Propionibacterium acnes-Derived Extracellular Vesicles Promote Acne-Like Phenotypes in Human Epidermis

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Acne vulgaris is an inflammatory disease occurring in the pilosebaceous unit and is the most common skin condition in young people. A gram-positive bacterium, *Propionibacterium acnes*, has been suspected to contribute to the development of acne. Here, we report that *P. acnes* constitutively releases extracellular vesicles (EVs) exhibiting typical EV morphology and size. Moreover, the *P. acnes*-derived EVs (PEVs) can induce acne-like phenotypes in human epidermal keratinocytes and a reconstituted human skin model. PEVs significantly induced inflammatory cytokines IL-8 and GM-CSF and dysregulated epidermal differentiation by increasing proliferating keratinocytes and decreasing epidermal keratin 10 and desmocollin 1 levels. PEVs showed strong effects, evoking these responses at earlier time points compared with *P. acnes* extract at the same protein concentration. We verified that PEVs were internalized via clathrin-dependent endocytosis into keratinocytes and that PEV-induced cellular responses occurred via Toll-like receptor 2-dependent signal cascades. Furthermore, PEVs showed a stronger effect than keratinocytes in inducing inflammatory cytokines in myeloid cells. Collectively, our study suggests that PEVs induce acne-like phenotypes in a unique way; therefore, inhibiting the release of EVs from *P. acnes* or targeting PEV-mediated signaling pathways could represent an alternative method for alleviating acne occurrence and phenotypes.

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INTRODUCTION

Acne vulgaris (simply acne) is a chronic inflammatory disease occurring in the pilosebaceous unit that initially begins with microcomedones, which develop into comedones and inflamed papules and pustules (Bhate and Williams, 2013; Gollnick et al., 2003). The resulting appearance, that is, inflammatory acne accompanied by scarring and hyperpigmentation, can affect quality of life, especially in young people, more than 85% of whom are affected by acne (Dunn et al., 2011; James, 2005). Based on family and twin studies, substantial heritable genetic factors contribute to acne susceptibility and determine its occurrence (Bataille et al., 2002; Evans et al., 2005; Williams et al., 2012). Recently, genetic loci associated with severe acne and linked genes were identified through genome-wide association studies (He et al., 2014; Navarini et al., 2014). In addition to a genetic cause, various factors such as ethnicity, hormones, diet, sunlight, smoking, stress, obesity, and infection are thought to be related to acne pathogenesis. Among these, the bacterium *Propionibacterium acnes* is detected in 68–79% of inflamed acne lesions (Leeming et al., 1988) and has been widely suspected to contribute to the development of acne by inducing inflammatory events (Bhate and Williams, 2013; Jeremy et al., 2003; Kistowska et al., 2014; Tanghetti, 2013). The predominant colonization of the sebaceous follicles with certain types of *P. acnes* is associated with moderate-to-severe inflammation in acne lesions (Higaki et al., 2000; McDowell et al., 2005), and through the increased proliferation of follicular keratinocytes, hyperkeratinization can create a preferable anaerobic environment for the colonization of *P. acnes* (Dessinioti and Katsambas, 2010; Hughes et al., 1996; Isard et al., 2011; Knaggs et al., 1994; Vowels et al., 1995). Although *P. acnes* is predicted to play roles in acne pathogenesis, the exact mechanism of action at the molecular level has not been clarified.

Bacteria secrete diverse factors to communicate with or evoke cellular responses from target cells. Like mammalian cells, in addition to soluble factors, most gram-negative and gram-positive bacteria release extracellular vesicles (EVs) known as outer membrane vesicles and membrane vesicles (Kim et al., 2015a; Lee et al., 2008, 2009; Mashburn-Warren and Whiteley, 2006). EVs are lipid bilayer-enclosed spherical

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Abbreviations: DSC1, desmocollin 1; EV, extracellular vesicle; FLG, filaggrin; KRT10, keratin 10; MAPK, mitogen-activated protein kinase; NHEK, normal human epidermal keratinocyte; PBMC, peripheral blood mononuclear cell; PEV, P. acnes-derived EV; siRNA, small interfering RNA; TLR, Toll-like receptor

