods. Results are representative of sections obtained from 4-5 mice. Individual symbols represent

individual mice with the line representing the mean value of the group of 4-5 mice. A significant additive effect of Vitamin D was observed ($p=0.016$); groups marked by the same letter are not, whereas those marked with different letters are, statistically significantly different ($p\leq0.05$).

FIGURE 5. **Soy PG in a penetration-enhancing vehicle suppressed TPA-induced ear edema.** **(**A) Ear thickness and weight were measured with a digital caliper prior to application of TPA in acetone (time zero) to both surfaces (inner and outer) of the ear. Vehicle with or without the indicated amounts of soy PG was applied to the ears 1h and 4h after TPA treatment. Ear thickness was determined again approximately 18h after the initial exposure to TPA, and the change in thickness calculated as described in the legend for Figure 1. (B) For the weight measurements punch biopsies were taken from each ear and weighed. Some mice received no TPA and the average weight of the ear punch biopsies of these mice was used for calculations as the time zero value. Individual symbols represent individual mice with the line representing the mean value of the group of 3-5 mice; groups marked by the same letter are not, whereas those marked with different letters are, statistically significantly different ($p\leq0.05$).

FIGURE 6. **Soy PG in a penetration-enhancing vehicle suppressed TPA-induced immune cell infiltration into the ear.** Multiple sections were cut from formalin-fixed, paraffin-embedded ear biopsies obtained from mice treated as in Figure 5 and stained for CD45 as described in Materials and Methods. Shown in panels A through E are representative sections from (A) control, (B) TPA-treated, (C) TPA- and vehicle-treated, (D) TPA- and 0.02% PG-treated and (E) TPA- and 0.2% PG-treated mice. (F) The number of CD45-positive cells were determined by counting immunostained (brown) cells in at least five random fields from a minimum of two

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sections from the left and right ear biopsies of treated mice as in Figure 5. Cells were counted in a blinded manner by at least two observers and the counts were averaged for each mouse. Individual symbols represent individual mice with the line representing the mean value of the group of 3-5 mice; groups marked by the same letter are not, whereas those marked with different letters are, statistically significantly different ($p \leq 0.05$).

FIGURE 7. **Vehicle alone and vehicle containing soy PG suppressed TPA-induced TNFα levels.** Multiple sections were cut from formalin-fixed, paraffin-embedded ear biopsies obtained from mice treated as in Figure 5 and stained for TNFα as described in Materials and Methods, with (A) control, (B) TPA, (C) TPA + vehicle, (D) TPA + vehicle containing 0.02% soy PG and (E) TPA + vehicle containing 0.2% soy PG. Results are representative of sections obtained from 3-5 mice; scale bars represent 20 μm.

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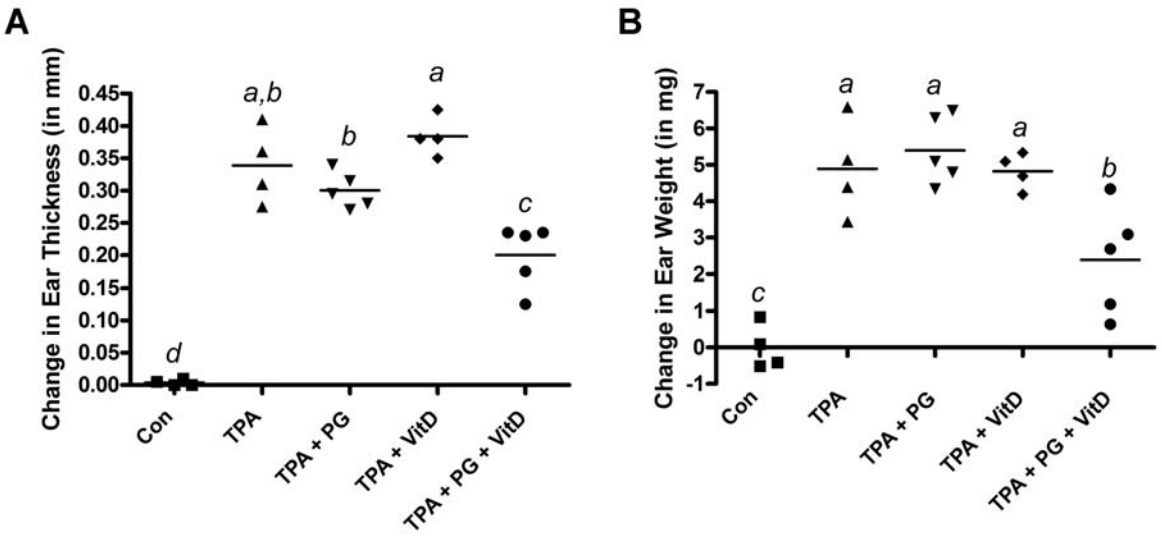