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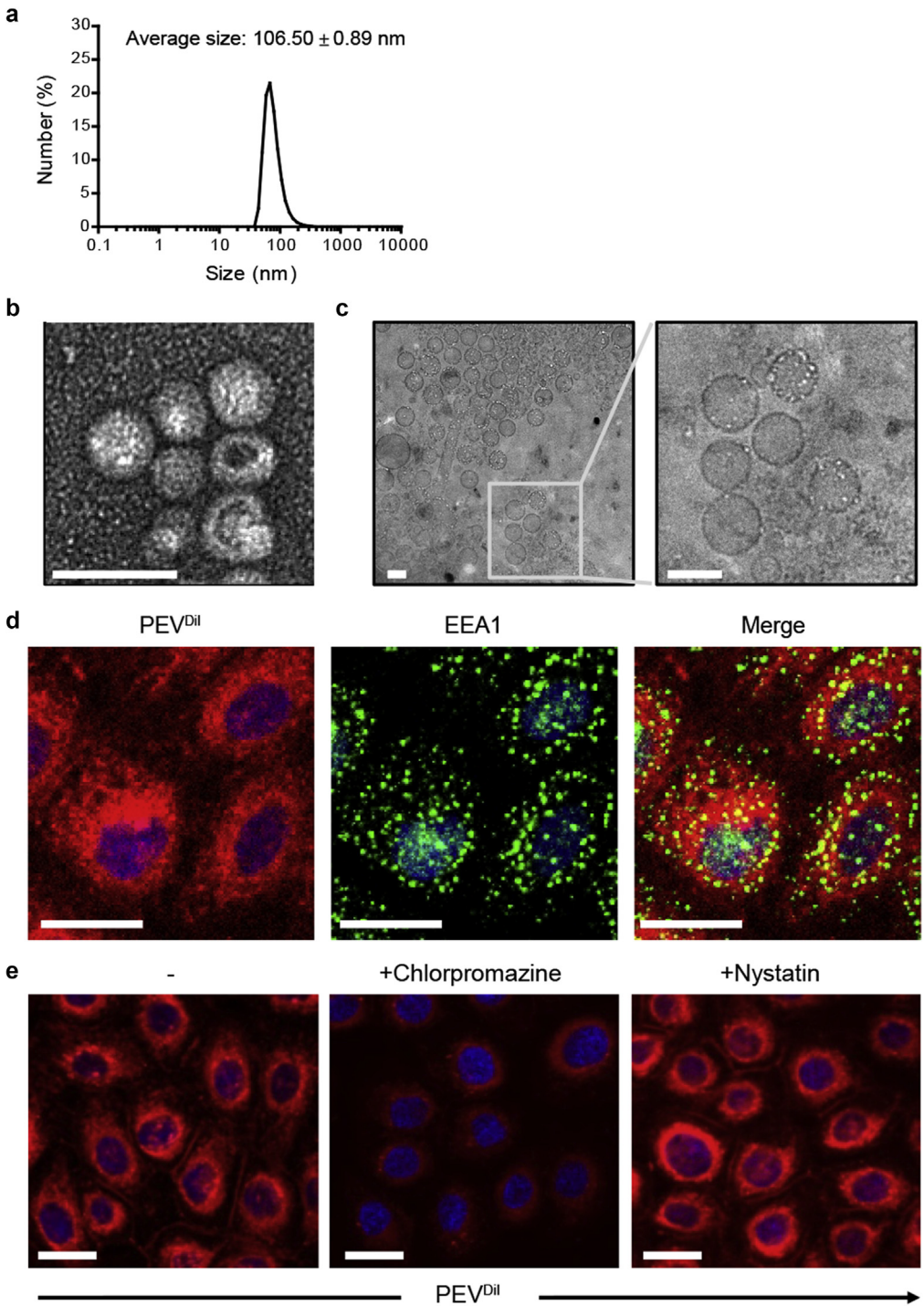

Figure 1. The gram-positive bacterium *P. acnes* secretes EVs. (**a**) PEVs were analyzed with dynamic light scattering. (**b**) Representative TEM image of PEVs. Scale bar, 50 nm. (**c**) Cryo-TEM image of PEVs. The boxed area is magnified. Scale bars, 100 nm. (**d**) Isolated PEVs were labeled with DiI (PEV^{DiI}, red) and used to treat NHEKs for 1 hour. Cells were stained with early endosome antigen 1 (EEA1; green) and observed under confocal microscopy. Nuclei were stained with DAPI (blue). Scale bars, 20 μm. (**e**) DiI-labeled PEVs were used to treat NHEKs supplemented with vehicle (−), 10 μg/ml chlorpromazine, or 10 μM nystatin for 6 hours. The DiI signal (red) was observed under confocal microscopy. Scale bars, 20 μm. EEA1, early endosome antigen 1; EV, extracellular vesicle; NHEK, normal human epidermal keratinocyte; PEV, *P. acnes*-derived EV; TEM, transmission electron microscopy.

vesicles approximately 30−1,000 nm in diameter that are constitutively or actively produced by most archaea, prokaryotes, and eukaryotes, indicating that EV release is an evolutionarily conserved process (Colombo et al., 2014; Kourembanas, 2015; Yanez-Mo et al., 2015). By harboring diverse proteins, lipids, nucleic acids, and metabolites originating from the parent cells, EVs transfer biologically active molecules to neighboring and distant cells for communication and influence (Brown et al., 2015). In particular, bacterial EVs are known to mediate pathophysiological functions in bacteria-bacteria and bacteria-host interactions by delivering toxins, inducing cellular inflammation, and evoking host cell death (Gurung et al., 2011; Hong et al., 2011; Kim et al., 2012; Rivera et al., 2010), leading to the occurrence and propagation of diseases such as atopic dermatitis, even in the absence of live bacterial cells (Hong et al., 2011; Kim et al., 2015b).

To the best of our knowledge, this is a previously unreported study demonstrating that *P. acnes* constitutively produces EVs showing typical morphology and size that are involved in acne pathogenesis. We found that *P. acnes*-derived EVs (PEVs) induce acne-like phenotypes such as