Figure 1

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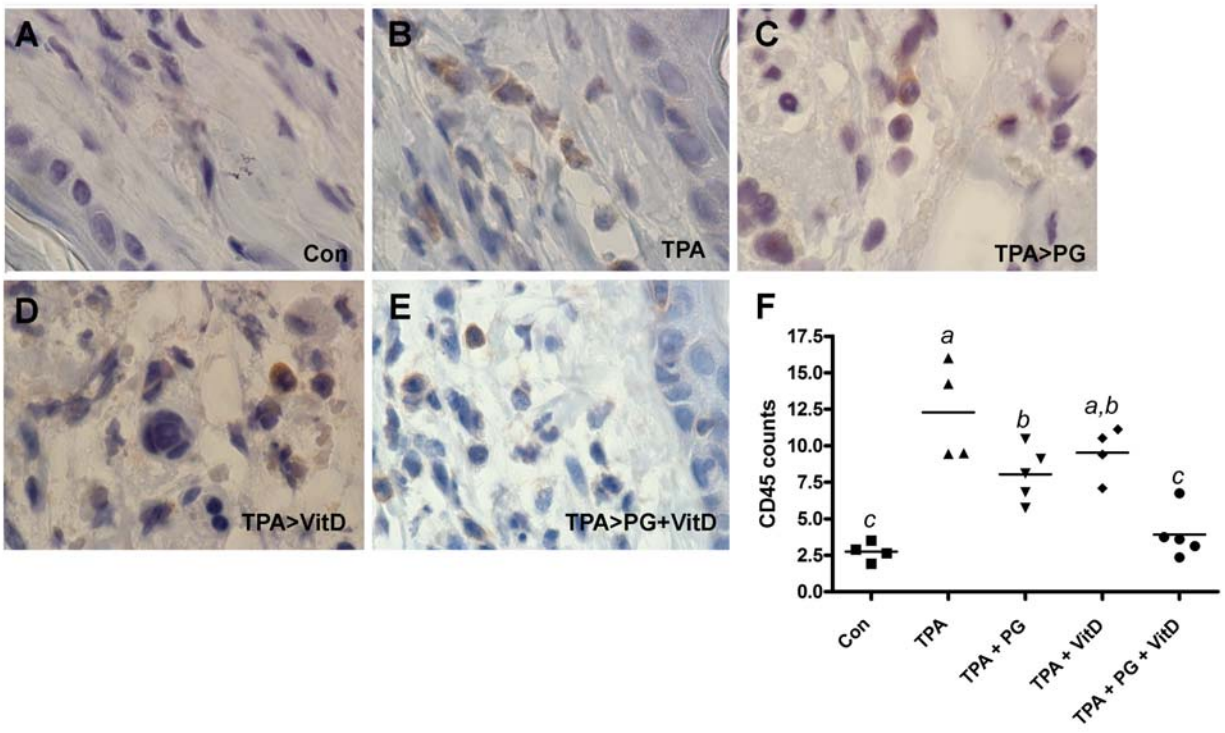

Figure 2

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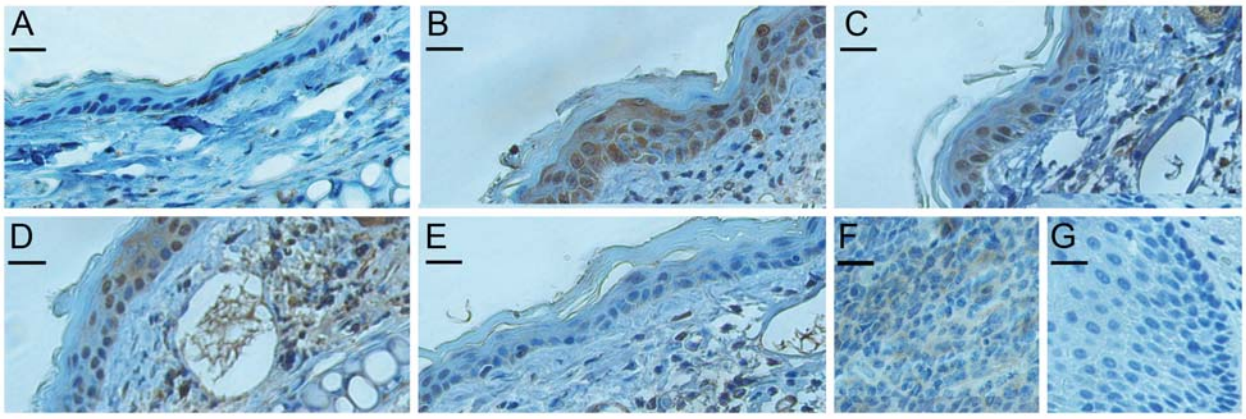

Figure 3

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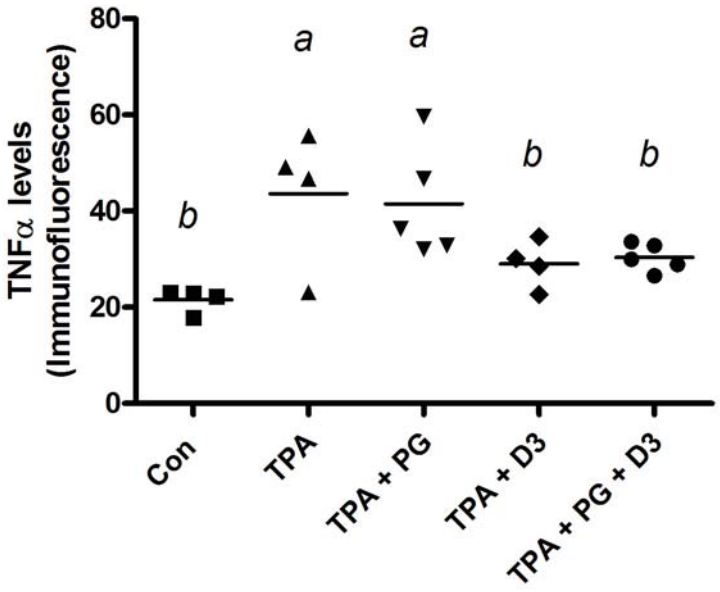

Figure 4

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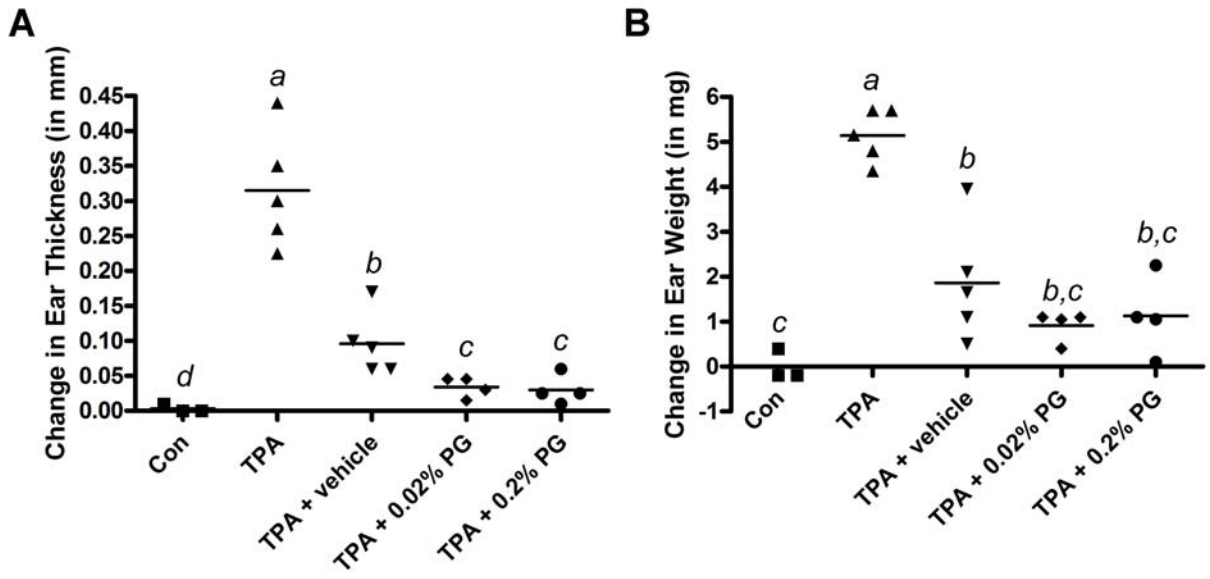