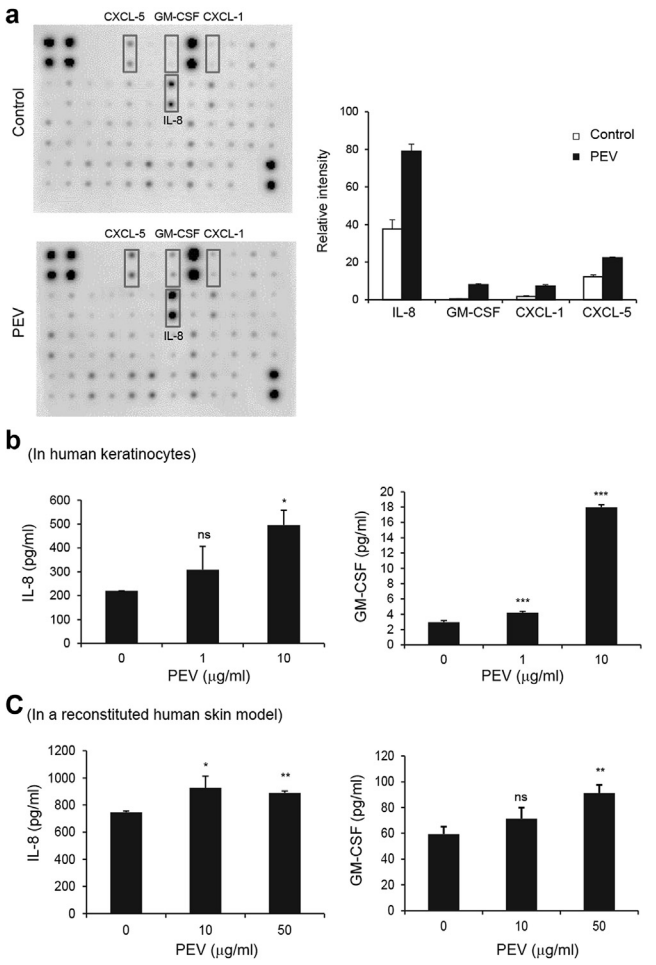

Figure 2. PEVs upregulate the expression of proinflammatory cytokines. (**a**) NHEKs were treated with vehicle or 10 μg/ml PEVs for 24 hours, and culture supernatants were harvested for cytokine array analysis (left). Representative altered spots were analyzed by densitometric analysis (right). Data are presented as mean ± SD of the intensities of spots from two independent experiments. (**b**) Secreted protein levels of IL-8 and GM-CSF were assessed in culture supernatants of PEV-treated NHEKs using ELISA. (**c**) A reconstituted human skin model was treated with indicated concentrations of PEVs every other day for 6 days. Culture supernatants were collected and analyzed using ELISA for IL-8 or GM-CSF. The data in (**b**) and (**c**) are presented as mean ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). NHEK, normal human epidermal keratinocyte; PEV, *Propionibacterium acnes*-derived extracellular vesicle; SD, standard deviation.

increased secretion of inflammatory cytokines and dysregulated epidermal differentiation in human keratinocytes and in a reconstituted human skin model. We also verified that PEVs evoke these responses via Toll-like receptor 2 (TLR2)-mediated signaling pathways and are more effective than keratinocytes at inducing inflammatory cytokines in myeloid lineage cells.

RESULTS

Gram-positive *P. acnes* secretes EVs

As a gram-positive anaerobic bacterium, *P. acnes* composes part of the commensal skin microbiota but is also considered one of the main causes of acne. As previously reported, gram-positive and gram-negative bacteria release EVs into the extracellular environment and evoke cellular responses (Brown et al., 2015). We examined whether *P. acnes* secretes EVs and whether these PEVs are involved in the development of acne vulgaris. According to a previously described isolation method for *Staphylococcus aureus*-derived EVs (Lee et al., 2009), we successfully isolated EVs from *P. acnes* culture supernatant. Based on dynamic light scattering analysis, the size of the PEVs was in the range of 30–200 nm with an average diameter of 106.50 ± 0.89 nm (Figure 1a). Morphological assessments revealed a spherical, bilayered, and closed membrane structure according to transmission electron microscopy and cryo-transmission electron microscopy image analyses (Figure 1b and c). These results indicate that *P. acnes* spontaneously releases EVs exhibiting similar morphology and size to previously described bacteria-derived EVs in the extracellular environment.

PEVs are internalized via clathrin-dependent endocytosis in human epidermal keratinocytes

To examine whether PEVs can be internalized into normal human epidermal keratinocytes (NHEKs), we labeled PEVs with DiI, a fluorescent dye incorporated into membranes, and traced the intracellular localization of PEVs by costaining with early endosome antigen 1, an early endosome marker. At 1 hour after treatment, most early endosome antigen 1-positive endosomes were colocalized with DiI-labeled PEVs, and the majority of PEVs were located in the perinuclear area (Figure 1d), indicating that PEVs were clearly endocytosed into NHEKs. To determine the endocytic mechanism, we treated NHEKs with PEVs in the presence of a clathrin-dependent (chlorpromazine) or caveolae-dependent (nystatin) endocytosis inhibitor (Tammi et al., 2001), and examined the degree of PEV endocytosis. Chlorpromazine markedly inhibited the internalization of PEVs into NHEKs when compared with internalization in nontreated or nystatin-treated cells (Figure 1e), suggesting that the entry of PEVs into keratinocytes is mediated by clathrin-dependent endocytosis and that the internal cargo of PEVs can be delivered into host cells.

PEVs increase the expression of proinflammatory cytokines in human epidermis

On the basis of earlier reports that inflammation is a fundamental process throughout the development of acne lesions and that *P. acnes* is considered a main causative inflammatory agent, we hypothesized that PEVs may evoke inflammatory responses in similar or different ways to *P. acnes*. To examine whether PEVs stimulate the secretion of inflammatory cytokines, NHEKs were treated with PEVs, and the culture supernatant was analyzed using a cytokine antibody array. Based on densitometric analysis, cytokines such as IL-8, GM-CSF, CXCL-1/GRO-α, and CXCL-5/ENA-78, which are known to promote the infiltration of neutrophils into acne lesions (Beylot et al., 2014), were increased in the supernatant of PEV-treated cells compared with those in nontreated cells (Figure 2a; Supplementary Figure S1a online). We validated the increased expression of these cytokines in terms of mRNA levels using quantitative real-time PCR analysis (Supplementary Figure S1b) and the increased secretion of IL-8 and GM-CSF proteins using quantitative ELISA (Figure 2b). Consistent with the above results, secreted IL-8 and GM-CSF levels were significantly

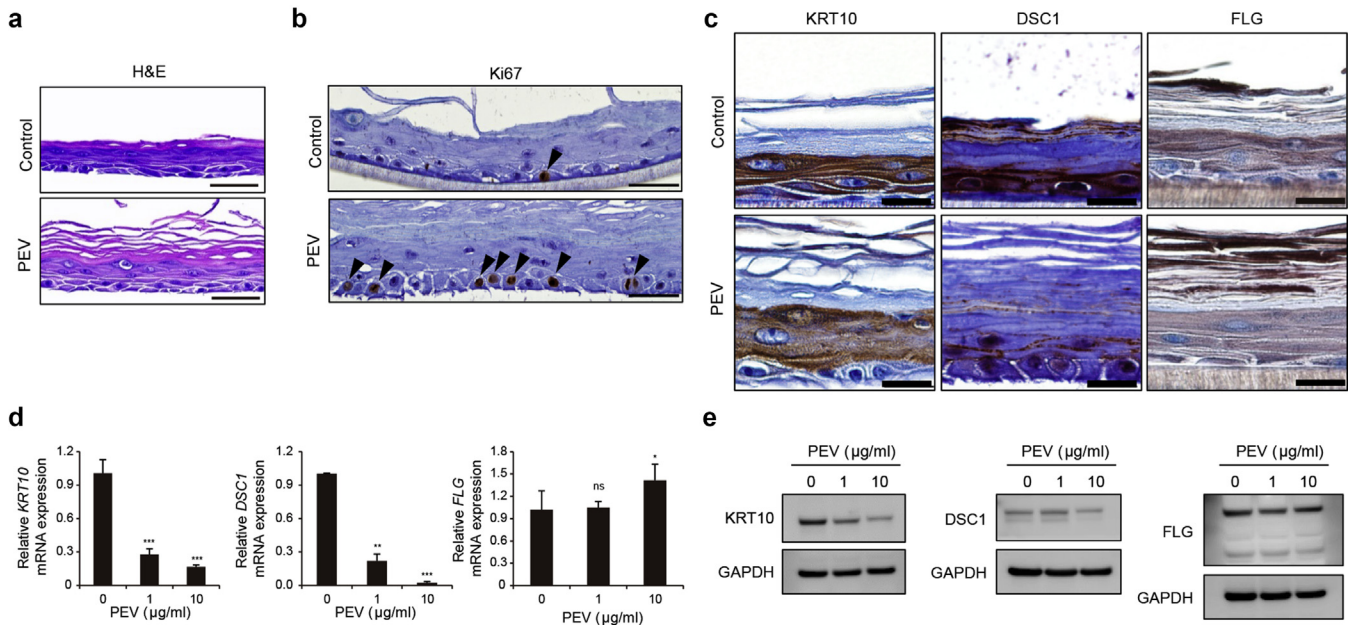

Figure 3. PEVs induce epidermal deformation by dysregulating the expression of epidermal markers. (**a**–**c**) A reconstituted human skin model was treated with 10 μg/ml PEVs or HEPES-buffered saline (control) for 6 days and fixed immunohistochemistry using relevant antibodies. (**a**) Hematoxylin and eosin staining. (**b**) Ki67 expression. The arrows indicate Ki67-positive cells. Scale bars in (**a**) and (**b**), 50 μm. (**c**) Expression of KRT10, DSC1, and FLG. Scale bars, 20 μm. (**d, e**) NHEKs were treated with PEVs for 4 days, and differentiation marker mRNA and protein expression were analyzed by (**d**) quantitative real-time PCR and (**e**) western blotting, respectively. mRNA levels were normalized to that of *RPL13A*, and GAPDH was used as a loading control for western blotting. Data are expressed as mean ± SD of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). DSC1, desmocollin 1; FLG, filaggrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; KRT10, keratin 10; NHEK, normal human epidermal keratinocyte; PEV, *Propionibacterium acnes*-derived extracellular vesicle.

increased by PEV treatment in a reconstituted human skin model (Figure 2c). These results suggest that PEVs, independently of *P. acnes*, upregulate the expression of proinflammatory cytokines in the human epidermis, thereby participating in inflammatory responses by stimulating keratinocytes in acne lesions.

PEVs induce epidermal deformation by dysregulating epidermal marker expression

In addition to the induction of inflammatory factors, *P. acnes* influences the differentiation of keratinocytes by altering the expression of differentiation markers (Akaza et al., 2009; Kurokawa et al., 2009). Hyperkeratinization is a precedent step for *P. acnes* colonization in the development of acne lesions, suggesting a positive feedback loop between keratinocytes and *P. acnes* in acne pathogenesis. We therefore investigated whether PEVs affect epidermal differentiation. A reconstituted human skin model (Desprez et al., 2015) was treated with PEVs or HEPES-buffered saline as a control every other day for 6 days and analyzed for determination of an epidermal structure. The PEV-treated skin model exhibited a thicker but less dense structure than the control group (Figure 3a). We performed immunohistochemical analysis using Ki67 for identification of proliferating cells, keratin 10 (KRT10) for the spinous layer, desmocollin 1 (DSC1) for desmosomal cell-cell adhesion structures, and filaggrin (FLG) for terminal differentiation of keratinocytes. Interestingly, the PEV-treated group showed a remarkable increase in Ki67-positive, proliferating keratinocytes compared with the control group (Figure 3b), consistent with an earlier report that hyperproliferating cells were observed in acne lesions (Knaggs et al., 1994). In addition, expression of KRT10 and DSC1 decreased considerably, but FLG expression seemed to increase (Figure 3c). We further validated the expression of these markers in NHEKs by treating cells with PEVs for 4 days. Similar to the results of the reconstituted skin model, PEVs prominently decreased the mRNA and protein expression of KRT10 and DSC1 (Figure 3d and e). Although PEVs did not severely affect the expression of Ki67 (data not shown) and FLG (Figure 3d and e), possibly due to the enriched culture conditions, these results suggest that PEVs affect the expression of epidermal markers related to proliferation and differentiation and result in epidermal deformation including hyperkeratinization, similar to the phenotypes observed in acne lesions.