Figure 5

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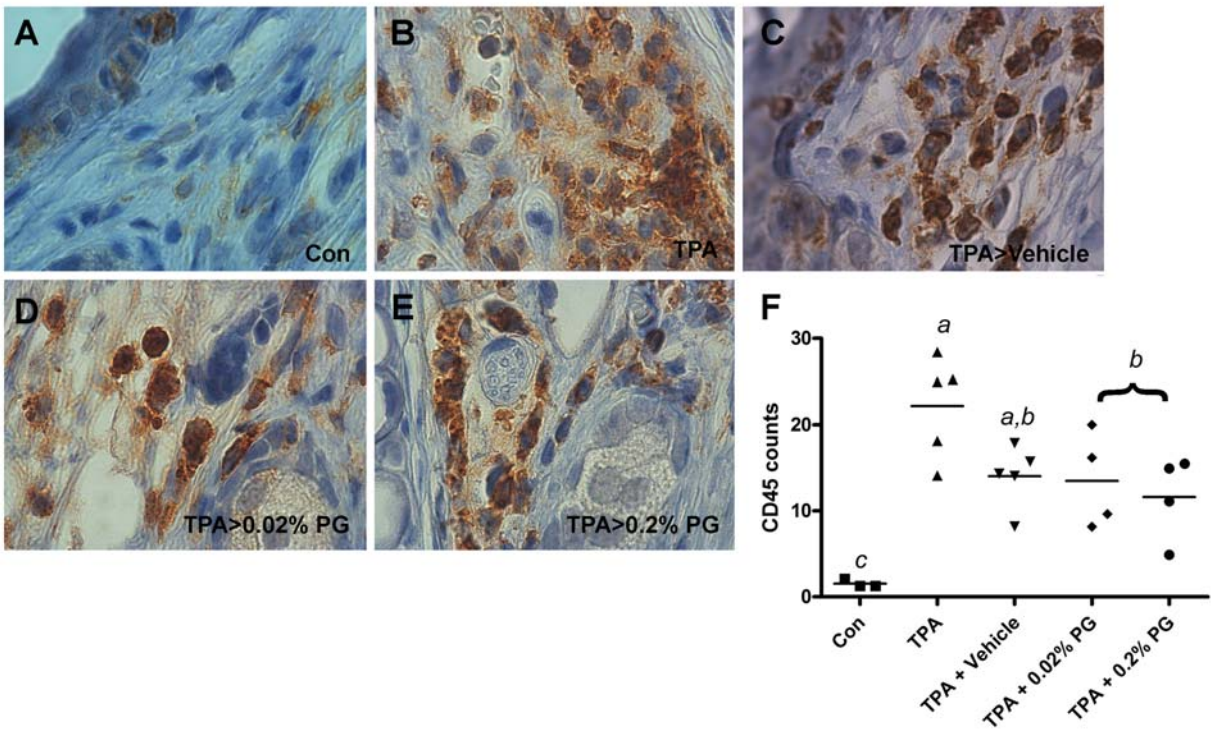

Figure 6

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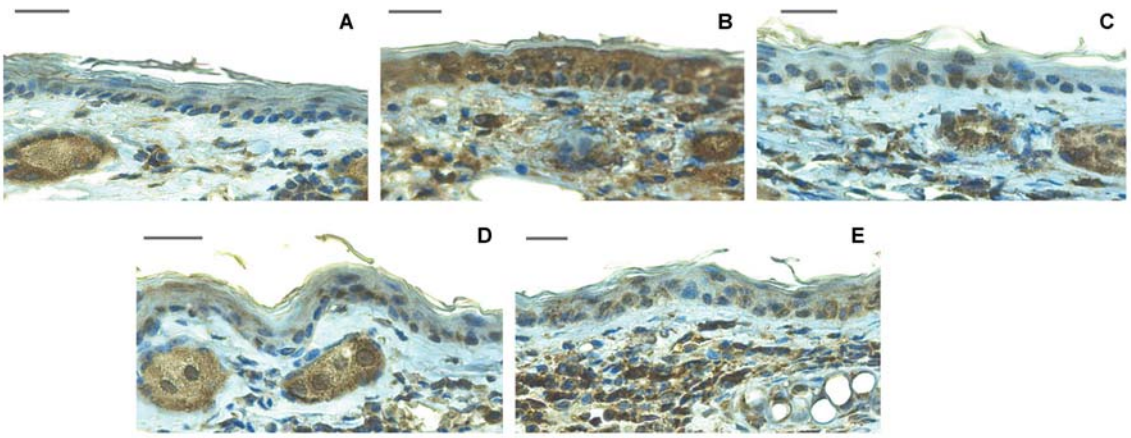

Figure 7