PEVs evoke rapid and strong cellular responses compared with *P. acnes* extract at the same protein concentration

In many cases, gram-positive bacteria-derived EVs are more cytotoxic to cells than purified toxins (Brown et al., 2015), suggesting that EV-mediated delivery of toxins and various cargo including (lipo)proteins and polysaccharides might contribute to bacterial pathogenesis. We compared the pathogenic activity of PEVs and *P. acnes* extracts in evoking acne-like phenotypes by treating NHEKs with the same protein concentrations. The mRNA levels of inflammatory cytokines were clearly higher after treatment with *P. acnes* extracts than those in nontreated controls; however, these effects were intensified by PEV treatment (Supplementary Figure S2 online). In terms of protein levels, secreted IL-8

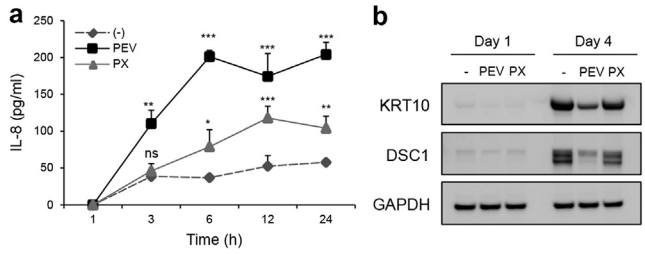

Figure 4. PEVs are more potent than *P. acnes* extracts in evoking cellular responses. NHEKs were treated with 10 μg/ml PEVs or 10 μg/ml *P. acnes* extract (PX). (**a**) Culture supernatants were harvested at the indicated time points after each treatment and quantitatively analyzed for IL-8 levels using ELISA. The data are expressed as the mean ± standard deviation of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). (**b**) Cells were harvested at day 1 or day 4 after each treatment and analyzed for the expression of KRT10 and DSC1 by western blotting using relevant antibodies. GAPDH was used as a loading control. DSC1, desmocollin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KRT10, keratin 10; NHEK, normal human epidermal keratinocyte; PEV, *P. acnes*-derived extracellular vesicle.

was markedly increased as early as 3 hours after treatment with PEVs and was maintained at considerably higher levels than those of nontreated controls, whereas treatment with *P. acnes* extract caused a significant but moderate release of IL-8 starting at 6 hours after treatment (Figure 4a). In addition, protein levels of KRT10 and DSC1 on day 4 after treatment were severely reduced by PEV treatment but only slightly influenced by *P. acnes* extract (Figure 4b). These results suggest that PEVs induce the rapid and intense production of inflammatory cytokines and decrease epidermal differentiation markers more effectively than *P. acnes* extracts at the same protein concentration; therefore, PEVs may be effective pathogenic factors in acne development.

PEVs induce cellular responses via TLR2-dependent signaling pathways

Next, we questioned how PEVs evoke or modulate these cellular responses. Previously, pathogen-associated molecular patterns on bacteria-derived EVs have been suggested to trigger cellular signaling pathways by binding to pattern recognition receptors such as TLRs (Brown et al., 2015; Kim et al., 2002, 2012, 2015b; Parker et al., 2010; Vanaja et al., 2016). Thus, we examined whether TLRs on keratinocytes can mediate PEV-induced cellular responses. To determine the major type of TLRs expressed in human keratinocytes, we analyzed the absolute expression level of each *TLR* mRNA using droplet digital PCR. *TLR2* and *TLR3* were considerably expressed in human keratinocytes, but other types of *TLR*s, including *TLR4*, were minimally expressed (Supplementary Figure S3a online). To determine whether TLR2 or TLR3 mediates PEV-induced cellular responses, we introduced small interfering RNAs (siRNAs) against *TLR2* or *TLR3* to keratinocytes and examined secreted cytokine levels and epidermal marker expression levels. The reduced mRNA expression and functional knockdown of TLRs by each siRNA was validated by quantitative real-time PCR (Supplementary Figure S3b) and the responses of receptor agonists after treatment (Supplementary Figure S3c). When cells were treated with *TLR2* siRNAs, PEV-induced IL-8 secretion was eliminated, and this effect was not observed after treatment with either nontargeting control or *TLR3* siRNAs (Figure 5a). In addition, the PEV-induced downregulation of KRT10 and DSC1 shown in nontargeting control siRNA-treated cells was recovered by the knockdown of *TLR2* but not of *TLR3* (Figure 5b), indicating that TLR2 is a major receptor for PEVs in human keratinocytes and mediates PEV-induced cellular responses.

TLR2 activates diverse signaling pathways including the mitogen-activated protein kinase (MAPK) and NF-kB signaling pathways (Brown et al., 2011). To examine whether these pathways are activated by PEVs via TLR2, we analyzed the phosphorylation of each signaling molecule in PEV-treated NHEKs using western blotting. The phosphorylated protein levels of p44/42 MAPK, c-Jun N-terminal kinase, p38, and p65 of the NF-kB complex were elevated within 3 hours after treatment with PEVs, and this was sustained until 12 hours after treatment, although there were differences in the degree and time point of activation of each signaling molecule (Figure 5c; Supplementary Figure S4 online). In contrast, PEV-induced phosphorylation of these signaling molecules was not upregulated when endogenous *TLR2* was knocked down in cells by siRNAs (Figure 5d), suggesting that PEVs stimulate various signaling pathways in a TLR2-dependent manner. To examine if these signaling pathway(s) are involved in PEV-induced cellular responses, we treated cells with PEVs in the presence of a specific inhibitor for each signaling molecule and analyzed the expression levels of the IL-8, KRT10, and DSC1 proteins. PD98059, SP600125, and BAY11-7082, specific inhibitors for p44/p42 MAPK, c-Jun N-terminal kinase, and NF-kB, respectively, significantly decreased PEV-induced IL-8 levels and recovered PEV-downregulated KRT10 and DSC1 levels. However, an inhibitor for p38, SB203580, neither reduced IL-8 levels nor recovered the expression of epidermal markers under PEV treatment (Figure 5e and f). Collectively, these results suggest that PEVs transmit signals via TLR2 and activate the downstream signaling molecules p44/p42 MAPK, c-Jun N-terminal kinase, and/or NF-kB, thereby inducing the secretion of inflammatory cytokines and dysregulating the expression of epidermal markers.

PEVs stimulate both human epidermal keratinocytes and myeloid cells

To determine whether there are differences in PEV-mediated effects on different cell types, we treated myeloid lineage cells, including human monocytic cell line THP-1 and peripheral blood mononuclear cells (PBMCs), with PEVs and examined the expression of various inflammatory cytokines. PEV treatment of THP-1 cells intensively increased IL-6, IL-8, tumor necrosis factor-α, and IL-1β protein levels in a dose-dependent manner (Supplementary Figure S5 online), whereas only IL-8 increased in PEV-treated NHEKs (Figure 2). A similar effect was observed in PBMCs, in which PEV treatment markedly induced the mRNA expression of all tested inflammatory cytokines (Supplementary Figure S6 online). Similarly, IL-6, IL-8, tumor necrosis factor-α, and IL-1β protein levels were markedly increased in PBMCs compared with those in vehicle-treated controls (Figure 6). These results suggest that

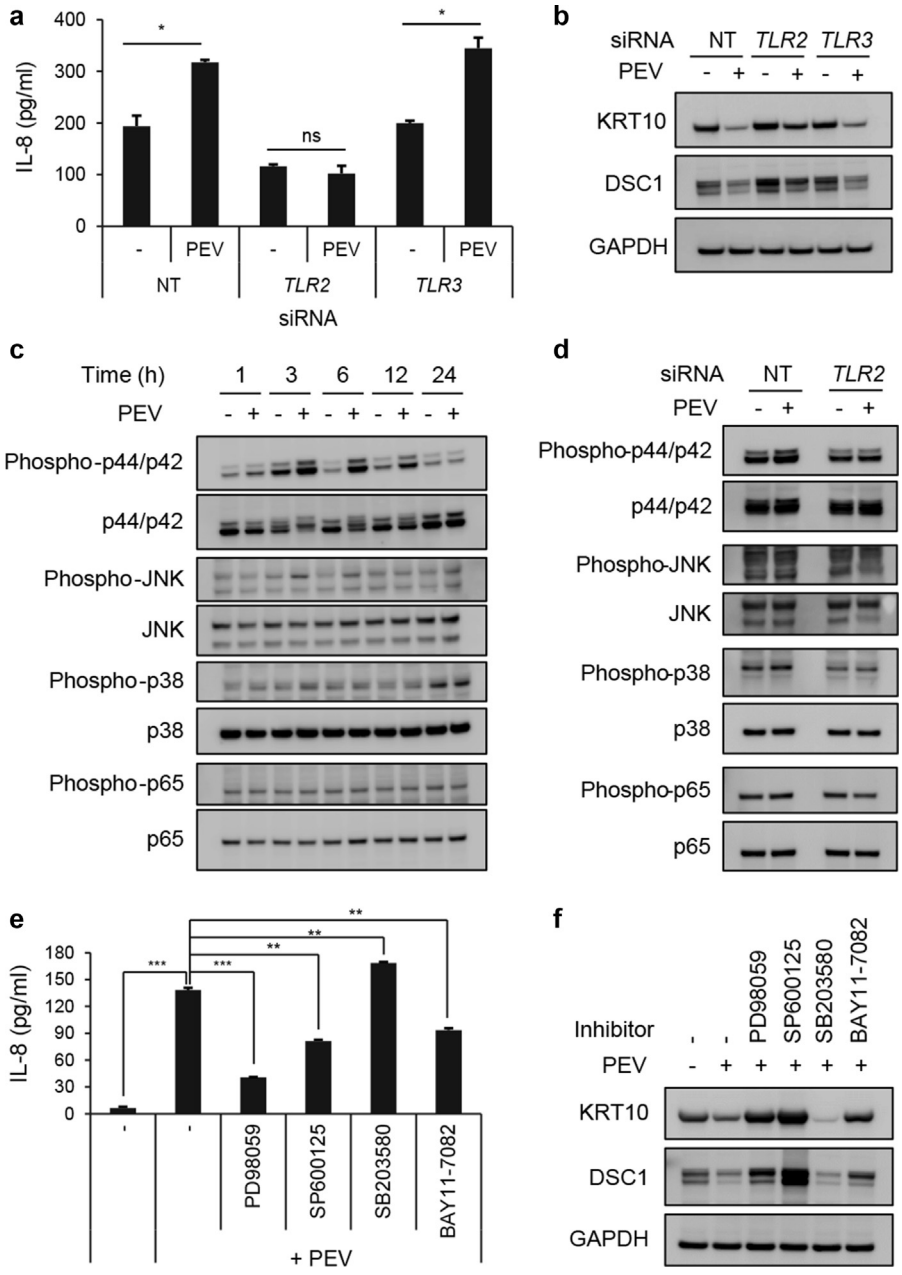

Figure 5. PEVs induce cellular responses via TLR2-dependent signaling pathways. (**a, b**) NHEKs were transfected with control (NT), *TLR2,* or *TLR3* siRNAs and treated with 10 μg/ml PEVs or HEPES-buffered saline (control, −). (**a**) IL-8 levels in culture supernatants at 24 hours after treatment. (**b**) Western blots of KRT10 and DSC1 at day 4 after treatment. (**c, d**) Western blots of phosphorylated and total forms of indicated proteins. (**c**) NHEKs were treated with PEVs and harvested at the indicated time points. (**d**) NHEKs were transfected with NT or *TLR2* siRNAs for 24 hours, treated with PEVs for another 3 hours, and then harvested. (**e, f**) NHEKs were treated with PEVs in the presence of 10 μM of each signaling inhibitor except BAY11-7082 (1 μM). (**e**) IL-8 levels in culture supernatants at 24 hours after treatment with PEVs. (**f**) Western blots of KRT10 and DSC1 at day 4 after treatment with PEVs. GAPDH was used as a loading control for western blotting. Data in (**a**) and (**e**) are expressed as mean ± standard deviation of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant). DSC1, desmocollin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KRT10, keratin 10; NHEK, normal human epidermal keratinocyte; NT, nontargeting control; PEV, *Propionibacterium acnes*-derived extracellular vesicle; siRNA, small interfering RNA; TLR, Toll-like receptor.

PEVs stimulate both keratinocytes and myeloid cells and that immune-related cells may be more sensitive to PEV stimulation than epidermal keratinocytes.

DISCUSSION

The skin is colonized by diverse types of microorganisms that are often related to skin disorders (Grice and Segre, 2011). Microorganisms secrete extracellular factors to communicate with and modulate host cells (Vowels et al., 1995). Among the secreted extracellular factors, EVs can mediate intercellular communication within or between living organisms (Kaparakis-Liaskos and Ferrero, 2015; Manning and Kuehn, 2013). A representative skin microorganism, *P. acnes*, has been thought to be involved in the development of acne; however, the pathogenic roles of EVs derived from *P. acnes* have not been investigated. To our knowledge, this is a previously unreported study demonstrating that *P. acnes* naturally secretes EVs and that these influence keratinocytes in a pilosebaceous unit by increasing the production of inflammatory cytokines and dysregulating the expression of epidermal markers. PEVs appear to evoke stronger and more rapid responses than *P. acnes* extracts at the same protein concentration through TLR2-dependent signaling pathways, and they also stimulate myeloid cells.

Increased numbers of neutrophils, hyperkeratinization, and less desquamation are frequently observed in acne lesions (Gollnick et al., 2003; Jeremy et al., 2003). Based on our study, PEVs significantly increase the secretion of cytokines such as IL-8, GM-CSF, CXCL-1, and CXCL-5, promoting the infiltration of neutrophils. Moreover, the potency of PEVs in inducing these inflammatory cytokines seems to be higher than that of *P. acnes* extracts at the same protein

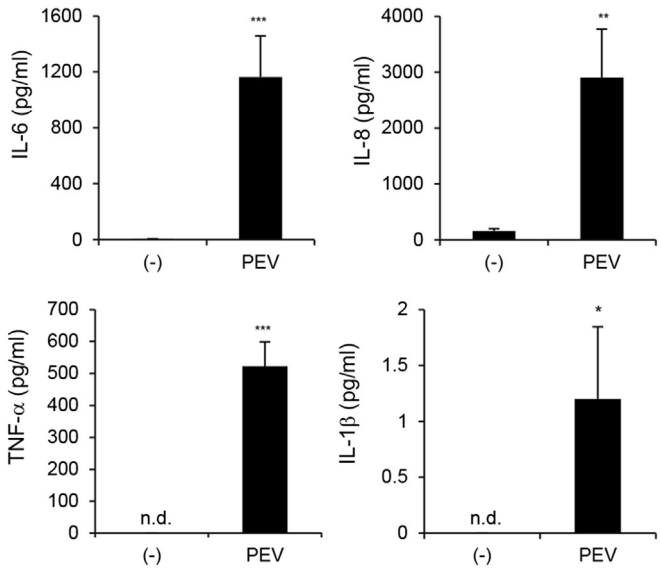

Figure 6. PEVs increase inflammatory cytokine protein levels in human PBMCs. Human PBMCs were treated with vehicle or 10 μg/ml PEVs for 48 hours. Culture supernatants were harvested and quantitatively analyzed for IL-6, IL-8, TNF-α, and IL-1β levels using specific ELISA kits. The data are expressed as the mean ± standard deviation of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.d., not detected). PBMC, peripheral blood mononuclear cell; PEV, *Propionibacterium acnes*-derived extracellular vesicle; TNF-α, tumor necrosis factor-α.

concentration in terms of time and effectiveness. These results suggest that PEVs may play pivotal roles in the early stages of the development of acne vulgaris by initiating inflammatory cytokine cascades in keratinocytes. Notably, despite the downregulation of KRT10 in the suprabasal layer, PEV-treated reconstituted human skin became thicker and showed increases in Ki67-positive cells in the basal layer and in FLG expression in the superficial layer compared with those in the control group. These phenomena are similar to the hyperkeratinization observed in the infundibula of acne lesions (Knaggs et al., 1994) and suggest that follicular hyperkeratinization can be partially attributed to the hyperproliferation of keratinocytes, possibly owing to PEV-induced cytokines or other factors.

Bacteria-derived EVs harbor bioactive materials, and these EVs can act as pathogenic agents by inducing inflammatory responses and changing the physiology of host cells through the transfer of EV contents and/or via TLR-dependent mechanisms (Bomberger et al., 2011; Hong et al., 2011; Kim et al., 2012; Park et al., 2010). EV contents can be transferred to recipient cells via various internalization methods depending on the size of the vesicles and the cell type (phagocytic vs. nonphagocytic) (Colombo et al., 2014; Yanez-Mo et al., 2015). The most frequently reported entry mode of bacteria-derived EVs into nonphagocytic cells involves lipid rafts (Kaparakis-Liaskos and Ferrero, 2015); however, PEVs seem to be internalized into keratinocytes through clathrin-dependent endocytosis, consistent with a recent finding regarding the entry mode for gram-negative bacteria-derived lipopolysaccharides into macrophages (Vanaja et al., 2016). Because clathrin-dependent endocytosis is mostly a receptor-dependent process, it is possible that TLR2, which is responsible for PEV-induced inflammatory responses and epidermal alterations, is involved in the clathrin-dependent endocytic process of PEVs, thus dually mediating PEV function in targeting keratinocytes.

As transmembrane receptors, TLRs recognize bacteria or bacteria-derived EVs and stimulate the signaling cascades necessary to produce inflammatory cytokines, contributing to the innate immunity of host cells. It was previously suggested that *P. acnes* stimulates the expression of both TLR2 and TLR4 and regulates the gene expression of human β-defensin 2 (*hBD2*) and *IL-8* in a dose-dependent manner in human keratinocytes (Jugeau et al., 2005; Nagy et al., 2005). Based on the analysis of absolute copy numbers of *TLR* mRNAs using droplet digital PCR in our culture conditions, however, *TLR4* was only minimally expressed compared with *TLR2* and *TLR3* in NHEKs. PEV-induced IL-8 release was completely blocked by *TLR2* knockdown, suggesting that TLR2 is a major receptor for PEVs and indispensable for PEV-mediated cytokine release in human keratinocytes. Contrary to the well-verified roles of TLRs in inflammatory responses, the involvement of TLRs and related signaling cascades in hyperkeratinization has not been clearly investigated. In our study, we demonstrated that TLR2 and the same downstream signaling pathways are also involved in the altered expression of epidermal differentiation markers.

Recently, it was reported that acne (P_A) and healthy (P_H) skin-associated *P. acnes* strains showed differential activity in stimulating PBMCs and T helper type 17 (Agak et al., 2018). Thus, we examined the pathogenic activity of EVs derived from acne or healthy skin-associated *P. acnes* strains. P_AEVs exhibited a stronger effect than P_HEVs in terms of inflammatory cytokine induction in PBMCs, in agreement with the above report, but a clear difference was not observed in keratinocytes (Supplementary Figures S7 and S8 online). Given that THP-1 and PBMCs were more sensitive to PEV stimulation than keratinocytes (Figure 6; Supplementary Figures S5 and S6), such immune-related cells may be more appropriate cell types for observing the differential effect of EVs derived from different *P. acnes* strains, at least in terms of inflammatory cytokine induction; however, PEV-induced epidermal dysregulation, another important acne phenotype, can be addressed by examining the effect of PEVs in keratinocytes.

In summary, our study suggests that lipid bilayer-enclosed and nanosized PEVs efficiently induce not only inflammatory responses but also epidermal deformation, that is, acne-like phenotypes. Therefore, the targeting of PEV-mediated pathogenesis using neutralizing antibodies against PEV-induced cytokines and TLR2 or the inhibition of the release of EVs from *P. acnes* could be alternative methods for alleviating acne occurrence and phenotypes.

MATERIALS AND METHODS

Cell culture and chemicals

Human primary keratinocytes (NHEKs; Lonza, Basel, Switzerland) were cultured in KBM-GOLD medium supplemented with the KGM-Gold BulletKit (Lonza). Chlorpromazine, nystatin, PD98059, SP600125, SB203580, and BAY11-7082 were purchased from Sigma (St. Louis, MO).

Isolation of PEVs

PEVs were purified from culture supernatant according to previously reported purification methods for gram-positive bacterial EVs (Lee et al., 2009). *P. acnes* extracts were prepared as previously described (Choi et al., 2008). Detailed protocols for PEV isolation are described in the Supplementary Material online.

Analysis of PEVs

PEV size was measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) and analyzed by the Dynamic V6 software. PEV morphology was analyzed by transmission electron microscopy (Tecnai G2 20; FEI, Hillsboro, OR) and cryo-transmission electron microscopy (Tecnai F20; FEI), for which detailed protocols are presented in the Supplementary Material.

PEV treatment in a skin model

A reconstituted human epidermis model (EPI-200) (Desprez et al., 2015) was purchased from MatTeK (Ashland, MA) and maintained following the manufacturer's instructions. In brief, a reconstituted skin model was maintained in standard maintenance medium (EPI-100-MM) for 3 days, followed by the application of HEPES-buffered saline or PEVs to the model every other day for 6 days. Culture media and reconstituted human skin were subjected to ELISA and immunohistochemical analyses, for which detailed protocols are presented in the Supplementary Material.

Immunocytochemistry and immunohistochemistry

PEVs were labeled with DiI (Molecular Probes, Eugene, OR) as described in the Supplementary Material. NHEKs were treated with DiI-labeled PEVs with or without endocytic inhibitors, stained with an anti-early endosome antigen 1 antibody (Abcam, Cambridge, MA), and analyzed under a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany). For immunohistochemical analysis, replicate sections from reconstituted human skin were stained with the following antibodies: anti-KRT10 (Biolegend, San Diego, CA), anti-DSC1 (Novus, Littleton, CO), anti-FLG (Abcam), and anti-Ki67 (Abcam). Detailed protocols for immunological analyses are described in the Supplementary Material.

Western blotting

NHEKs were lysed in Radioimmunoprecipitation assay buffer (Millipore). After centrifugation at 13,000 rpm for 10 minutes at 4°C, the supernatant was loaded onto a 5–20% gradient gel and stained with the following antibodies: anti-KRT10 (Biolegend), anti-DSC1 (Novus), anti-FLG (Abcam), anti-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated and total forms of p44/42 MAPK, c-Jun N-terminal kinase, p38, and p65 of the NF-kB (Cell Signaling Technology, Beverly, MA).

Quantitative real-time PCR

Total RNAs were prepared using TRIzol (MRC, Cincinnati, OH), and cDNA was synthesized using a Revertaid RT kit (Thermo Fisher Scientific, Waltham, MA). Quantitative real-time PCR was performed using TaqMan probes (described in the Supplementary Material; Applied Biosystems, Foster City, CA) and the 7500 Fast Real-Time PCR system (Applied Biosystems).

siRNA experiments

NHEKs were transfected with 50 nM of nontargeting control siRNA or siRNAs against *TLR2* or *TLR3* (Dharmacon, Lafayette, CO) using a Lipofectamine RNAimax transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

CONFLICT OF INTEREST

E-JC, HGL, I-HB, WK, TRL, and E-GC are employees of AmorePacific Corporation. JP states no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.01.007.

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